CONSERVATION OF THE ENDANGERED HAWAIIAN FERN ʻIHIʻIHILAUĀKEA (*MARSILEA VILLOSA*): A SYNTHESIS OF EXPERIMENTAL RESTORATION, COMMUNITY ECOLOGY, AND POPULATION GENETICS

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For Tom Ranker and Whitney Reyes
without whom this endeavor would not have been possible

And for my family
for their constant love, support, and encouragement

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ABSTRACT

Conservation of endangered plants is a critical step in maintaining and restoring global biodiversity. Management efforts such as *in situ* conservation and restoration through plant reintroduction are more likely to be successful if decisions are based on carefully designed scientific research. *Marsilea villosa* is an endangered, endemic Hawaiian fern with only seven remaining populations on the islands of Oʻahu and Molokaʻi restricted to ephemerally flooding dry lowlands. Among its uncommon traits are long-lived sporocarps (i.e., highly modified leaves with drought resistant walls containing sporangia and spores), a requirement of flood and drought to complete its sexual life cycle, and extensive vegetative growth. In this dissertation I conducted three studies to answer the following questions: 1) Which management techniques best facilitate growth of *M. villosa* in outplanting for reintroduction? 2) Which ecological factors affect the growth of *M. villosa* under field conditions? 3) How much genetic variation exists within and among *M. villosa* populations? 4) What are the implications of these studies for how *M. villosa* is managed? I conducted a restoration experiment to evaluate the effects of light, flooding, weeding, and their interactions on the growth of *M. villosa* outplanted in a common-garden. I found that the combination of flooding and shade treatments promoted the greatest increase in *M. villosa* growth, and that the effects of this interaction grew stronger over time. After drought occurred, shade also increased *M. villosa* growth in the absence of weeding. In a three-year field study, I examined ecological factors that influenced *M. villosa* growth and confirmed that shade and flooding have positive synergistic effects, while the negative effects of associated non-native species differ with functional groups. In a population genetic study, the majority of genetic variation was found at the subpopulation level, but there was also genetic structure that showed strong differentiation among some populations and between the two islands. This research provides several explicit management recommendations that will increase the chances of success in conservation and restoration of *Marsilea villosa*, and a model upon which to base restoration of the more resilient endangered species in Hawaiʻi and worldwide.

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PREFACE

The following data chapters (Chapters 2, 3, and 4) were written in the format of scientific papers for submission to peer-reviewed journals. Chapter 2 (Effects of light, flooding, and weeding on experimental restoration of an endangered Hawaiian fern) has been submitted to *Restoration Ecology* with coauthor Whitney Reyes and is in review. Chapter 3 (Ecological factors influencing growth of the endangered Hawaiian fern *Marsilea villosa* and implications for conservation management) is in preparation for submission to *American Journal of Botany* with coauthors Tom Ranker and Whitney Reyes. Chapter 4 (Bottlenecks and founder effects in the endangered Hawaiian fern ʻihiʻihi [*Marsilea villosa*]) is in preparation for submission to *Biodiversity and Conservation* with coauthors Tom Ranker and Clifford Morden.

CHAPTER 1
INTRODUCTION

Conservation of biodiversity is key to the continued existence of life on earth, including humans. We rely on an abundance of species that provide services such as food, medicine, pollination, clean water, and many others (Bullock et al., 2011). With the world in an extinction crisis driven by human activity, we must mitigate our damage to global biodiversity through conservation and ecological restoration. Conserving endangered plant species is a complex task, and on-the-ground management is often trial and error, with managers doing their best to address immediate needs. Ecologists and botanists try to improve this process by conducting sound science upon which to base management, but there is often a disconnect between the findings of scientists and their practical application (Hobbs, 2007). My goal with this study was to conduct scientific inquiry on an endangered plant species that would both contribute to scientific knowledge and have applied results that would be easily translated into methods for conservation practitioners.

Humans have had a dramatic effect on plant species distributions throughout the world, but the isolated ecosystems of Hawai‘i have been especially prone to the negative impacts of development. Human influence in Hawai‘i has led to the increase of non-native species richness to levels greater than that of native plants (Jakobs et al., 2010), and to a high percentage of endangered species (~25%) in the Hawaiian flora (Sakai et al., 2002). The introduction of invasive species such as grasses has also resulted in dramatic reductions of native species. Most Hawaiian dry forests are now degraded and dominated by non-native fire-promoting grasses (Cabin et al., 2002). Management of such non-native grasses and reintroduction of native species that formerly thrived in particular locations can restore native biodiversity and reverse habitat degradation (Daehler et al., 2005).

The reintroduction of rare and endangered plant species can be an effective strategy for maintaining biodiversity and helping to restore degraded ecosystems if there are also management systems in place. However, Godefroid et al. (2011) found that the success rate of plant reintroductions is moderate, with an average survival rate of 52%, with failures often due to lack of long-term monitoring. In addition to better monitoring efforts, strategies that may increase the success of reintroduction are a better understanding of basic species biology

and ecological interactions, and better site preparation prior to outplanting. Site quality of translocations and conditions that maximize population growth are key to long-term persistence (Rout et al., 2007), and providing suitable habitat is crucial to the reintroduction of rare and threatened plant species (Hobbs & Cramer, 2008).

Another critical step in the reintroduction of endangered species is gaining an understanding of the genetic makeup of populations that might serve as sources of propagules for restoration efforts. It is important to ensure that enough variation is present to allow for adaptability in new populations through natural selection (Lesica et al., 1999), particularly with clonally growing plants (Fant et al., 2008), and this often means sampling from multiple source populations (Godefroid et al., 2011). The capacity for evolutionary adaptation is critical in light of global climate and environmental changes, and the presence of genetic variation may facilitate the adaptation of many species to climate change, even over relatively short timeframes (Weeks et al., 2011). In what would be considered a genetically successful restoration, an introduced population would maintain levels of genetic diversity similar to those of wild populations (Menges, 2008; Ramp et al., 2006). In order to accomplish this, sufficient numbers of individuals must be sampled from one or more source populations and newly introduced populations must expand sufficiently within a few generations (Weeks et al., 2011). However, practitioners must find a balance between maximizing genetic variation in introduced populations and minimizing the risk of outbreeding depression that leads to decreased local adaptation (Vergeer et al., 2004). The prevailing attitude among restoration practitioners and ecologists has been a 'better safe than sorry' approach of favoring local populations for source transplant material, but some argue that these risks are overstated and have unduly restricted the use of translocation as a much needed restoration strategy (Weeks et al., 2011).

It is also critical that we overcome gaps in knowledge of basic species biology, especially of environmental factors that limit or facilitate establishment, including ecological interactions (Drayton & Primack, 2012; Guerrant & Kaye, 2007). More comparisons should be made with reference populations in the field, and reintroductions should be carefully designed as experiments based on prior ecological understanding (Kaye, 2008; Menges, 2008). Conducting these types of studies and experiments is especially important when working with unique ecosystems and understudied taxa.

Ephemeral pools are distinctive ecosystems characterized by small spatial scale, isolation, transience, high dependence on precipitation patterns, and biota that are uniquely adapted to these often stressful conditions. Although ephemeral pools are fairly abundant worldwide, many are also threatened by human development and exotic species invasion, and native species that flourish there are often endemic or endangered (Bauder, 2005; Collinge et al., 2011; Deil, 2005). However, there have been relatively few studies that have targeted ephemeral pool ecology and conservation. Small scale ecosystems such as ephemeral pools, which vary considerably in habitat characteristics and therefore beta diversity, should be studied not only for their own sake, but also because they make excellent model systems for hypothesis-testing in ecology, conservation, and evolutionary biology (Blaustein and Schwartz, 2001; De Meester et al., 2005).

Ferns and lycophytes are ecologically important but have also been understudied with respect to conservation and restoration. Worldwide, only about 2% of all 11,000 species have been evaluated for extinction risk, but 89% of those evaluated were found to be at risk. Furthermore, most risk assessments are based only on abundance and geographic range, and there is a need to examine intrinsic biology and ecology of ferns to better understand and evaluate species for conservation purposes (Mehltreter, 2010). Although it is not uncommon for studies of ecosystem restoration to account for regeneration of native fern species (e.g., Burns et al., 2011; Jager & Kowarik, 2010; Weller et al., 2011), very few studies target rare or endangered ferns for restoration (but see Aguraiuja, 2011; Zenkteler, 2002). There is a need for ecological studies on which to base conservation and restoration of fern species.

Marsilea villosa (‘ihi‘ihi) is an endangered, endemic Hawaiian fern that is found in only four surviving populations on the island of O‘ahu and three on the island of Moloka‘i (Bruegmann 1996; Dan Palmer, Bill Garnett, personal communication). ‘Ihi‘ihi is unusual among ferns in being heterosporous, producing sporocarps, growing in ephemeral pool habitats in dry lowland areas, and requiring both flood and drought to complete its sexual life cycle (Palmer 2002). *Marsilea villosa* produces regular photosynthetic leaves when rain is abundant enough to keep soil moist (typically December to March but varying among years and among populations; Wester et al., 2006; Bruegmann, 1996; Chau, personal observations), and during the dry summer season, the leaves die and the rhizomes are dormant. The plants produce sporocarps (i.e., highly modified leaves with thickened, drought-resistant walls

containing sporangia and spores) when the soil begins to dry at the end of the rainy season, but require standing water for sporocarp germination and sexual reproduction. Mature sporocarps can detach from rhizomes, intact, and potentially form a "sporocarp bank" with a function similar to a seed bank in angiosperms (though not analogous, as it represents dispersal of a different life stage). Sporocarps in the soil may be viable for up to a century, as found in the closely related species *M. oligospora* (Johnson 1985). The sporocarp is also the most likely stage for dispersal, which may occur via water or via movement by wetland bird species (Carlquist, 1976). When soil is moist enough for leaf production but flooding does not occur to allow for sexual reproduction, which may last several years, ʻihiʻihi grows vegetatively by resprouting leaves and extensive rhizome elongation.

Three studies have been conducted on the ecology of ʻihiʻihi at Koko Head, Oʻahu. The first was a study of ecophysiology, phenology, and taxonomy of the species in the field and in the greenhouse, which found no significant differences in water potential, leaf resistance, and osmotic potential at full turgor under varying moisture conditions, suggesting that *M. villosa* evolved drought resistance to adapt to specific environments (Bruegmann 1986). A second study mapped the population within ʻIhiʻihilauakea Crater at Koko Head and conducted a weeding experiment within the population (Wester 1994). Wester found that management through labor-intensive weed removal had no lasting effects and that periodic flooding was sufficient to exclude most competitors. A study using the same methods as Wester (1994) surveyed the same population over the following decade and found the population in severe decline, probably due to lack of flooding for thirteen consecutive years (Wester et al. 2006). They also found ecological changes, such as a decline in canopy cover of kiawe (*Prosopis pallida)* and invasion of alien grasses that may have influenced hydrology and flooding frequency in ʻIhiʻihilauakea Crater.

Restoration through outplanting has been studied experimentally for a few endangered Hawaiian plants. Cabin et al. (2002b) tested effects of light, weed control methods, and native (including two endangered) species addition through outplanting and seeding in a Hawaiian dry forest. They found an increase in native plant cover in all treatments, with the greatest increase in shade, bulldozed, and outplanted treatments, but noted that results were highly species-specific. Cabin et al. (2002a) also tested canopy microsite, watering, and weeding treatments on plots seeded with a similar suite of

native/endangered plants. The results of this study indicated that initial clearing of alien grass provided a sufficient window for native establishment, and that direct seeding is a promising method for dry forest restoration in Hawai‘i. Efforts to restore the endangered Mauna Kea silversword (*Argyroxiphium sandwicense*) have been successful with outplanted seedlings initially grown under controlled conditions. Walker and Powell (1999) experimentally tested seeding at different elevations, soil conditions, and microsites. Their results showed that outplanting, though labor intensive, had higher success rates than seeding, but that seeding could be more broadly applied and, presumably, allowed for natural selection of the most favorable genotypes. They suggested combining the two restoration strategies. Several experimental reintroductions of endangered species outside of Hawai'i have also been successful in identifying effective planting and management techniques (Alley & Affolter, 2004; Falk et al., 1996; Guerrant & Kaye, 2007; Jusaitis, 2005; Rowland & Maun, 2001), including two with endangered ferns (Aguraiuja, 2011; Zenkteler, 2002).

Although studies of fern reintroductions are few, there have been three successful ‘ihi‘ihi translocations on O‘ahu. In 2004, a community group outplanted *M. villosa* from the center of ‘Ihi‘ihilauakea Crater to an adjacent area under the shade of kiawe cover, and two patches have survived and grown with supplemental weeding (Larry Abbot, personal communication). At Hanauma Bay Nature Preserve, an occasionally flooding depression on the lawn outplanted in 2002 has been successful, and an even larger population grows on Kamehameha Schools/Bishop Estate land near Makapu‘u, outplanted in the 1960s (Alan Hong, personal communication). These populations were all started from Koko Head plant material and are now larger and healthier than their parent population, particularly the Makapu‘u site (personal observation).

No genetic studies of any kind have focused on *M. villosa*, however, its phylogenetic relationships within the cosmopolitan genus *Marsilea* have been examined, placing it in a clade with two North American species, *M. vestita* and *M. oligospora* (Nagalingum et al. 2007). Population genetics have been studied for *Marsilea strigosa*, another endangered species from the Mediterranean basin, and it was found to have high differentiation among populations, suggesting highly restricted gene flow and reproduction predominantly through selfing (Vitalis et al. 2001).

In the following studies, I aimed to address the following questions regarding the biology of *Marsilea villosa* in order to make conservation, restoration, and management recommendations. 1) Which management techniques best facilitate growth and expansion of *M. villosa* in outplanting for reintroduction? 2) Which ecological factors affect the growth of *M. villosa* under field conditions? 3) How much genetic variation exists within and among *M. villosa* populations? 4) What are the implications of these studies for how *M. villosa* is managed, both *in situ* and for future restoration efforts? To answer these questions, I conducted three studies. The first was a restoration experiment in which I outplanted *M. villosa* in a common-garden, using combinations of different management techniques (Chapter 2). I tested the effects of light (full sun or 50% shade), flooding (once or none), weeding (twice monthly or none), and their interactions on the growth of *M. villosa* over time. The second was a field study of ecological factors influencing *M. villosa* growth, including associated vegetation cover, canopy cover, flooding depth, soil nitrogen, and soil particle size distribution (Chapter 3). The study took place in three subpopulations at Lualualei Naval Base, O‘ahu from 2008 to 2011. I collected and analyzed soil samples in 2009, conducted vegetation surveys over three rainy seasons, and measured vegetation cover, canopy cover, and flooding depth every three weeks over the course of the last season. For the first two studies, I used mixed models ANOVAs to determine the models that best explained variation in percent cover of *M. villosa* in the experiment or in the field. For the third study, I employed random amplified polymorphic DNA (RAPD) methods to analyze genetic variation within and among the seven populations of *M. villosa* (Chapter 4). I used several statistical methods to determine the structure of genetic variation, analyze differentiation within and between islands, and make hypotheses about biogeographical relationships among populations. With each of these studies, I developed several explicit management recommendations that, taken together, will provide a wide-ranging plan for informed conservation and restoration of *Marsilea villosa*, with the ultimate goal of de-listing this endangered species.

CHAPTER 2
EFFECTS OF LIGHT, FLOODING, AND WEEDING ON EXPERIMENTAL RESTORATION OF AN ENDANGERED HAWAIIAN FERN

Abstract

Conservation of rare plants can be accomplished by the restoration practice of reintroduction, but subsequent management is often required. In species with narrow habitat requirements, it is difficult to predict which management methods will be successful at new locations. *Marsilea villosa* is an endangered, endemic Hawaiian fern with only seven remaining populations in ephemerally flooding drylands. Among its uncommon traits are long-lived sporocarps, a requirement of flood and drought to complete its sexual life cycle, and the potential for extensive vegetative growth. An experiment was performed to determine which restoration techniques might best facilitate growth of outplanted *M. villosa*. The following effects were tested in a split-plot factorial design: flooding (once/none), light (50% shade/full sun), weeding (bi-monthly/none), and all interactions. I hypothesized that flooding would have the largest single-factor effect and that there would be interactions among treatments. As hypothesized, flooding had the greatest positive effect on percent cover and sporocarp production. However, shade also increased cover over full sun when the plants began to experience drought. There was an interaction of light×flooding because *M. villosa* grew best in flooded, shaded plots. Weeding had no significant effect except in combination with flooding. Beyond protected status, current management of *M. villosa* populations consists entirely of weed management. This study shows that labor-intensive weeding may be unnecessary if reintroduced *M. villosa* is planted under conditions of flooding and moderate shade and, if planted at the start of a rainy season, will require minimal management to become a self-sustaining new population.

Introduction

The reintroduction of rare and endangered plant species can be an effective strategy for maintaining biodiversity and helping to restore degraded ecosystems if there are also ecological management techniques in place to monitor changes and ensure plant establishment and survival. However, Godefroid et al. (2011) found that the success rate of plant reintroductions is moderate, with an average survival rate of 52%, and lower rates of reproduction measures. Among strategies that may increase the success of reintroduction are a better understanding of species biology, better site preparation prior to outplanting, and consistent long-term monitoring. Site quality of translocations and conditions that maximize population growth are key to long-term persistence (Rout et al., 2007), and providing suitable habitat is crucial to the reintroduction of rare and threatened plant species (Hobbs & Cramer, 2008).

Humans have had a dramatic effect on plant species distributions throughout the world, but negative effects can be especially challenging in isolated ecosystems such as Hawai'i. Development and other human activities have led to the increase of non-native species richness to levels greater than that of native plants (Jakobs et al., 2010). The introduction of invasive species such as grasses has also resulted in dramatic reductions of native species. Most Hawaiian dry forests are now degraded and dominated by non-native fire-promoting grasses (Cabin et al., 2002). Management of such non-native grasses and reintroduction of native species that formerly thrived in particular locations can restore native biodiversity and reverse habitat degradation (Daehler et al., 2005). However, restoration efforts may never return an ecosystem to its original state, because exotic species cannot be entirely excluded, and management of invasive species will require a long-term commitment of resources (Norton, 2009).

Reintroduction has been studied experimentally for several endangered Hawaiian plant species. Often the most important factors in the success of native species are competition with alien grasses and exploitation of favorable microsites (Cabin et al., 2002). Efforts to restore the endangered Mauna Kea silversword (*Argyroxiphium sandwicense* DC. subsp. *sandwicense*) have been successful with outplanted seedlings (Walker & Powell, 1999). Several experimental reintroductions of endangered species outside of Hawai'i have been successful in identifying effective planting and management techniques (Alley &

Affolter, 2004; Falk et al., 1996; Guerrant & Kaye, 2007; Jusaitis, 2005; Rowland & Maun, 2001), including two with endangered ferns (Aguraiuja, 2011; Zenkteler, 2002). Though published studies of fern reintroductions are few, two outplanted *Marsilea villosa* Kaulf. (Marsileaceae; ʻihiʻihi) populations in Hawai'i have survived for several years (Alan Hong, Hanauma Bay Nature Preserve, Honolulu, HI, personal communication; Chau, personal observation).

Marsilea villosa is an endangered, endemic Hawaiian fern with only seven surviving populations on the islands of O'ahu and Moloka'i (Bruegmann, 1996; W. Garnett, Rare Plant Species Recovery, Moloka'i, HI, personal communication; Chau & Reyes, personal observations). Species of *Marsilea* are unusual among ferns in being heterosporous, producing sporocarps (i.e., highly modified leaves with thick drought-resistant walls that contain sporangia and spores), and requiring flood and drought to complete their sexual life cycle (Palmer, 2003). *Marsilea villosa* produces photosynthetic leaves when rain is abundant enough to keep soil moist (typically December to March, but varying among years and among populations; Chapter 3; Wester et al., 2006; Bruegmann, 1996) and produces sporocarps when the soil begins to dry, but requires standing water (i.e., during the next flooding event) for sporocarp germination and sexual reproduction. The extent of *M. villosa* cover is positively related to rainfall and fluctuates with supra-annual rainfall variation. Within populations that have been studied, flooding does not occur every year, but populations or subpopulations are limited to areas that have had some observed instance of flooding (Chapter 3; Wester, 1994; Wester et al., 2006; M. Bruegmann 2008, U.S. Fish & Wildlife Services, Honolulu, HI, personal communication). In the dry season, the leaves die and the rhizomes are dormant. Sporocarps that matured at the end of the rainy season can detach from rhizomes, intact, and potentially form a "sporocarp bank" with a function similar to a seed bank in angiosperms (though not analogous, as it represents dispersal of a different life stage). Sporocarps may be viable for up to a century, as found in the closely related species *Marsilea oligospora* Goodd. (Johnson, 1985). When conditions are wet enough for plant growth (moist soil) but not for sexual reproduction (standing water), which may last several years, *M. villosa* grows vegetatively by resprouting new leaves from old rhizomes and potentially growing new rhizomes. Production of long-lived sporocarps and abundant vegetative growth likely contribute to the ability of *M. villosa* to recover from stressful

conditions, such as a drought of a year or more, as long as flooding occurs in subsequent rainy seasons (Chapter 3). This resilience of *M. villosa* makes it an excellent candidate for restoration through reintroduction.

I have conducted the first experiment to evaluate the potential of management techniques for restoration of this endangered species. The goal of this study was to test the effects of flooding, light levels, and weed management on growth of outplanted *M. villosa*. My first hypothesis was that flooding would be the greatest factor affecting *M. villosa* growth and sporocarp production, based on earlier reports (Bruegmann, 1996; Wester, 1994; Wester et al., 2006) and my firsthand account that *M. villosa* expands to the boundaries of newly flooded areas within two weeks of flooding subsidence. Second, I hypothesized an interaction between flooding and weeding, where *M. villosa* growth would be higher in weeded than non-weeded plots in the absence of flooding, but would not differ between weed treatments within flooded plots. The rationale for this second hypothesis is that seasonal flooding suppresses weeds, allowing *M. villosa* to form mats and dominate (personal observations), while prolonged drought allows weed establishment, leading to *M. villosa* decline (Wester et al., 2006). Third, I hypothesized that in non-flooded plots there would be an interaction between light and weeding, where weeding would increase growth of *M. villosa* in sun but not in shade. When flooding is absent for long periods, *M. villosa* grows more vigorously in shade, where most invasive species show suboptimal growth, than it does in sun, where invasive species thrive (L. Abbott 2008, U.S. Army Natural Resources, Honolulu, HI, personal communication; personal observations). Fourth, I hypothesized that sporocarp production would increase in non-flooded/sun treatment plots because these treatments would increase levels of water stress, which stimulates sporocarp production in the field (Tryon & Tryon, 1982). Finally, I hypothesized that the effects of flooding and the strength of interactions would increase over the time period of this experiment, given the fast-growing nature of *M. villosa*. My aim was to provide baseline resource management knowledge that will stimulate and inform efforts to restore *M. villosa* through reintroduction.

Materials and Methods

Study Site

The experiment was conducted at the University of Hawai'i at Mānoa on the top floor balcony of the St. John Plant Sciences Building. The balcony receives full sun and limited wind - conditions comparable to the natural habitat of *M. villosa*. Mean annual rainfall at this location is 999.8 mm, which is higher but similar to *M. villosa* populations on O'ahu such as Lualualei (619.1 mm) and Koko Head (724.0; Giambelluca, 2011). I chose to conduct a common-garden experiment because manipulative experiments are not permitted at the sites of natural *M. villosa* populations.

Experimental Design

I tested the following effects: two flooding levels (once or none), two light levels (50% shade or full sun), two weeding levels (bi-monthly or none), and the interactions of these factors. Percent cover was measured for *M. villosa* to determine relative growth in response to different treatment combinations, and sporocarps were counted after the experiment ended to quantify sexual reproduction potential. I used a split-plot design with repeated measures and a factorial arrangement of the three treatments. Light levels were in the main plots, and a 2×2 factorial combination of flooding and weeding levels were in the subplots, for an overall 2×2×2 factorial design (Fig. 2.1). Main plots were placed so that shaded plots would not cast shadows on sun plots. There were six replicates, which were blocked for variation along the length of the building (Fig. 2.1), due to the physical structure and space available. Since there was potential variation in conditions along the building-wall-to-balcony-wall gradient (within each block), I measured the distance from the building wall to the center of each plot. When wall distance was analyzed as a covariate, it was not found to be significant. The split-plot design was chosen because using light levels as a main plot was most logistically feasible, as sun or shade was more easily applied to a larger plot. If a similar experiment were done in the field, flooding would also have to be in the main plot for logistical reasons, but in this experiment the plots were constructed allowing flooding in the subplot, increasing precision for that factor. The factorial arrangement was chosen to maximize the use of resources and to enable testing for all interactions between factors, since these were all of interest.

Since *M. villosa* has a shallow root system, plots were constructed using 0.87 cm sanded pine untreated plywood (Georgia Pacific; Atlanta, GA) as a base and untreated 2 × 4 pine boards (5.08 × 10.16 cm; Home Depot; Atlanta, GA) as rims, and 150 micron Husky Plastic Sheeting (Poly-America; Grand Prairie, TX) was stapled inside each subplot to waterproof flooding treatment plots. Holes were drilled through plastic and plywood bases to provide ample drainage in the subplots receiving the no flooding treatment. Each main plot (containing four subplots) was placed on cinder blocks to allow full drainage. For shaded main plots, a frame of 1.27 cm polyvinyl chloride (PVC) pipe was constructed to a height of 70 cm above the plot, and the frame was covered with 50% shade cloth on its top and sides. This ensured that the plots were fully shaded but did not shade any other plots. Plots were monitored every 1-2 days. Since 2010 was an exceptionally dry year and rainfall was not sufficient to keep soil moist enough for plant growth, the plots were watered using the building's water source twice a week in March and April 2010. The flooded main plots were manually flooded on 26 February 2010. Once weed species emerged, weeded subplots were hand weeded on a bi-monthly basis from 22 February to 19 April 2010. The experiment ended when subplots began to reach 100% cover of *M. villosa*. Although the emergent season of *M. villosa* in the field tends to fall between December and March (M. Bruegmann 2008, U.S. Fish & Wildlife Services, Honolulu, HI, personal communication), rainy seasons at Lualualei can extend into June (Chapter 3), so the timing of the experiment (February through April) was within the natural range.

Plant Material and Soil

One of the natural subpopulations at Lualualei Valley, O'ahu, was the source of dormant *M. villosa* rhizomes that were grown in 1-liter pots at the experimental site while plots were being constructed. Soil from the top 10 cm of the ground surface was also collected from Lualualei Valley, adjacent to the natural population of *M. villosa*. Soil in 1-liter pots was watered and observed to confirm that a weed seed bank was present and that seeds were viable. The soil was sifted for particles larger than 5 cm diameter and thoroughly mixed to maximize homogeneity, and then soil was placed in each subplot to a depth of 8 cm. Forty-eight clumps of *M. villosa* with 10 to 15 fully expanded leaves each were chosen randomly and transplanted into experimental plots in Lualualei soil on 13 February 2010. A

threatened and endangered species permit was obtained from the U.S. Fish and Wildlife Service (Permit No. TE096741-1) prior to all collection activities.

Response Measurement

Plant response measurements were taken bi-monthly, alternating with bi-monthly weeding treatments. Percent cover of *Marsilea villosa* and all other vegetation was measured by image analysis, using a digital camera secured to a 1.4 m high photo-quadrat built from PVC pipe. Shade cloth was detachable from PVC frames to allow photographs to be taken with the photo-quadrat, and immediately replaced. Images were analyzed with PhotoGrid 1.0 software (Bird, 2003) using 200 randomly generated points per photograph that were each scored as *M. villosa*, grass species, other plant species, or litter/soil. These point counts were converted into percent cover for each class. This method was used for photographs taken on the day of planting to confirm no statistical differences between outplantings. Subsequent measures were taken every two weeks for ten weeks. Three months after the end of the experiment (allowing time for full drought to occur and for sporocarp development), all sporocarps were counted in each plot to quantify sexual reproduction potential. Number of sporocarp-producing nodes on rhizomes and number of sporocarps per node were also quantified. Aborted sporocarps, which are easily identified by their small size or flattened appearance, were assumed to be non-viable and were not counted.

Data Analysis

Arcsine square root transformation was used on all percent cover data to normalize the data. Transformed data for percent cover were analyzed using linear mixed model ANOVAs with repeated measures. Data for sporocarps per subplot, sporocarps relative to percent cover, and sporocarps per node were normally distributed without transformation and were analyzed in the same manner as percent cover but without repeated measures. The largest models contained all terms in the fully crossed factorial of light (L), flood (F), weed (W), and time (T) as fixed effects. Block (B) and the interactions of B×L and B×L×F×W were chosen as random effects because these terms have physical counterparts (block, main plot, and subplot, respectively; Fig. 2.1), based on an *a priori* assumption that these would be the only meaningful random effects. I used a top-down strategy for model selection,

beginning with the full model and selecting appropriate estimation methods and covariance structures (West et al., 2007). Models were systematically reduced by elimination of non-significant random effects, followed by elimination of non-significant fixed effects, from highest to lowest order interactions. Best fitting models were selected using the Akaike Information Criterion corrected for small sample size (AICc), and these models were used to determine significant differences ($P< 0.05$). Between the fourth and final repeated measure, a sudden decrease in rainfall caused an unintentional drought stress event in the experiment, and the data for the final measure had a higher variance than the first four measures. Therefore, I treated the final measure as a separate dataset and did not include it in the repeated measures analysis. The same methods of model selection were used on the data from this single measure. All ANOVAs were followed by Tukey-adjusted least squares post hoc tests to determine *P* values for pairwise significant differences between treatment combinations. All statistical analyses were run in SAS using the Mixed Procedure (SAS Institute, 2006).

Results

The model that best explained variation in growth of *M. villosa* over the first four measurements (2 - 8 weeks) had no random effects, was reduced in number of fixed effects, and used unstructured covariance structure and maximum likelihood estimation (Table 2.1). The effect of time was significant, with mean percent cover more than tripling over eight weeks (Table 2.2; Fig. 2.2). Over this eight-week period, flooding was the only treatment with a significant single-factor effect on percent cover of *M. villosa*. In flooded plots compared to non-flooded plots, cover was 1.75 times higher at six weeks and was 1.64 times higher at eight weeks (Table 2.2). There was an interaction of time×flood because the positive effect of flooding on growth increased over time (Table 2.2; Fig. 2.2b). A significant interaction of time×light occurred because at four weeks percent cover was higher in sun plots ($P = 0.0067$) than in shaded plots, at six weeks there was no difference between light treatments, and at eight weeks shaded plots had higher cover than sun plots ($P = 0.0061$; Fig. 2.2a). There was also an interaction of time×light×flood (Table 2.2) because although flooded plots increased percent cover more than non-flooded plots over time, the difference between flood treatments in sun plots was only significant at six weeks ($P = 0.0106$; Fig.

2.3a), while the difference between flood treatments in shade plots was only significant at eight weeks ($P = 0.0062$; Fig. 2.3b).

A sudden drop in rainfall to levels at which supplementary watering was inadequate caused an unintentional drought event in the experiment at approximately nine weeks. The final 10-week measurement was analyzed separately from the repeated measures, and the model that best explained variation in this final measure included all fixed effects, no random effects, unstructured covariance structure, and maximum likelihood estimation (Table 2.1). Flooding again had a significant single-factor effect with percent cover twice as high in flooded plots than in non-flooded plots (Table 2.2, Fig. 2.2b). Light also had a significant effect with percent cover 3.4 times higher in shade plots than in sun plots (Table 2.2; Fig. 2.1a). There was an interaction of flood×light because there was no difference between flood treatments grown in sun, while percent cover was over twice as high in flooded plots than in non-flooded plots grown in shade ($P = 0.0001$, Fig. 2.4a). A flood×weed interaction also occurred because in weeded plots there was no difference between flood treatments, but in non-weeded plots percent cover was 3.5 times higher in flooded than in non-flooded plots ($P = 0.0006$, Fig. 2.4a).

The model that best explained variation in sporocarp counts included all fixed effects, no random effects, diagonal covariance structure, and restricted maximum likelihood estimation, and this model was the best fit for all sporocarp response variables (Table 2.1). Results for number of sporocarps per subplot were parallel to percent cover of *M. villosa* after the drought. There was a single-factor effect of flooding with more than double the number of sporocarps in flooded plots relative to non-flooded plots (Table 2.3). There was also a flood×light interaction with no difference between flood treatments grown in sun, but more than four times more sporocarps in flooded plots than non-flooded plots when grown in shade ($P = 0.0002$; Fig. 2.4b). The flood×weed interaction was also significant, with no difference in sporocarp numbers by flood treatment in weeded plots, but without weeding there were 7.6 times more sporocarps in flooded plots than in non-flooded plots ($P = 0.0004$; Fig. 2.4b). When I divided number of sporocarps by percent cover of *M. villosa* within plots, there were no significant differences between any treatments or interactions (Table 2.3). The number of sporocarps per sporocarp-producing node showed significant single-factor effects of flooding and light, with flooded plots averaging just less than one more sporocarp per

node than non-flooded plots, and shaded plots averaging just over one more sporocarp per node than sun plots (Table 2.3).

Associated species present in the experiment were all found in the field at Lualualei, in the subpopulation adjacent to which soil was collected for the experimental plots and weed seed bank. All species besides *M. villosa* were non-native species. All species with greater than 3% cover in the field in 2010 were also present in the experimental plots (Table 2.4). A 3% threshold was chosen because all other associated species had less than 1% cover in the field, and since 2010 was a drought year, no species had greater than 15% cover in the field, including *M. villosa* (Chapter 2). Two species found in the field in 2010 are invasive, according to either the Hawai'i Department of Agriculture or the Hawai'i-Pacific Weed Risk Assessment (Table 2.4) and all of these invasive species were also found in the experiment.

Discussion

This experiment strongly supported my hypothesis that flooding is the factor with the greatest influence on reintroduction success. However, interactions among treatments showed that light levels and weed management could also affect restoration of *M. villosa* over time. The first eight weeks of the experiment showed support for my first and last hypotheses, with flooding having the greatest single-factor positive effect and having an increased positive effect over time (Fig. 2.1b). The doubling of *M. villosa* cover in flooded vs. non-flooded plots after the unplanned drought event was not predicted, but it is not surprising given the biology of *Marsilea* and other plant species that thrive in ephemeral pool habitats (Deil, 2005).

I did not make hypotheses about the effect of light or interactions of light and flood over time, but these were some of the strongest effects in the experiment. Although *M. villosa* is considered a sun-loving plant (Bruegmann, 1996), I have observed it thriving in both sun and shade conditions. Since water availability is so critical to the growth and life cycle of *M. villosa*, its increased growth in shade might be explained by the decrease in rate of plant transpiration and soil water loss relative to full-sun conditions (Mejia-Dominguez et al., 2011). This hypothesis is supported by the interactions of light and flood over time because in full sun, flooding made less difference over time (Fig. 2.3a), and even after drought flooding did not make a difference in full sun (Fig. 2.4a). However, in shade

conditions flooding did increase percent cover over time (Fig. 2.3b), and these gains were still present and even stronger after drought (Fig. 2.4a). It appears that the benefits of flooding are prolonged by the water-conserving properties of shade even after the onset of drought conditions. My results indicate that the combination of flooding and shade produces optimal conditions for *M. villosa* growth, and that reintroductions would greatly benefit from being planted in areas with seasonal flooding and partial shade, regardless of subsequent management activities.

Although I did not make predictions about the single-factor effect of weeding, it was surprising that there were no differences at any time, including after the drought (Fig. 2.2c). However there were some interactions involving weeding, as I predicted. The percent cover of *M. villosa* supported my second hypothesis after the drought event, which was higher in weeded than non-weeded plots in the absence of flood (Fig. 2.4b). An even greater difference was found between flooded and non-flooded plots in the absence of weeding; however, this also lent support to my hypothesis that flooding helped suppress weeds in the absence of weed control (Wester, 1994; Wester et al., 2006). My third hypothesis that weeding would increase percent cover of *M. villosa* in sun and non-flooded plots was not supported. No three-way effects were detected, and both two-way interactions involved flooding, further confirming the central role of flooding in the growth of *M. villosa*.

The results of sporocarp counts following the experiment contradicted my hypothesis that more sporocarps would be produced in the stressful conditions of sun and absence of flood. The doubling of sporocarps per plot with flooded compared to non-flooded plots (Table 2.3) and the similarity of the significance and direction of flood×light and flood×weed interactions (Fig. 2.4c, d) suggests that production of sporocarps is correlated with growth of *M. villosa*, rather than a strategy to increase dispersal and reproduction potential under stressful conditions. Indeed, the lack of difference in sporocarps produced per percent cover of *M. villosa* reinforces this hypothesis (Table 2.3). Thus, it seems important to target restoration practices that maximize vegetative growth of *M. villosa*, because losses in growth are not likely to be compensated by gains in sexual reproduction potential.

An unexpected result of sporocarp counts was the variation in number of sporocarps per sporocarp-producing node. More sporocarps were produced per node in both flooded and shaded plots, and although these effects were not strong enough to create an interaction

effect, these results suggest that optimal growth conditions tend to produce more sporocarps per node. During the experiment, I observed that plants that were fully submerged in water could produce sporocarps, despite the accepted view that sporocarps are produced at the end of the dry season when plants are water-stressed (Tryon & Tryon, 1982). In the first few weeks of the experiment, when individual rhizomes could still be distinguished in plots, I also observed that plants in sun tended to produce longer rhizomes while plants in shade tended to grow more densely, so that even when percent cover did not differ, distribution of growth may have. Therefore, I hypothesize that *M. villosa* grown under stressful conditions will spread rhizomes farther and faster, producing fewer sporocarps per node, while plants in optimal conditions will grow more densely and produce more sporocarps per node. Thus, I hypothesize that in a field situation, *M. villosa* grown in flooded, shaded conditions may ultimately produce more sporocarps per area, gaining a potential advantage in sexual reproduction.

Although I initially had some concerns about variability within the study site, using the model selection process alleviated these concerns. The elimination of all random variables in the best fitting models and the lack of any block effects indicated that the variation detected by the experiment was due to treatment effects. Since this experiment produced robust results, I expect that these can be applied to reintroductions in a field setting even with moderate spatial variability. Although the average length of a *M. villosa* emergent season is unknown, the length of this experiment did fall within the range observed at Lualualei Naval Base (Chapter 2). Therefore, my results are relevant to potential reintroduction locations even if they are flooded only once a season.

This study has important implications for future ecological restoration involving *M. villosa*. First, it has expanded the range of potential reintroduction locations. Conventional wisdom based on simple observation would have reintroduction take place in flooding but sunny locations. However, based on my results I recommend outplanting new populations of *M. villosa* in areas with both flooding and partial shade. Further research may be required to determine which canopy species would best coexist with *M. villosa*, as there is considerable variation in the ways that tree species alter understory microsites (Mejia-Dominguez et al., 2011). Weed control is the only form of management currently used at natural populations of *M. villosa,* either by applying herbicide to surrounding areas and targeted hand weeding (L.

Abbott 2008, U.S. Army Natural Resources, Honolulu, HI, personal communication) or by mowing in the dry season (A. Hebshi 2010, U.S. Navy Environmental Planning Division, Honolulu, HI, personal communication). If outplanted at the beginning of a rainy season, reintroduced populations should not require labor-intensive weed management, except perhaps in years of extreme drought. However, extended lack of flooding (approximately 18 yr) in a natural *M. villosa* population that used to flood on average every 6.5 yr has led to severe decline of that population, despite moderate weed control efforts (Wester et al., 2006). Although ephemeral pools tend to be more resistant than many other ecosystems to exotic species invasions, extreme climatic events can provide opportunities for invasive species to establish (Collinge et al., 2011). Thus I add the caveat that potential reintroduction sites should have a consistent record of flooding, and that occasional weed management, long-term monitoring, and assessment would be ideal. If reintroductions of *M. villosa* are implemented and managed as I recommend, they are likely to establish well, require minimal management, and become self-sustaining in the long term, reflecting the goals of successful ecological restoration.

TABLES

Table 2.1 Selected models tested and used for ANOVAs on repeated measures, percent cover at 10 weeks, and sporocarp count variables. AICc = Akaike Information Criterion corrected for small sample size, smaller numbers indicate better fit of the model; w_i = AICc weights. For estimation methods, REML = restricted maximum likelihood, ML = maximum likelihood. For model components, L = light, F = flooding, W = weeding, T = time. Models in bold font were selected as the best fits for the repeated measures analysis. For the remaining analyses, only the selected best fitting model(s) are shown.

Covariance Structure	*Estimation*	*Components*	*Model*	*AICc*	*Δ AICc*	*w_i*
Repeated measures of percent cover for times 2, 4, 6, and 8 weeks						
Compound Symmetry	REML	Fixed	L + F + W + LxF + LxW +FxW + LxFxW + T + TxL + TxF + TxW + TxLxF + TxLxW + TxFxW +TxLxFxW	-246.5	114.8	6×10^{-26}
		Random	B + BxL + BxLxFxW			
Autoregressive (AR)	REML	Fixed	L + F + W + LxF + LxW +FxW + LxFxW + T + TxL + TxF + TxW + TxLxF + TxLxW + TxFxW +TxLxFxW	-246.8	114.5	7×10^{-26}
		Random	B + BxL + BxLxFxW			
Heterogeneous AR	REML	Fixed	L + F + W + LxF + LxW +FxW + LxFxW + T + TxL + TxF + TxW + TxLxF + TxLxW + TxFxW +TxLxFxW	-253.3	108.0	2×10^{-24}
		Random	B + BxL + BxLxFxW			
Unstructured	REML	Fixed	L + F + W + LxF + LxW +FxW + LxFxW + T + TxL + TxF + TxW + TxLxF + TxLxW + TxFxW +TxLxFxW	-253.6	107.7	2×10^{-24}
		Random	B + BxL + BxLxFxW			
Unstructured	REML	Fixed	L + F + W + LxF + LxW +FxW + LxFxW + T + TxL + TxF + TxW + TxLxF + TxLxW + TxFxW +TxLxFxW	-255.9	105.4	6×10^{-24}
		Random	B + BxL			
Unstructured	REML	Fixed	L + F + W + LxF + LxW +FxW + LxFxW + T + TxL + TxF + TxW + TxLxF + TxLxW + TxFxW +TxLxFxW	-255.9	105.4	6×10^{-24}
		Random	B			
Unstructured	REML	Fixed	L + F + W + LxF + LxW +FxW + LxFxW + T + TxL + TxF + TxW + TxLxF + TxLxW + TxFxW +TxLxFxW	-255.9	105.4	6×10^{-24}
Unstructured	ML	Fixed	L + F + W + LxF + LxW +FxW + LxFxW + T + TxL + TxF + TxW + TxLxF + TxLxW + TxFxW +TxLxFxW	-328.4	32.9	4×10^{-08}
Unstructured	ML	Fixed	L + F + W + LxF + LxW +FxW + LxFxW + T + TxL + TxF + TxW + TxLxF + TxLxW + TxFxW	-335.9	25.4	2×10^{-06}
Unstructured	ML	Fixed	L + F + W + LxF + LxW +FxW + LxFxW + T + TxL + TxF + TxW	-340.0	21.3	2×10^{-05}
Unstructured	ML	Fixed	L + F + W + LxF + LxW +FxW + T + TxL + TxF + TxW + TxLxF	-349.9	11.4	0.0017
Unstructured	**ML**	**Fixed**	**L + F + W + T + TxL + TxF + TxLxF**	**-361.3**	**0**	**0.4992**
Unstructured	**ML**	**Fixed**	**F + T + TxL + TxF + TxLxF**	**-361.3**	**0**	**0.4992**

Table 2.1 (Continued) Selected models tested and used for ANOVAs on repeated measures, percent cover at 10 weeks, and sporocarp count variables.

Covariance Structure	*Estimation*	*Components*	*Model*	*AICc*	*Δ AICc*	w_i
Percent cover at time 10 weeks						
Unstructured	ML	Fixed	L + F + W + LxF + LxW +FxW + LxFxW	7.8	0.3	0.4079
Unstructured	ML	Fixed	L + F + W + LxF + LxW +FxW	7.5	0	0.4740
Sporocarps per plot						
Diagonal	REML	Fixed	L + F + W + LxF + LxW +FxW + LxFxW	474.9	0	0.6587
Sporocarps per percent cover						
Diagonal	REML	Fixed	L + F + W + LxF + LxW +FxW + LxFxW	198.1	0	0.5721
Sporocarps per node						
Diagonal	REML	Fixed	L + F + W + LxF + LxW +FxW + LxFxW	148.2	0	0.5573

Table 2.2 Mixed-model ANOVA of the effect of light, weeding, and flooding on percent cover of *M. villosa* using the two best fitting models each for repeated measures (weeks 2-8) and a single measure (week 10) after drought. w_i = AICc weights, which represent the relative likelihood of each model within response variables.

Factor	*df*	*F*	*P*	*Factor*	*df*	*F*	*P*
% Cover (weeks 2-8)				% Cover (week 10)			
Model 1, w_i=0.4992				*Model 1, w_i=0.4079*			
Light	1	0.12	0.7267	Light	1	47.78	<0.0001
Weed	1	2.37	0.1304	Weed	1	1.52	0.2246
Flood	1	5.40	0.0245	Flood	1	16.36	0.0002
Time	3	67.54	<0.0001	Light×Weed	1	0.07	0.7985
Time×Light	3	12.70	<0.0001	Light×Flood	1	8.55	0.0057
Time×Flood	3	3.84	0.0156	Flood×Weed	1	4.17	0.0478
Time×Light×Flood	4	4.46	0.0041	Light×Flood×Weed	1	2.77	0.1041
Model 2, w_i=0.4992				*Model 2, w_i=0.4740*			
Flood	1	5.15	0.0277	Light	1	45.17	<0.0001
Time	3	67.54	<0.0001	Weed	1	1.44	0.2372
Time×Light	4	9.59	<0.0001	Flood	1	15.47	0.0003
Time×Flood	3	3.84	0.0156	Light×Weed	1	0.06	0.8039
Time×Light×Flood	4	4.38	0.0045	Light×Flood	1	8.08	0.0069
				Flood×Weed	1	3.94	0.0538

Table 2.3 Relative sporocarp counts three months after the experiment ended. Values are means ± SE, $n = 24$ for single-factor effects, $n = 12$ for two-way interactions. Response variables and interactions with significant effects in bold font. For all F tests, $df = 1$.

Treatment effect	*Number of sporocarps*		
	Per subplot	*Per % cover*	*Per node*
Sun	69.0 ± 13.8	2.7 ± 0.5	**2.0 ± 0.3**
Shade	100.2 ± 21.9	3.0 ± 0.4	**3.3 ± 0.3**
F	2.09	0.14	**12.95**
P	0.1556	0.7111	**0.0009**
Flood	**118.9 ± 19.6**	3.4 ± 0.5	**3.1 ± 0.3**
No flood	**50.3 ± 14.4**	2.3 ± 0.4	**2.3 ± 0.3**
F	10.17	2.47	**5.27**
P	**0.0028**	0.1242	**0.0270**
Weed	91.9 ± 14.7	2.9 ± 0.5	2.9 ± 0.3
No weed	77.3 ± 21.7	2.7 ± 0.4	2.4 ± 0.3
F	0.46	0.10	1.93
P	0.5022	0.7500	0.1720
Light×Flood			
F	6.33	0.41	1.38
P	**0.0160**	0.5273	0.2472
Flood×Weed			
F	5.37	0.46	0.06
P	**0.0257**	0.5014	0.8039
Light×Weed			
F	2.91	0.56	0.59
P	0.0957	0.4599	0.4471
Light×Flood×Weed			
F	1.22	1.18	1.54
P	0.2767	0.2847	0.2221

Table 2.4 Comparison of associated species present in field and experiment. All species listed were present in the Lualualei subpopulation adjacent to which soil was collected for the experiment. Marks (x) indicate species with greater than 3% cover in the field in 2010 and species present in the experiment.

Family	Genus/species	Field > 3%	Exp present	Invasive
Asteraceae	*Sonchus oleraceus*		x	
Boraginaceae	*Heliotropium currasavacum*			
Chenopodiaceae	*Atriplex suberecta*			
Convolvulaceae	*Ipomoea* sp.			
Cyperaceae	*Cyperus rotundus*	x	x	
Euphorbiaceae	*Chamaesyce hypericifolia*	x	x	
Fabaceae	*Desmanthus virgatus*	x	x	x
Fabaceae	*Macroptilium lathyroides*		x	
Malvaceae	*Sida acuta*		x	
Malvaceae	*Sida ciliaris*			
Phylanthaceae	*Phyllanthus debilis*		x	
Poaceae	*Chloris barbata*	x	x	
Poaceae	*Dichanthium aristatum*	x	x	
Poaceae	*Digitaria* sp.			
Poaceae	*Echinochloa colona*	x	x	
Poaceae	*Megathyrsus maximus*	x	x	x
Portulacaceae	*Portulaca* sp.		x	
Solanaceae	*Solanum lycopersicum*			

FIGURES

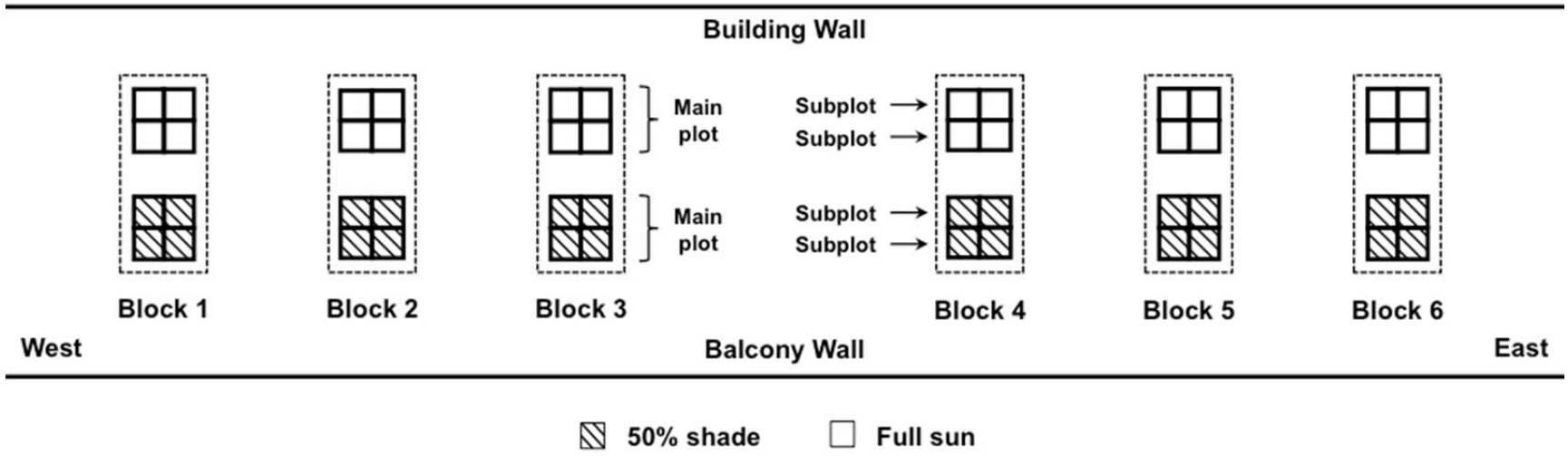

Figure 2.1 Experimental design showing location and arrangement of blocks, main plots, and randomized subplots. A factorial of flood and weed treatments was applied to subplots using randomly determined positions within the main plots.

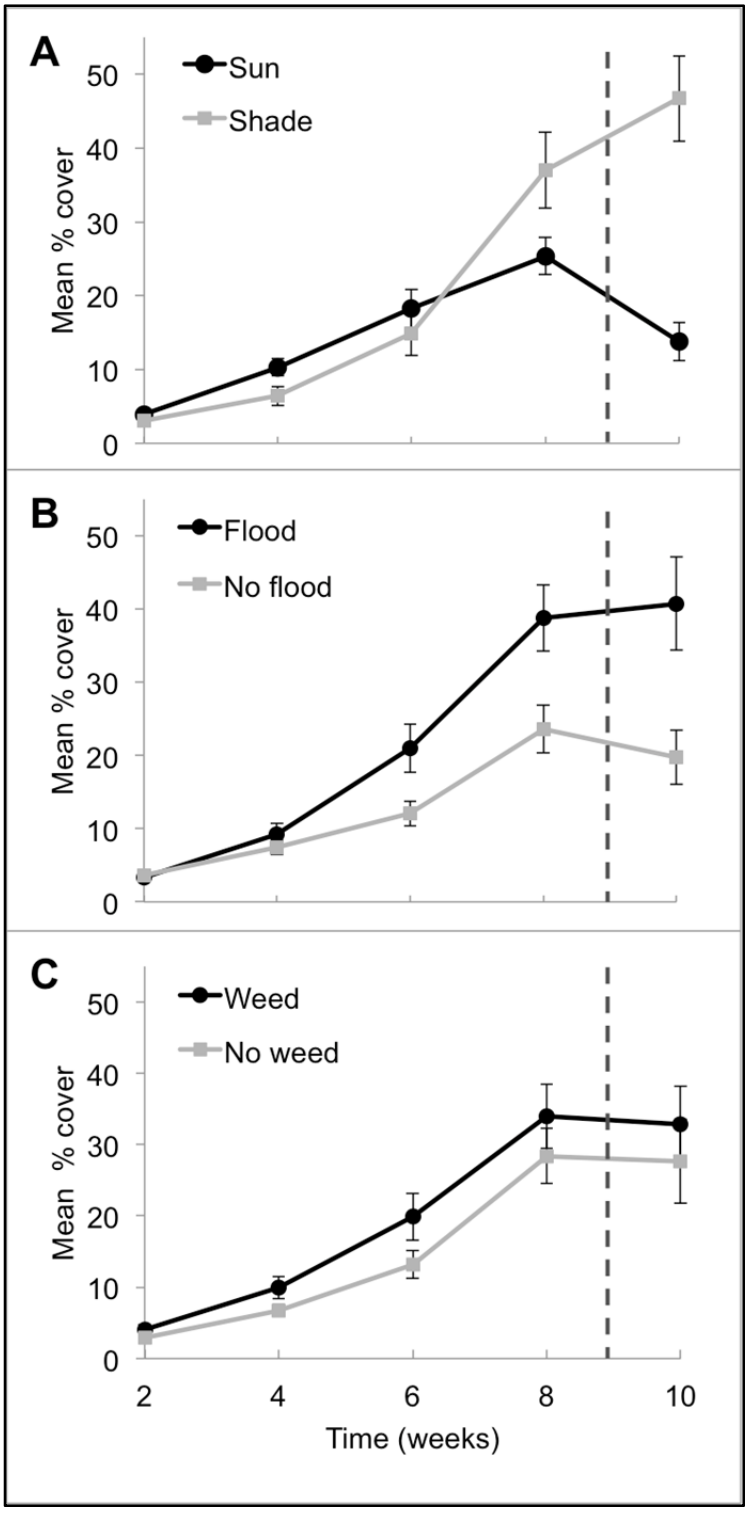

Figure 2.2 Single-factor effects of light (a), flooding (b), and weeding (c) on mean percent cover of *M. villosa* over time. Dashed lines indicate a drought event that occurred between the last two measures. The final measurement was treated as a separate dataset but is presented here for comparison. Error bars indicate ± 1 SE.

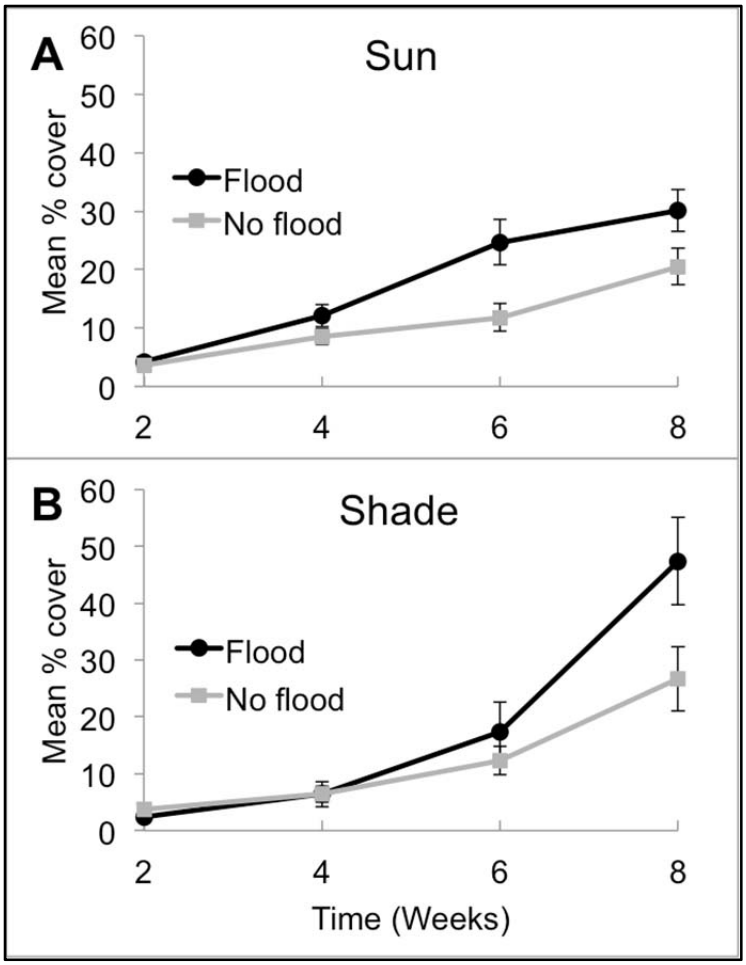

Figure 2.3 Three-way interaction of time×light×flood on mean percent cover of *M. villosa*. Interactions of time and flooding are shown within the sun treatment (a) and the shade treatment (b). Error bars indicate 1 SE.

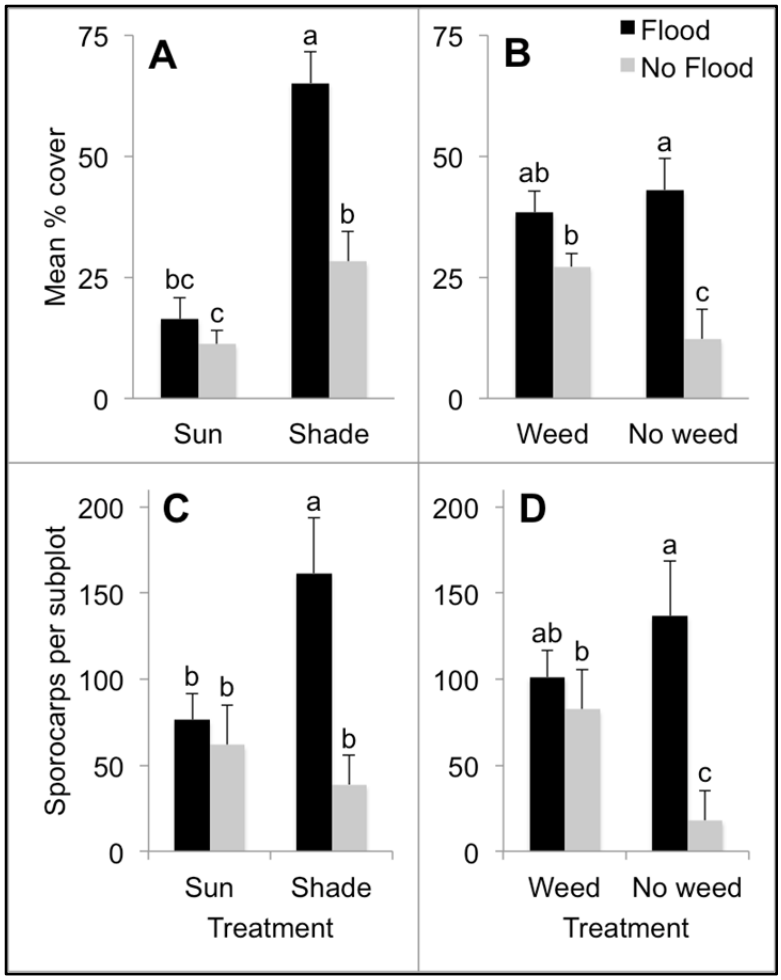

Figure 2.4 Two-way interactions of flood×light (a) and flood×weed (b) on mean percent cover of *M. villosa* at the end of the 10-week experiment, and interactions of flood×light (a) and flood×weed (b) on number of sporocarps per subplot after the experiment. Error bars indicate 1 SE. Lowercase letters indicate significant differences among treatments within each graph.

CHAPTER 3

ECOLOGICAL FACTORS INFLUENCING GROWTH OF THE ENDANGERED HAWAIIAN FERN *MARSILEA VILLOSA* AND IMPLICATIONS FOR CONSERVATION MANAGEMENT

Abstract

Conserving endangered plant species is a complex task, and on-the-ground management is often trial and error, with managers doing their best to address immediate needs. Botanists can improve this process by conducting sound science upon which to base management practices. *Marsilea villosa* is an endangered, endemic Hawaiian fern with seven remaining populations in ephemerally flooding drylands. Among its uncommon traits are long-lived sporocarps, a requirement of flood and drought to complete its sexual life cycle, and extensive vegetative growth. I conducted a three-year field study on the ecology of *M. villosa* to identify ecological factors with the greatest impact on its growth. I found that maximum flooding depth and canopy cover had strong positive relationships with *M. villosa* growth, and that all plots with over a 50% threshold of either variable reached 100% cover of *M. villosa* by the end of the study. Interaction effects explained the nuances of these relationships and confirmed some degree of synergy between the two variables. Percent cover of non-native understory species by functional groups (grass and non-grass species) each had negative relationships with percent cover of *M. villosa*, but interactions showed that percent cover of non-grass species was driven by a particular species over time, and that time since flooding had a greater influence on *M. villosa* growth than percent cover of grasses. I recommend several specific management strategies that will optimize *in situ* conservation, guide reintroduction practices to promote self-sustaining new populations, and reduce the need for labor-intensive management.

Introduction

Restoration of endangered plant species is becoming more and more critical as we face global loss of biodiversity. For many species, both management of existing populations and reintroduction of the species to new locations may be necessary to protect them from extinction. Reintroductions are sometimes successful, but often fail to become sustainable in the long-term (Fahselt, 2007; Godefroid et al., 2011). It is critical that we overcome gaps in knowledge of basic species biology, especially environmental factors that limit or facilitate establishment, including ecological interactions (Drayton & Primack, 2012; Guerrant & Kaye, 2007). More comparisons should be made with reference populations in the field, and reintroductions should be carefully designed as experiments based on prior ecological understanding (Kaye, 2008; Menges, 2008).

Ephemeral pools are distinctive ecosystems characterized by their small spatial scale, isolation, transience, high dependence on precipitation patterns, and biota that are uniquely adapted to these often stressful conditions. Although ephemeral pools are fairly abundant worldwide, many are also threatened by human development and exotic species invasion, and native species that flourish there are often endemic or endangered (Bauder, 2005; Collinge et al., 2011; Deil, 2005). However, there have been relatively few studies that have targeted ephemeral pool ecology and conservation. Small scale ecosystems such as ephemeral pools, which vary considerably in habitat characteristics and therefore beta diversity, should be studied not only for their own sake, but also because they make excellent model systems for hypothesis-testing in ecology, conservation, and evolutionary biology (Blaustein and Schwartz, 2001; De Meester et al., 2005).

Ferns and lycophytes are ecologically important but have also been understudied with respect to conservation and restoration. Worldwide, only about 2% of all 11,000 species have been evaluated for extinction risk, but 89% of those evaluated were found to be at risk. Furthermore, most risk assessments are based only on abundance and geographic range, and there is a need to examine intrinsic biology and ecology of ferns to better understand and evaluate species for conservation purposes (Mehltreter, 2010). Although it is not uncommon for studies of ecosystem restoration to account for regeneration of native fern species (e.g., Burns et al., 2011; Jager & Kowarik, 2010; Weller et al., 2011), very few studies target rare

or endangered ferns for restoration (but see Aguraiuja, 2011; Zenkteler, 2002). There is a need for ecological studies on which to base conservation and restoration of fern species.

Marsilea villosa Kaulf. is an endangered, endemic Hawaiian fern with only seven surviving populations on the islands of Oʻahu and Molokaʻi (Bruegmann, 1996); W. Garnett, Rare Plant Species Recovery, Molokaʻi, HI, personal communication; Chau & Reyes, personal observations). Species of *Marsilea* are unusual among ferns in being heterosporous, producing sporocarps (i.e., highly modified leaflets with thick drought-resistant walls that contain sporangia and spores), and requiring flood and drought to complete their sexual life cycle (Palmer, 2003). *Marsilea villosa* produces photosynthetic leaves when rain is abundant (typically November to March) and produces sporocarps when the soil begins to dry out, but requires standing water for sporocarp maturation and sexual reproduction. In the dry season, the leaves die and rhizomes are dormant. Sporocarps may be viable for up to a century, as found in the closely related species *Marsilea oligospora* Goodd. (Johnson, 1985). When conditions are wet enough for leaf production but not for sexual reproduction, which may last several years, *M. villosa* grows vegetatively. Production of long-lived sporocarps and vegetative growth likely contribute to the ability of *M. villosa* to recover from stressful conditions, such as a drought of a year or more, as long as flooding occurs in subsequent rainy seasons (personal observations). This combination of characters makes *M. villosa* a resilient species and therefore an ideal candidate for restoration efforts.

I performed an ecological field study of the largest population of *M. villosa* over the course of three growing seasons. My objectives were to answer the following questions: 1) How is *M. villosa* growth affected by biotic factors (e.g., associated vegetation and canopy cover), abiotic factors (e.g., flooding characteristics and soil properties), and temporal and spatial variation in these factors? 2) Do the results of this study support those of an experimental restoration study I conducted during the second year of the field study? (Chapter 2) and 3) What management recommendations can I make to the U.S. Navy Environmental Division, the owners of the land on which this population occurs?

I sought to test six hypotheses about single-factor effects on *M. villosa* growth and four hypotheses on interactions between ecological factors (Table 3.1). I hypothesized that increased flooding (1) and shade (2) would have positive relationships with growth of *M. villosa*, while variables related to associated non-native species (3) would have negative

effects, based on observations of growth in the field. Among associated species, I hypothesized that functional groups would have different effects (4), with non-native grasses having a more negative impact on growth of *M. villosa* than other non-native (mostly forb) species, due to the fibrous root systems, often perennial habit, and possible clonal growth via rhizomes of grasses that might compete with *M. villosa* root and rhizome systems. Among soil characteristics, I hypothesized that nitrogen (N) content (5) would have a positive relationship with growth of *M. villosa,* based on potential facilitation effects of N on plant growth, and that soils with a greater percentage of clay (6) would increase *M. villosa* growth by increasing duration of flooding and retaining more moisture with smaller particle size. With regard to interactions, I hypothesized that there would be interactions with time (1), specifically that some effects might only be significant in relation to time. I also made the following hypotheses about interactions among environmental factors. (2) *Marsilea villosa* growth would increase with combined higher levels of flooding and canopy cover, based on an experiment I conducted in the second year (Chau and Reyes, in review). (3) Non-native species would have a greater effect under lower canopy cover, since many grasses and other non-native species in dryland areas thrive under high light conditions. (4) The cover of non-native species would have a lesser effect on *M. villosa* growth with increased flooding, since flooding would kill most non-native species (at least temporarily, via above-ground biomass), providing a chance for *M. villosa* to establish.

Materials and Methods

The study population was located on the Lualualei Naval Base on O‘ahu, the entrance to which is located at 21°26’19. 15 N, 158°08’39. 29 W. Three subpopulations, distinct but located within 2 km of each other, were monitored over the course of three winter rainy seasons from Dec 2008 through June 2011. Since the majority of each rainy season occurred mostly after December, seasons will hereafter be referred to as 2009, 2010, and 2011. Field seasons varied considerably in monthly and total precipitation (Table 3.2.) For reference I named the subpopulations with numbers: LUA1, which covered 2288 m^2, LUA2, which was 266 m^2, and LUA3, which was 340 m^2 (Vanessa Pepi, U.S. Navy Environmental Planning Division, 2008, personal communication). In January 2009, before the start of this study, I observed a major flooding event, after which LUA1 expanded by an estimated 200-300 m^2.

The subpopulations also varied in microsite conditions, with LUA1 partially shaded, LUA2 in full sun, and LUA3 mostly shaded. All shaded areas were under a monotypic canopy of kiawe (*Prosopis pallida* [Humb. & Bonpl. ex Willd.] Kunth; Fabaceae).

Prior to any work with *M. villosa* plants, I obtained a Permit for Threatened and Endangered Species (P-121) from the State of Hawai‘i Department of Land and Natural Resources, Division of Forestry and Wildlife, and was added as an authorized individual on the Pacific Naval Facilities Federal Fish and Wildlife Permit (TE096741). In 2009, I established permanent plots at each subpopulation. I set up 1 × 1 m grids throughout each subpopulation and then randomly chose 20 of the plots at each subpopulation to monitor for three growing seasons. These permanent plots were marked with 12.7 cm galvanized steel nails (with no more than 5 cm above ground) wrapped in flagging tape, positioned at the upper left corner of the plot (relative to the road) in each subpopulation. I also used Glo Orange Flag Stakes (Empire; Mukwonago, WI) to more easily locate plots during the growing season, but removed these during the dry season so that groundskeepers could mow over the dormant subpopulations at LUA1 and LUA2 to control weeds. Mowing occurred monthly throughout the dry season, in several years prior to and during each year throughout this study (personal communication, Vanessa Pepi, U.S. Navy Environmental Planning Division, 2008). During each season, I began monitoring from the time the first rain occurred at the site and continued until the vegetative growth had subsided. The first season lasted from mid-December to early April, the second from late January to late April, and the third from mid-December to late May (Table 3.2). In February 2009, I took soil cores from the west corner (LUA1), south corner (LUA2), or north corner (LUA3) of each of the 60 plots using a Signature Soil Core Sampler (AMS, Inc.; American Falls, Idaho) with an inner diameter of 5 cm and a sample volume of ~196 cm^3. The 20 soil samples from each of the subpopulations (60 total) were taken to the Agricultural Diagnostic Service Center at the College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa for analysis. Soils were analyzed for total N by dry combustion (Burt, 2004) and for particle size distribution (% sand, silt, and clay) using a combination of sieving and sedimentation steps following Kettler et al. (2001).

Vegetation surveys were conducted once in 2009, twice in 2010, and seven times at three-week intervals over the course of the entire 2011 season (Table 3.2). I used the point-

frequency (or 'pin-drop') method for determining percent cover of understory plant species in overlapping tiers (Mueller-Dombois and Ellenberg, 1974). I constructed a 1 m tall frame of polyvinyl chloride pipe (1.9 cm diameter), and six holes were drilled across the top of the horizontal piece of the frame at 10 cm intervals. Metal pins (Crown Bolt Round Rods; Aliso Viejo, California) of 3.2 mm diameter and 91.4 cm length were fitted through the holes in the frame. The frame was placed over the plot to be surveyed, each pin was raised, and then as it was lowered each new plant species touched by the pin was recorded. This was repeated six times at 10 cm intervals, so that each plot had 36 data points in a 50 × 50 cm area (approximately centered within the 1 × 1 m plot). The pins were lowered slowly and did not harm the vegetation, and surveying materials were lightweight to minimize disturbance to plants. From the survey data I calculated the percent cover of *M. villosa* and all associated species in each plot.

Canopy cover was also measured during the 2011 season, using a Spherical Crown Densiometer (Forestry Suppliers, Inc.; Jackson, Mississippi). The densiometer had a grid of 24 squares etched on a convex mirror, with a bubble in the corner for leveling. Each of the squares etched on the mirror was visually split into four quadrants (total of 96 quadrants) and given a value of 0 to 4 estimating how much of the quadrant showed canopy openness (a value of 0 represented 0% canopy openness and value of 4 represented 100% canopy openness). This was done facing north, east, south, and west at each plot. All values within a cardinal direction were summed, the average was taken for the four directions and multiplied by 1.04 (providing percent canopy openness for that plot), and then this value was subtracted from 100 to obtain the percent canopy cover.

In 2011 I also measured the depth of any standing water present in plots during the regular surveys. During the largest flooding event, I measured water depth in the permanent plots twice, at one-week intervals between the second and third surveys (Jan 2011). Using a plastic ruler, water depth to the nearest 0.1 cm was recorded for the deepest area that fell within the plot boundary.

I also calculated percent cover of all non-native grass species, percent cover of all other non-native species (including mostly forbs, but also rare woody plants, vines, algae, and fungi; Table 3.3), species richness (number of species per plot), change in flooding depth from the previous repeated measure, maximum flooding depth per plot, flooding duration per

plot, and time since flooding. Time since flooding required special consideration, since some plots did not flood in 2011. From personal observations and rainfall records, I was confident that no flooding occurred in 2010, when a severe drought year affected the whole state of Hawai‘i, and I also observed a large flood in the 2009 season that inundated all subpopulations well beyond their previous boundaries, and thus all of the plots. Although I did not have plot-specific flooding data for 2009, since that flood subsided in late Jan 2009, I assigned a value of 100 weeks since flooding at the first repeated measure for all plots that did not flood in 2011. Data for the 2011 season were analyzed using linear mixed model ANOVAs with repeated measures (Table 3.4). The starting model included all independent single factors as fixed effects. All models used site (block) as a random effect, with variance components covariance structure. Restricted maximum likelihood was used to estimate the best covariance structure for random effects, followed by maximum likelihood to test fixed effects. Since all flooding variables were calculated from the same data, they were removed from the model one at a time by highest P value, unless significant. I then used a top-down strategy for model selection (West et al., 2007) within two-way interactions of interest. Two-way interactions with time were introduced then removed from the model one at a time by highest P value, unless significant, followed by two-way interactions with maximum flooding depth and two-way interactions with time since flooding, each reduced in the same manner. The best fitting model was selected using the Akaike Information Criterion corrected for small sample size (AICc), and these models were used to determine significant differences ($P< 0.05$) for fixed effects (Table 3.5). All statistical analyses were run in SAS using the Mixed Procedure (SAS Institute, 2006).

Results

Associated species at Lualualei (2009-2011)

There were 32 associated species recorded within the Lualualei survey plots over the three years of this study (Table 3.3). These included 15 flowering plant families, a green alga (Chlorophyta, Cladophoraceae), and a slime mold (Amoebozoa, Physaridae). Seventeen of the species (53%) were found within the plots in all three years of the study. All angiosperm species were non-native, and six (19%) are considered invasive species according to the Hawai‘i Department of Agriculture or the Hawai‘i-Pacific Weed Risk Assessment (Table

3.3; Division of Plant Industry, 2003; Daehler et al., 2004). Eight species (25%) were in the Poaceae, thus associated species were divided into two functional groups for analyses: non-native grasses and other non-native species. Average percent cover of *M. villosa*, total non-native species, and each functional group are shown in Fig. 3.1a, and all differences among years were significant (*M. villosa* $P < 0.0001$, grasses $P < 0.0001$, other species $P = 0.0004$). The four most abundant species by average percent cover were *Dichanthium aristatum* (Poir.) C. E. Hubb. (Poaceae), *Echinochloa colona* (L.) Link (Poaceae), *Leonotis nepetifolia* (L.) W. T. Aiton (Lamiaceae), and *Macroptilium lathyroides* (L.) Urb. (Fabaceae). Percent cover of these species also changed between years, particularly in 2010 when all species cover had declined more than 50% relative to 2009 or 2011 (Fig. 3.1b). *Dichanthium aristatum* was the only species averaging over 10% cover in all three years. The other three associated species each had over 10% cover in only one year. With regard to functional groups, grasses overtook *M. villosa* in terms of percent cover only in 2010, which was a drought year (Table 3.2), but non-grass species never did so (Fig. 3.1a).

In 2011, average percent cover of all non-native species together exceeded that of *M. villosa* for the first three weeks, but following a major flooding event after the third week, average percent cover of all non-native species together was equal to that of *M. villosa* (Fig. 3.2a). The three most abundant species by average percent cover were *D. aristatum* (15.8 ± 3.3), *E. colona* (12.9 ± 2.8), and *M. lathyroides* (10.7 ± 2.7). The total cover of these three species together increased over time in a pattern parallel to that of *M. villosa* cover, but the cover of the individual species was more variable (Fig. 3.2b). *Macroptilium lathyroides* showed an increase from 6 to 12 weeks and then leveled at just under 20% cover. The two grass species actually showed a shift in dominance between 9 and 12 weeks, with *E. colona* more abundant early in the season and *D. aristatum* dominant after the shift (Fig. 3.2b). *Leonotis nepetifolia* was relatively abundant at time zero (10.2 ± 2.1 average percent cover) and at three weeks (23.3 ± 4.6), but sharply decreased and remained low following flooding that occurred after three weeks. Thus *L. nepetifolia* was largely responsible for the early spike in percent cover of total non-native species seen in Fig. 3.2a. The only canopy species present was *Prosopis pallida*, and it ranged from 0 to 86% cover, with an overall average of 27.6 ± 4.2% cover.

Ecological patterns across one season (2011)

The model I selected for the 2011 season included time as a repeated measure, all independent single factors, with maximum flooding depth and time since flooding selected as flood-derived variables, several two-way interactions with time, and the interactions of maximum flooding depth × percent cover of canopy and of time since flooding × percent cover of non-native grass species (Table 3.4). Site (block) was included as a random effect, even though the covariance parameter estimate for site was zero, since investigating potential site effects of the Lualualei subpopulations was part of the experimental design. Single factors showed variability, but several of their effects were significant (Table 3.5). Maximum flooding depth had a positive relationship with percent cover of *M. villosa*, explaining 13% of the variation (Fig. 3.3a), while time since flooding had a negative relationship with *M. villosa* cover, explaining 21% of the variation (Fig. 3.3b). Percent cover of *M. villosa* increased with increasing canopy cover, which explains 5% of the variation (Fig. 3.3c). The vegetation related variables of percent cover of non-native grasses and percent cover of non-native other species both had a negative relationship with *M. villosa* percent cover, and explain 9% and 14% of the variation in this model, respectively (Fig. 3.3d, e). Time had a single-factor effect on *M. villosa* percent cover, which showed linear expansion until 9 weeks into the study, after which average growth slowed and reached a plateau between 75 and 80% cover (Fig. 3.2a). No effects related to soil characteristics were significant.

There were several interactions between time and different environmental variables (Fig. 3.4). Percent cover of non-native non-grass species had an increasingly negative relationship with *M. villosa* percent cover over time. Canopy cover did not have a strong relationship with *M. villosa* cover for the first three weeks of the study, but showed a slight negative relationship at six weeks, and then a continual and increasingly positive relationship with *villosa* percent cover during the last nine weeks. Maximum flooding depth had an increasingly positive relationship with *M. villosa* percent cover over time, while time since flooding had an increasingly negative effect.

Other significant two-way interactions included maximum flooding depth × percent canopy cover (Fig. 3.5). When canopy cover was zero, maximum flooding depth had a positive relationship with percent cover of *M. villosa*, but when there was any canopy cover there was no relationship between maximum depth and *M. villosa* cover. Another interaction

present was that of time since flooding × percent cover of non-native grass species (Fig. 3.6). For those plots that flooded during the 2011 season (less than 20 weeks since flooding), percent cover of grasses had a negative relationship with percent cover of *M. villosa*; however, for plots that had not flooded since the 2009 season (over 100 weeks since flooding), there was no relationship between grass and *M. villosa* percent cover.

Discussion

I have produced a model that explains variation in percent cover of *M. villosa* based on a suite of biotic and abiotic environmental variables. This model will be applicable to *M. villosa* in locations beyond the Lualualei population, despite observations of obvious differences between subpopulations. Since the covariance parameter estimate for the random effect of site was zero, the variation among sites is explained by the variation in the significant fixed effects in the model.

The single-factor effects supported several of my hypotheses. I did not make explicit predictions about which aspects of flooding would be significant, but the effects of maximum depth and time since flooding both support my hypothesis that flooding would increase *M. villosa* growth. The positive relationship between maximum depth and *M. villosa* cover, along with the lack of significance of flooding depth at the time of each repeated measure (eliminated from the model), indicates that flooding is likely a long-term scale (i.e., multiple years) effect rather than an instantaneous one. This idea is also supported by the decrease in percent cover of *M. villosa* with time since flooding, because the negative relationship is driven by the difference between plots that flooded sometime in 2011 and those that had not flooded since 2009. The positive relationship between canopy cover and *M. villosa* cover also supports my hypothesis about increased *M. villosa* growth in shade; however, the effect of canopy cover is better understood in the context of its interaction with time (see below).

As I hypothesized, both functional groups within non-native species had a negative effect on the growth of *M. villosa*, but the data do not support my hypothesis that grasses would have a stronger effect than other non-native species, as more of the variation in percent cover of *M. villosa* is explained by percent cover of non-grass species. However, the strength of this relationship is driven in large part by several of the observations with the highest value of percent cover of non-grass species. These in turn are driven by the species

Macroptilium lathyroides, which reached 80-100% cover in nine out of the ten plots with the highest percent cover of non-grass species. *Macroptilium lathyroides* is a biennial or short-lived perennial N-fixing legume with erect but twining branches (Wagner et al., 1999), and at Lualualei it was able to form thickets that made surveying some plots difficult. Although *Leonotis nepetifolia* never exceeded 25% cover and disappeared entirely by the end of the season, it should be noted that it did reach nearly 25% prior to the largest flood in Jan 2011. Additionally, *L. nepetifolia* was very abundant in the LUA3 population in 2009, with an average of 91.7% cover in those 20 plots, while *M. villosa* had only 36.1% cover. However, *L. nepetifolia* had < 1% cover in those same 20 plots on the same date in 2011, while *M. villosa* had 96.8% cover (data not shown). While *M. villosa* appears to be resilient to *L. nepetifolia* invasion over the years of this study, *L. nepetifolia* has been documented to cause significant ecological or economic harm in Hawai‘i according to the Hawai‘i-Pacific Weed Risk Assessment (Daehler et al., 2004) and cannot be discounted as an individual species that may have significant effects on *M. villosa* growth under certain conditions.

I did not find support for my hypotheses about the effects of soil N content or particle size distribution as I found no significant differences among any soil variables. Since the extant populations occur in a variety of soil types (including vertisols, mollisols, and andisols on O‘ahu, and vertisols, aridisols, and inceptisols on Moloka‘i; Deenik and McClellan, 2007), it may be that soil characteristics are not as important as topography that allows for flooding, along with the other significant environmental factors. A limitation of this study is that I collected soil samples only once (2009) and thus do not have data on how soil characteristics, particularly N content, change over time.

As I hypothesized, there were several interactions with time, all of which were also significant as single factors. All interactions with time have a slope that increases or decreases with time, partially driven by the fact that *M. villosa* cover was low at the beginning of the season, as would be expected with its seasonal growth pattern. However, there are several more subtle patterns in the interactions that are interesting and useful for understanding drivers of *M. villosa* growth. The interaction of time × maximum flooding depth shows that all plots with a maximum depth of over 50 cm reached 100% cover of *M. villosa* by or before 18 weeks, and those plots with less than 20 cm maximum depth never reached 100% cover (Fig. 3.4a). A strikingly similar pattern occurs in the time × canopy

cover interaction; although plots without any canopy have values that range over the full spectrum of percent cover of *M. villosa*, plots with over 50% canopy cover all reach 100% *M. villosa* cover by 15 weeks (Fig. 3.4c). These two findings provide strong support for my hypothesis that flooding and shade would be the factors most likely to increase *M. villosa* growth. The interaction of time × time since flooding (TSF) provides another way of examining the effect of flooding. The plots that had flooded at some point during the 2011 season have TSF < 20 weeks, and those that had not flooded since 2009 have TSF >100 weeks. There is not a strong difference between these categories at times zero and three weeks, but from six weeks on, only plots that had flooded at some time in 2011 were able to reach or maintain over 50% cover of *M. villosa* (Fig. 3.4b). This reinforces the conclusion that flooding related factors have long-term effects for *M. villosa* growth. The increasingly negative slope of the interaction of time × percent cover of non-grass species reflects not only the low percent cover of *M. villosa* in early weeks, but also the reduction of percent cover of non-grass species after 12 weeks (Fig. 3.4d). Some plots reached 150 to 225% cover of non-grass species at week 12, and all of these observations had below 50% cover of *M. villosa*. In week 15, non-grass species cover was reduced to less than 175%, but some plots with over 100% also had over 90% cover *M. villosa*. In week 18, non-grass species cover was further reduced to below 125%, but for the upper range of non-grass species cover, *M. villosa* cover remained below 80%. These results are again strongly influenced by the high percent cover of *Macroptilium lathyroides* in several plots, which in a few cases was able to support 50-100% cover of other non-grass species below its thicket-canopy, especially *Ageratum conyzoides* L. and *Emilia fosbergii* Nicolson (both Asteraceae), as well as the grass species *E. colona*. Although the negative relationship in this interaction is consistent, the changes over time indicate that the relationship between non-grass species cover and *M. villosa* growth is complex. Taken into consideration with the changes of grass species percent cover over time, which did not show a significant interaction effect on *M. villosa* cover, effects of non-grass species are likely more species-specific than the effects of grasses as a functional group.

With regard to interactions among variables besides time, I hypothesized that the combination of higher flooding levels and greater canopy cover would lead to the greatest *M. villosa* growth. While there was an interaction of maximum flooding depth × percent canopy

cover, it was not the relationship I expected. Instead, maximum depth only shows a positive relationship with *M. villosa* percent cover in the absence of canopy cover (Fig. 3.5a), which suggests that shade may actually be more beneficial to *M. villosa* growth than the extent of flooding. Additionally, in the presence of any amount of canopy cover, all plots flooded and had no less than 20 cm maximum depth (Fig. 3.5b). This lends support to a component of my hypothesis that there would be some degree of synergy between the two variables. I also hypothesized that the negative effects of non-native species would be stronger in the absence of canopy, but this was not the case with either functional group.

My last hypothesis was that increased flooding would decrease the negative effects of non-native species cover. Although I did not find this relationship between maximum depth and either functional group, I did find an interaction of time since flooding (TSF) × percent cover of non-native grasses. For plots with TSF < 20 weeks (flooded in 2011), there is a clear negative relationship between percent cover of grasses and that of *M. villosa*. However, this relationship seems to be driven by plots with especially high grass cover, i.e., over 100%, in which *D. aristatum* and *E. colona* are able to coexist and both maintain over 50% cover. For those plots with TSF > 100 weeks (no flooding since 2009), there was no relationship between percent cover of grasses and *M. villosa*, primarily because the large majority of plots had less than 20% cover of *M. villosa*, regardless of grass percent cover. Thus it appears that the negative effects of grass species are outweighed by the negative effects of time since flooding in the long term.

Conservation and restoration management implications

Clearly, aspects of flooding, as single factors or in various complex relationships with other variables, have a central role in promoting growth of *M. villosa*. The presence of flooding effects that depend more on long-term processes than short-term ones suggests that management efforts that target flooding parameters might have long-term effects and lead to self-sustainable populations. While this may not be applicable to most extant populations (i.e., in most locations manual flooding would not be logistically feasible even if cost were not prohibitive), I recommend that new reintroductions of *M. villosa* only be outplanted into areas with a consistent record of seasonal flooding, or possibly in areas where it would be feasible to control flooding manually.

Effects of shade (i.e., increased canopy cover) provide some of the most interesting results of this study. *Marsilea villosa* clearly survives well in full sun conditions since many of its natural populations occur where there is little to no canopy cover. Aside from the single factor effect of canopy cover, its positive influence on *M. villosa* growth is seen in several complex interactions. First, all plots in at least 50% shade eventually reach 100% cover. Second, maximum flooding depth does not affect *M. villosa* cover under any percent canopy cover (as opposed to no canopy cover). Finally, all plots flood to at least 20 cm with any canopy cover. Together, these effects indicate that canopy cover could be just as influential on growth of *M. villosa* as is flooding. Moderate shade (50%) also had a net facilitative effect on 46 temperate grassland species in Estonia, especially those species from dry or nutrient poor habitats, despite being adapted to high irradiance (Semchenko et al., 2012). Facilitation has often been overlooked in ecological theory, but there is much evidence that it affects population and community dynamics in a variety of ecosystems (Bruno et al., 2003). Though we currently do not know how frequently the benefits of shade outweigh the cost of reduced irradiance, there is a growing body of literature (much of which reported results of shade facilitation only incidentally) that suggests it is more common than previously assumed (Semchenko et al., 2012), and this study certainly provides evidence for this.

Not only does shade facilitate *M. villosa* growth, but it likely also has positive synergistic effects with flooding. This has obvious implications for potential reintroductions: if new populations are outplanted in shade, they are much more likely to become self-sustaining and to require less labor-intensive management. Based on these results, I also propose that "shade management" could be considered for *in situ* conservation. While I would not recommend major modifications to any populations of an endangered plant, managers might consider trials of outplanting native dry forest tree species on the margins of some populations, particularly if they are already in decline, such as Koko Head, or heavily invaded, such as the LUA2 subpopulation. If efforts to reintroduce *M. villosa* to new sites were made, I would highly recommend designing such reintroductions as experiments with permanent plots for continued monitoring. Outplanting of *M. villosa* with and without various native tree species (alone and/or in combinations) could serve as controlled trials for testing prior to tree outplanting at any natural populations and, if continued as a long-term

experiment, would be a valuable contribution to the science and practice of ecological restoration.

Non-native grass species tended to behave as a true functional group at Lualualei, with the possible interchange of one dominant species for another over time, which could simplify weed management efforts. However, as a caveat, practitioners should not discount the possibility of grass species that are exceptionally problematic. Although *Megathyrsus maximus* (Jacq.) B. K. Simon & S. W. L. Jacobs was present at Lualualei in only a few plots with low percent cover, this species is considered a noxious weed and also occurs at Koko Head where it is more of a threat to the *M. villosa* population, perhaps due to the absence of deep flooding for nearly 20 years (Wester et al. 2006). Another caveat is that grasses at Lualualei were already under minimal weed management, i.e., the mowing of subpopulations LUA1 and LUA2 during the *M. villosa* dormant season. However, although I did not explicitly compare sites in my model (since I considered site a random effect), it is worth noting the following: LUA3, which was never mowed but had the highest average canopy cover, maximum flooding depth, and flooding duration, also had the lowest average percent cover of non-native grasses and reached 100% cover of *M. villosa* by the end of this study.

Since effects of non-grass species cover on *M. villosa* growth were more species-specific than those of grasses, more attention should be given to the monitoring of non-grass forbs at the species or genus level rather than as a functional group. The relationship of non-grass species cover was driven in large part by *Macroptilium lathyroides*, which had an increased negative effect toward the end of the season despite decreases in overall non-grass species cover. *Leonotis nepetifolia* also showed potential as a species abundant enough to affect *M. villosa* growth. However, *M. lathyroides* has a biennial or perennial habit, whereas *L. nepetifolia* is an annual (Gill & Conway, 1979), which may explain why the former has a greater advantage than the latter in an ephemerally flooding habitat. Therefore, I recommend *M. lathyroides* as a target weed species for early control if it should appear within *M. villosa* populations, and the same for *L. nepetifolia* if resources allow; if not, *L. nepetifolia* should be carefully monitored. If target species are removed, *M. villosa* populations should continue to be monitored for any invasive species that may become more abundant in the absence of targeted species.

Since I found no significant effects of soil factors in this study, I conclude that flooding and vegetation factors are of the highest management priority. The lack of an effect of soil N content also confirms that the effects of canopy cover are due to shade, rather than increased N from *Prosopis pallida*, a N-fixing legume. Further research into soil biogeochemistry in *M. villosa* populations would be useful to investigate how soil characteristics may affect *M. villosa* growth, but given the potential limitations of such studies, such as the cost of soil analyses and the need to minimize impacts on populations of endangered plants, I recommend basing management decisions on the clear effects of flooding, shade, and vegetation related variables. Since *M. villosa* grows in several soil types, reintroduction efforts could aim to outplant new populations in sites with one of those soil types and the other favorable environmental characteristics discussed here.

The biotic and abiotic environmental factors affecting growth of *M. villosa* are quite complex, but I have identified probable factors and their interactions that play the most important roles. My model is robust and can be applied to any population of *M. villosa*, even new ones that result from reintroduction efforts, with the caveat that populations should always be monitored for any threats that may be unique to their specific localities. These results also may be useful for modeling growth for conservation management of other rare or endangered plant species, particularly other fern species with similar life cycles or any species that occur in ephemeral pool type habitats. Although *Marsilea villosa*, like any endangered species, will continue to require management and monitoring for the foreseeable future, the recommendations I have made here are likely to increase the cost effectiveness and ecological success of both *in situ* conservation and future restoration efforts.

TABLES

Table 3.1 Hypotheses for single factor effects on *M. villosa* growth, and interactions

Hypothesis	*Effect*	*Rationale*
Increased flooding	+	Based on field observations
Increased canopy cover	+	Based on field observations
Increased non-native species	-	Based on field observations
Non-native grasses > non-native other species	-	Growth habits may be more competitive with *M. villosa*
Increased soil N	+	N may facilitate growth of *M. villosa*
Increased soil percent clay	+	Smaller particle size may retain more moisture in soil
Interactions		
Some factors would only be significant in relation to time		Based on field observations
Combined increases in flooding & canopy cover would increase *M. villosa* growth		Based on experiment results (Chapter 2) and field observations
Non-native species would have a more negative effect with lower canopy cover		Based on field observations
Non-native species would have a less negative effect with greater flooding		Based on previous study (Wester et al., 2006)

Table 3.2 Summary of field seasons with monthly and seasonal rainfall. Summary of the three seasons in this study, including rainfall, season duration, flooding events, and sampling times. Field seasons are named for the calendar year in which the majority of the growing season occurred. Previous dry season totals included July-November immediately prior to the start of the field season, and field/rainy season totals include December-June as shown. Start and end of seasons were determined by observation of the first and last leafy vegetative growth.

	Field Seasons					
Month	*2009*		*2010*		*2011*	
	Rain (mm)	Events	Rain (mm)		Rain (mm)	
Previous dry season totals	99.06		80.52		60.71	
Dec	163.07	Start Flood	30.23		264.92	Start Vegetation survey Flood
Jan	21.08	Flood (continued) Mapping Plots established	38.86	Start	207.01	Vegetation survey Flood Vegetation survey
Feb	21.59	Soil sampling	12.70	Vegetation survey	133.10	Vegetation survey
Mar	38.35	Vegetation survey	35.56		43.18	Vegetation survey
Apr	31.24	End	35.56	Vegetation survey End	45.72	Vegetation survey Vegetation survey
May	5.84		35.31		90.93	
Jun	6.86		14.48		21.34	End
Field/rainy season totals	288.04		202.69		806.20	

Table 3.3 Associated species present within Lualualei survey plots. No associated species were native to Hawai'i. Invasive species were so designated by either the Hawai'i Department of Agriculture or the Hawai'i-Pacific Weed Risk Assessment.

Family	*Genus/species*	*2009*	*2010*	*2011*	*Invasive*
Asteraceae	*Ageratina* sp.			x	x
Asteraceae	*Ageratum conyzoides*	x		x	
Asteraceae	*Emilia fosbergii*	x	x	x	
Asteraceae	*Lactuca serriola*	x	x	x	
Asteraceae	*Sonchus oleraceus*	x	x	x	
Boraginaceae	*Heliotropium currasavacum*		x	x	
Chenopdiaceae	*Atriplex suberecta*	x	x		
Commelinaceae	*Tradescantia* sp.	x	x	x	
Convolvulaceae	*Ipomoea* sp.		x	x	
Cucurbitaceae	*Coccinea grandis*	x			x
Cyperaceae	*Cyperus rotundus*	x	x	x	
Euphorbiaceae	*Chamaesyce hypericifolia*	x	x	x	
Fabaceae	*Desmanthus virgatus*	x	x	x	x
Fabaceae	*Macroptilium lathyroides*	x	x	x	
Fabaceae	*Prosopis pallida*	x	x	x	
Lamiaceae	*Hyptis suavolens*	x			x
Lamiaceae	*Leonotis nepetifolia*	x	x	x	x
Malvaceae	*Sida acuta*	x	x		
Malvaceae	*Sida ciliaris*		x		
Phylanthaceae	*Phyllanthus debilis*	x	x	x	
Poaceae	*Cenchrus ciliaris*	x	x	x	
Poaceae	*Chloris barbata*	x	x	x	
Poaceae	*Dichanthium aristatum*	x	x	x	
Poaceae	*Digitaria* sp.		x	x	
Poaceae	*Echinochloa colona*	x	x	x	
Poaceae	*Eragrostis amabilis*	x			
Poaceae	*Megathyrsus maximus*	x	x		x
Poaceae	*Setaria verticillata*	x	x	x	
Portulacaceae	*Portulaca* sp.		x	x	
Solanaceae	*Solanum lycopersicum*	x	x	x	
Chlorophyta/Cladophoraceae	*Pithophora* sp.			x	
Amoebozoa/Physaridae	*Physarum* sp.			x	

Table 3.4 Models tested for within-season (2011) repeated measures ANOVA. # = model number; AICc = Akaike Information Criterion corrected for small sample size, smaller numbers indicate better fit of the model; w_i = AICc weights; ML = maximum likelihood; TSF = time since flooding. All models used Site as a random effect, with variance components covariance structure. Full models are shown at the end of each model reduction process.

#	*Covariance structure*	*Model for fixed components*	*AICc*	*Δ AICc*	w_i
1	Unstructured (Unst.)	*Time + %Grass + %Other + SppRichness + Canopy + FloodDepth + ΔDepth + MaxDepth + FloodDuration + TSF + SoilN + Sand + Clay*	-244.8	208.9	1×10^{-46}
2	Unst. w/correlations	As above	-244.8	208.9	1×10^{-46}
3	Autoregressive (AR)	As above	-108.9	344.8	5×10^{-76}
4	Heterogeneous AR	As above	-218.5	235.2	3×10^{-52}
5	Ante-dependence	As above	-252.3	201.4	6×10^{-45}
6	Ante-dependence	As above with ML test of fixed effects	-365.4	88.3	2×10^{-20}
7	Ante-dependence	As above - ΔDepth	-367.3	86.4	6×10^{-20}
8	Ante-dependence	As above - FloodDepth	-369.9	83.8	2×10^{-19}
9	Ante-dependence	As above – FloodDuration *Time + %Grass + %Other + SppRichness + Canopy + MaxDepth + TSF + SoilN + Sand + Clay*	-371	82.7	4×10^{-19}
11	Ante-dependence	Model 9 + all Time 2-way interactions	-387.1	66.6	1×10^{-15}
12	Ante-dependence	As above - Time×Sand	-402.8	50.9	3×10^{-12}
13	Ante-dependence	As above - Time×%Grass	-416.9	36.8	4×10^{-09}
14	Ante-dependence	As above - Time×SppRichness	-430.5	23.2	3×10^{-06}
15	Ante-dependence	As above - Time×Clay	-439.2	14.5	0.0002
16	Ante-dependence	As above - Time×SoilN *Time + %Grass + %Other + SppRichness + Canopy + MaxDepth + TSF + SoilN + Sand + Clay + Time×%Other + Time×Canopy + Time×MaxDepth +Time×TSF*	-446.8	6.9	0.0109
17	Ante-dependence	As above + all MaxDepth 2-way interactions	-436.8	16.9	7×10^{-05}
18	Ante-dependence	As above -MaxDepth×%Other	-439.6	14.1	0.0003
19	Ante-dependence	As above -MaxDepth×TimeSinceFlood	-442.2	11.5	0.0011
20	Ante-dependence	As above -MaxDepth×Clay	-444.7	9	0.0038
21	Ante-dependence	As above -MaxDepth×SoilN	-447.2	6.5	0.0133
22	Ante-dependence	As above -MaxDepth×%Grass	-449.2	4.5	0.0363
23	Ante-dependence	As above -MaxDepth×Sand	-449.7	4	0.0466
24	Ante-dependence	As above -MaxDepth×SppRichness *Time + %Grass + %Other + SppRichness + Canopy + MaxDepth + TSF + SoilN + Sand + Clay + Time×Other + Time×Canopy + Time×MaxDepth + Time×TSF + MaxDepth×Canopy*	-450.1	3.6	0.0569
25	Ante-dependence	As above + all TimeSinceFlood 2-way interactions	-440.9	12.8	0.0006
26	Ante-dependence	As above - TSF ×SoilN	-443.6	10.1	0.0022
27	Ante-dependence	As above - TSF ×%Other	-446.3	7.4	0.0085
28	Ante-dependence	As above - TSF ×Clay	-449	4.7	0.0328
29	Ante-dependence	As above - TSF ×Canopy	-451.5	2.2	0.1146
30	Ante-dependence	As above - TSF ×SppRichness *Time + %Grass + %Other + SppRichness + Canopy + MaxDepth + TSF + SoilN + Sand + Clay + +Time×Other + Time×Canopy + Time×MaxDepth + Time×TSF + MaxDepth×Canopy + TSF×Grass + TSF×Sand*	-453.7	0	0.3442
31	Ante-dependence	As above - TSF ×Sand	-453.6	0.1	0.3275

Table 3.5 ANOVA table for fixed effects on percent cover of *M.villosa* in the within-season (2011) repeated measures model. Bold font indicates significant factors and interactions.

Factor	*df*	*F*	*P*
Time	**6**	**11.54**	**<0.0001**
% Grass Cover	**1**	**6.30**	**0.0125**
% Other Cover	**1**	**6.95**	**0.0088**
Spp Richness	1	0.15	0.7011
Canopy	**1**	**4.44**	**0.0358**
MaxDepth	**1**	**12.86**	**0.0004**
Time Since Flood	**1**	**11.08**	**0.0010**
Soil N	1	0.85	0.3563
% Sand	1	0.42	0.5198
% Clay	1	3.39	0.0662
Time × % Other Cover	**6**	**6.78**	**<0.0001**
Time × Canopy	**6**	**5.90**	**<0.0001**
Time × Max Depth	**6**	**4.00**	**0.0007**
Time × Time Since Flood	**6**	**3.73**	**0.0013**
Max Depth × Canopy	**1**	**8.06**	**0.0048**
Time Since Flood × % Grass Cover	**1**	**7.49**	**0.0065**
Time Since Flood × % Sand	1	2.83	0.0933

FIGURES

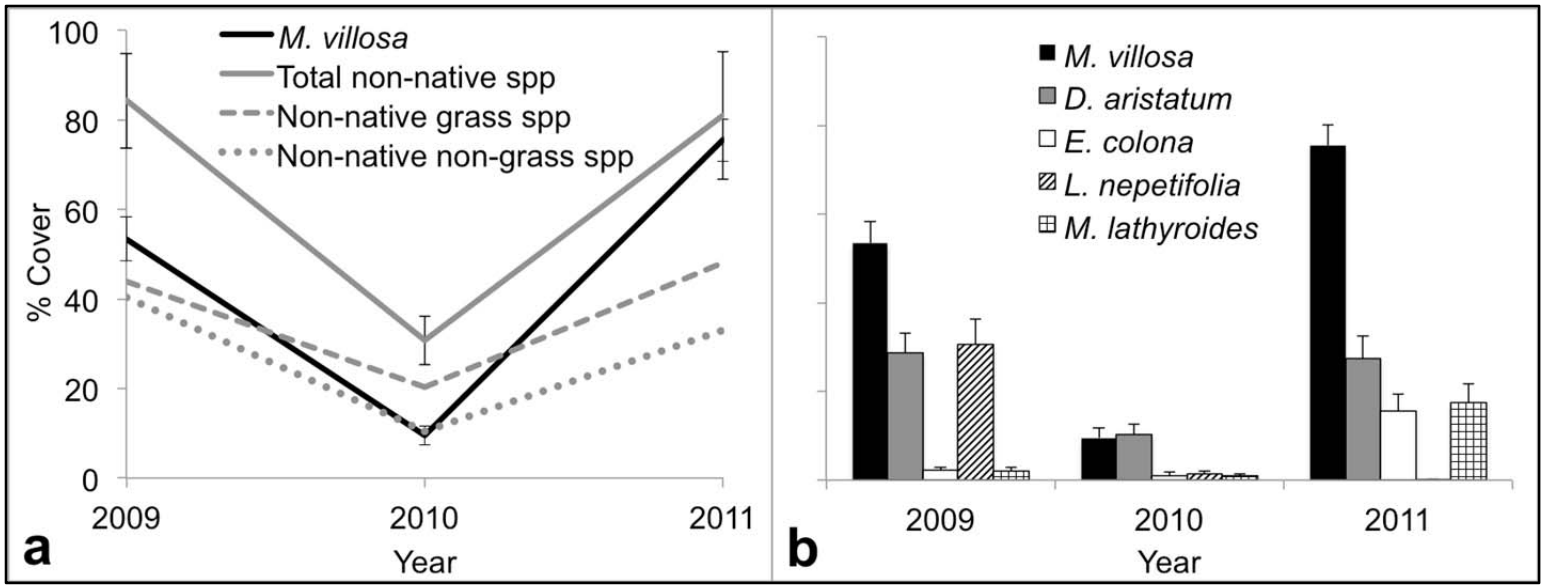

Figure 3.1 Percent cover of *M. villosa* and a) functional groups and b) the four associated species with the highest average percent cover, including two grass species (filled gray or white) and two non-grass species (filled with patterns), over the three years of this study. Data are means ± SEs, N=60 within each year.

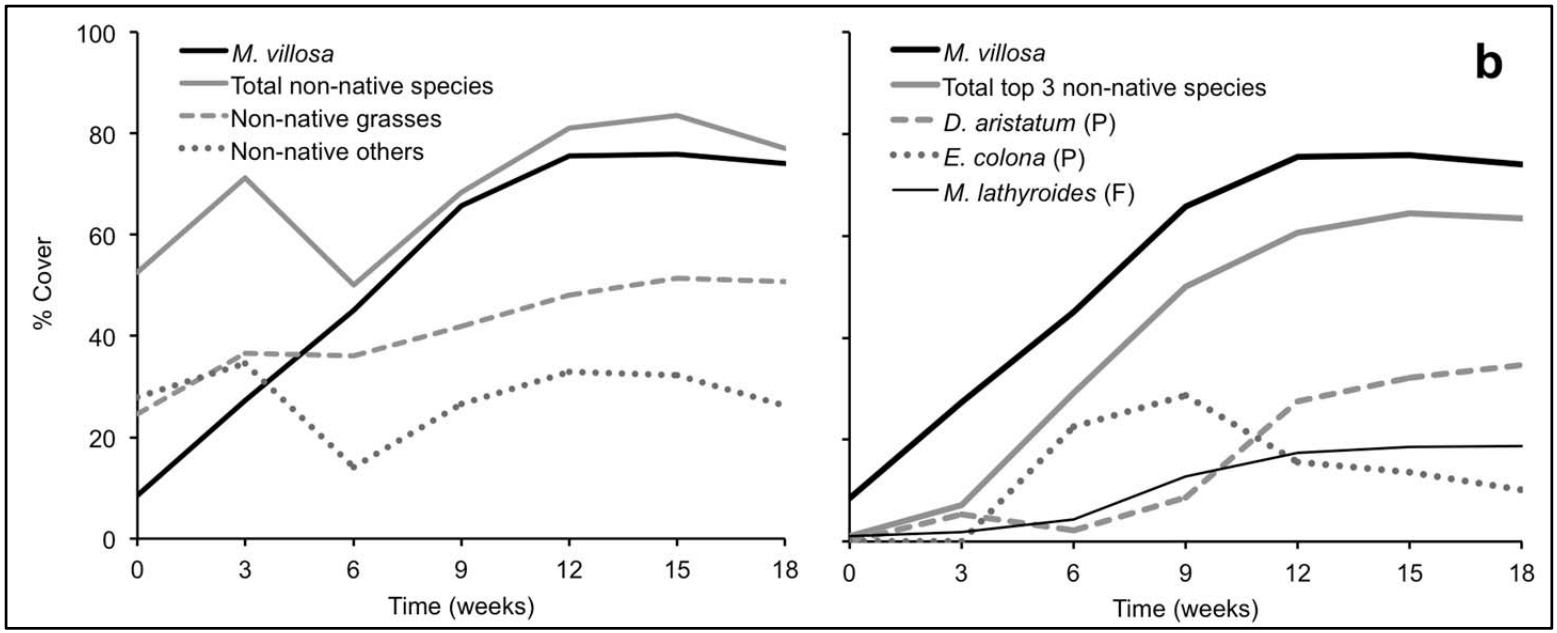

Fig. 3.2 Average percent cover of *M. villosa* and (a) non-native species by functional groups and (b) the three non-native species with highest average percent cover, over time in the 2011 season. P = Poaceae; F = Fabaceae.

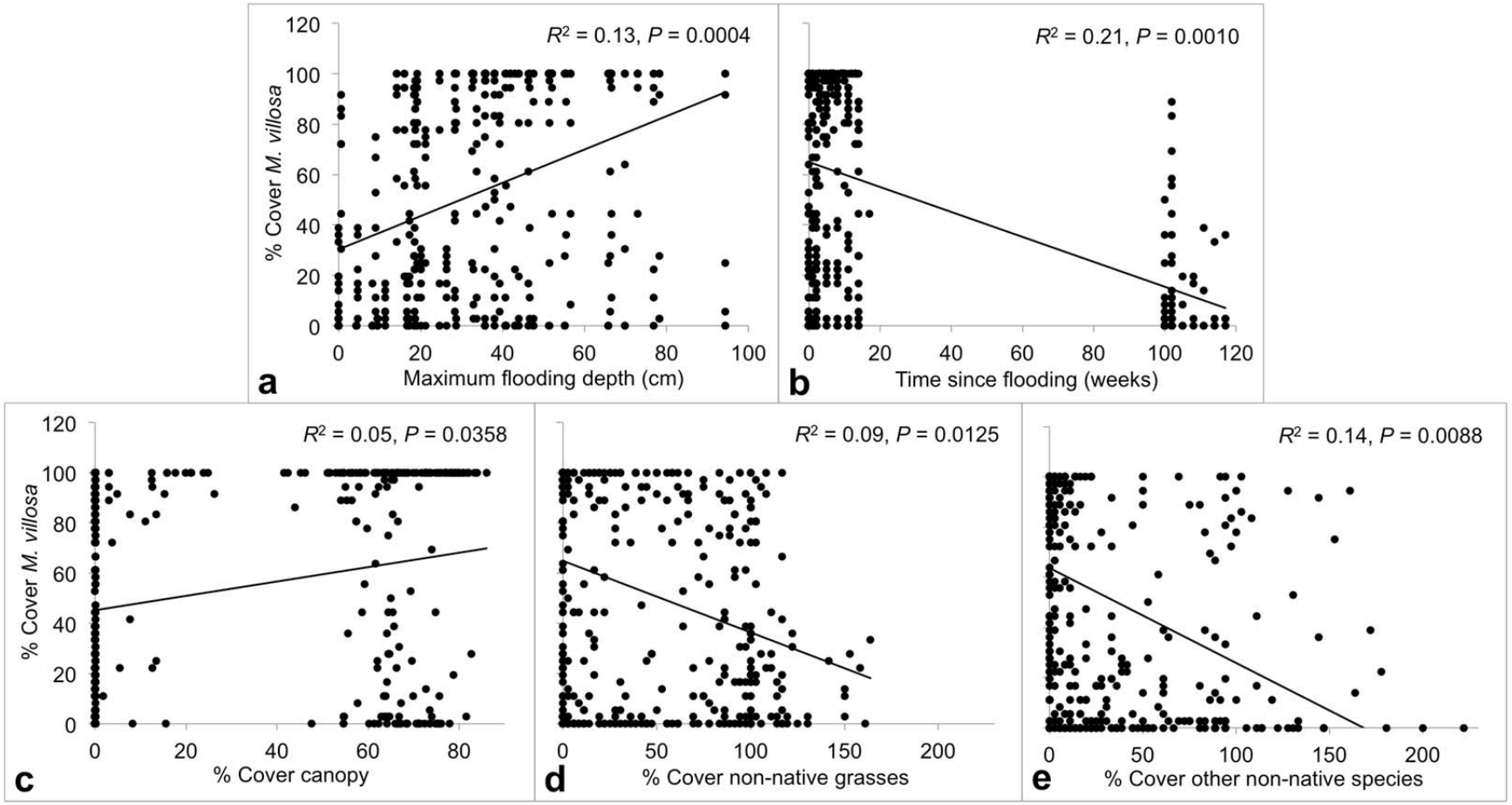

Figure 3.3 Linear regressions of single-factor effects of a) maximum flooding depth, b) time since flooding, c) percent cover of canopy, d) percent cover of non-native grasses, and e) percent cover of other non-native species, on percent cover of *M. villosa* in the 2011 season.

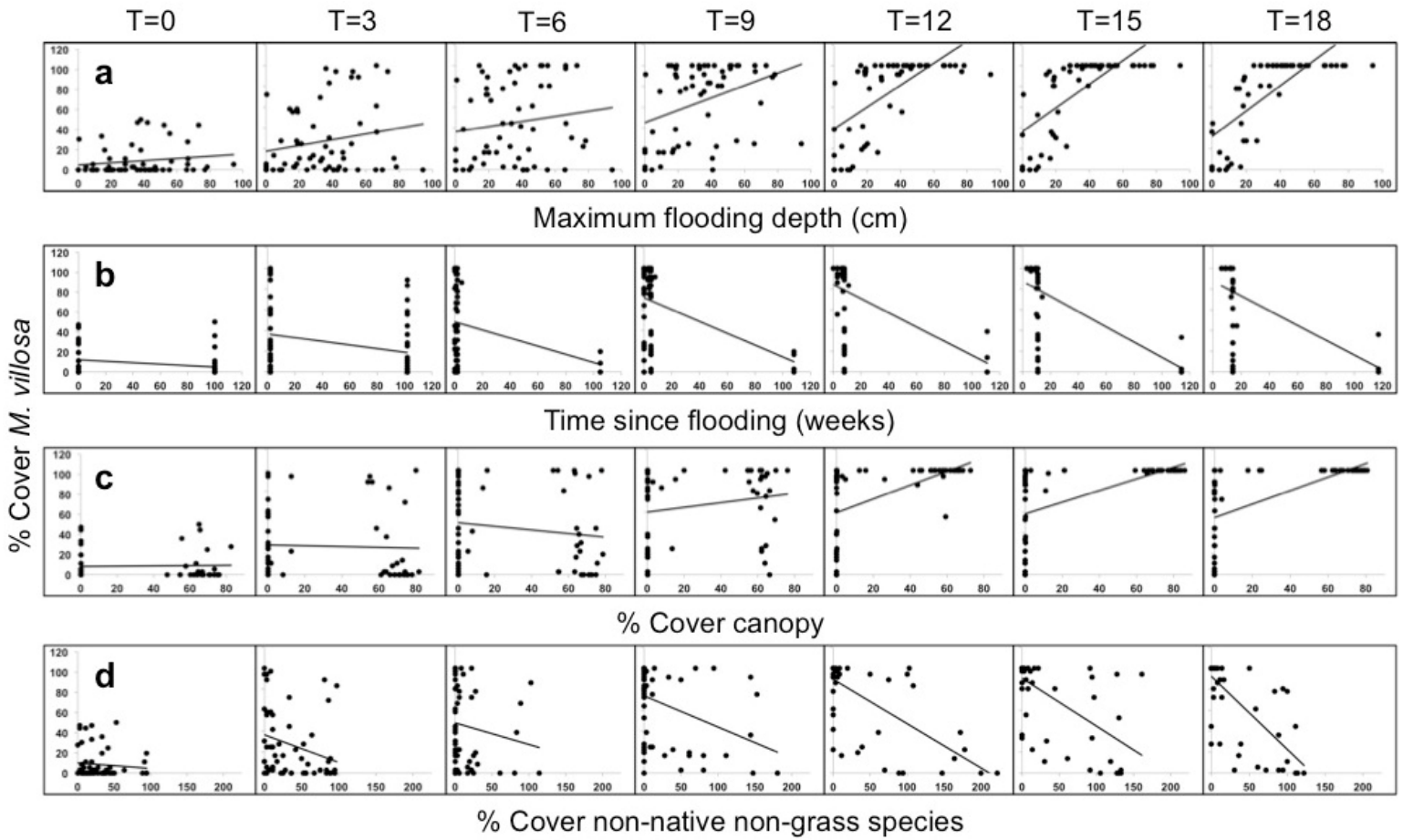

Figure 3.4 Interaction effects of time by maximum flooding depth, time since flooding, percent cover canopy (of *Prosopis pallida*), and percent cover non-native non-grass species on percent cover of *M. villosa* in the 2011 season. T = time, in weeks.

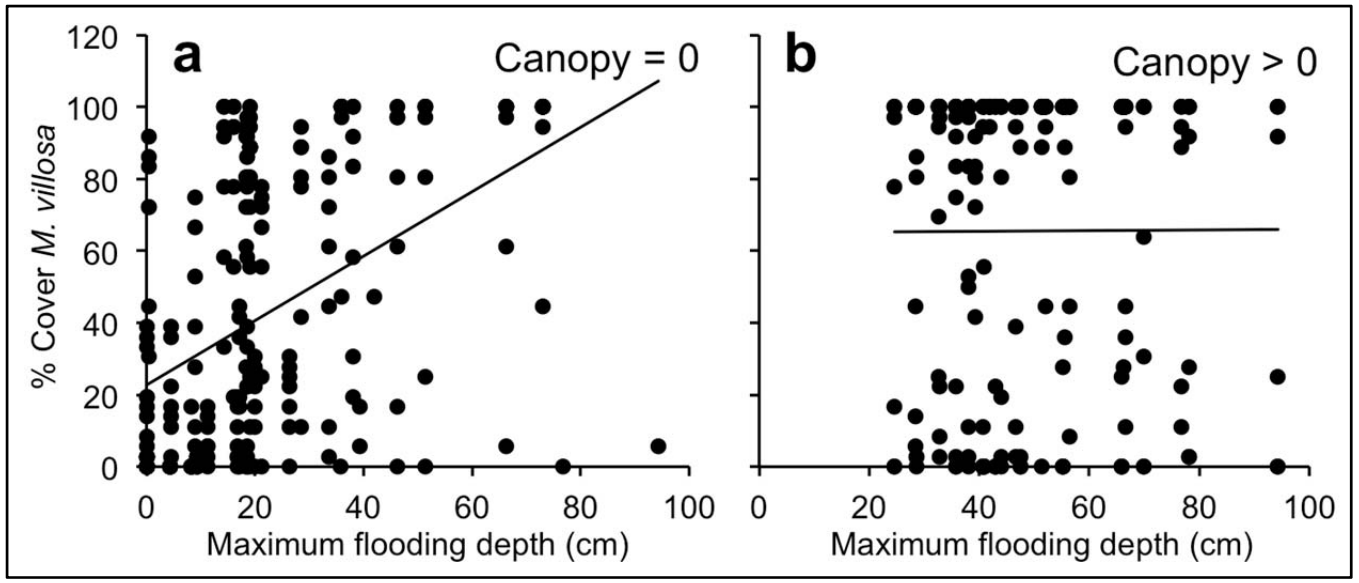

Figure 3.5 Effect of maximum flooding depth on percent cover of *M. villosa* when a) percent cover of canopy = 0, and b) percent cover of canopy > 0, during the 2011 season.

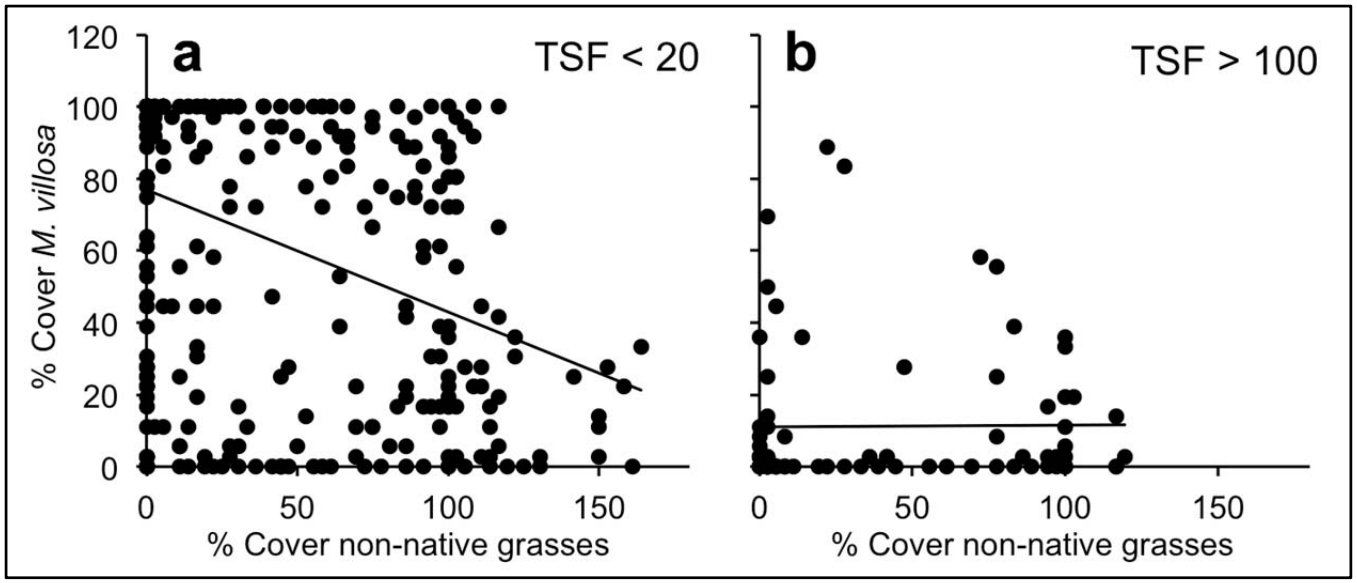

Figure 3.6 Effect of percent cover of non-native grass species on percent cover of *M. villosa* when a) time since flooding < 20 weeks, and b) time since flooding > 100, during the 2011 season. TSF = time since flooding.

CHAPTER 4

BOTTLENECKS AND FOUNDER EFFECTS IN THE ENDANGERED HAWAIIAN FERN ʻIHIʻIHI (*MARSILEA VILLOSA)*

Abstract

Reintroduction of plants or propagules to suitable areas within a natural historical range can be an effective strategy for endangered species restoration. Understanding the genetic structure of plant populations is critical to ensure that sampling for reintroduction captures sufficient genetic diversity to allow for environmental adaptation in new populations. *Marsilea villosa* (ʻihiʻihi) is an endangered, endemic Hawaiian fern with seven remaining populations in ephemerally flooding drylands on the islands of Oʻahu and Molokaʻi. Among its uncommon traits are long-lived sporocarps (i.e., highly modified leaves containing sporangia and spores), a requirement of flood and drought to complete its sexual life cycle, and extensive vegetative growth. I used RAPD markers to assess population genetic structure across the full range of *M. villosa*. Using a Bayesian modeling approach, I inferred three optimal clusters. Cluster assignment was mixed in most Oʻahu populations, while Molokaʻi populations and one small recently outplanted population on Oʻahu (Hanauma Bay) fell distinctly within clusters. Within Molokaʻi, populations from the northwest region were distinct from a population in the southwest region. The Hanauma Bay population distinction is likely due to a genetic bottleneck since it is known to have been recently established from few individuals. Strong population structure within Molokaʻi is also consistent with founder effects. A higher degree of genetic variation on Oʻahu and the distinction between regions within Molokaʻi suggest two separate colonizations from Oʻahu to Molokaʻi. Restoration efforts that include *M. villosa* reintroduction should take into account the results of this study and consider that best sampling practices for outplanting may differ between islands.

Introduction

Translocation of rare or endangered plants is increasingly being used as a management strategy for species and ecosystem restoration, and these efforts will become increasingly important as we continue to see increases in human population, development pressures, and global climate change. Before translocating endangered plants, it is ideal to know the genetic makeup of source populations to ensure that enough variation is present to allow for adaptability in new populations through natural selection (Lesica et al., 1999), particularly with clonally growing plants (Fant et al., 2008), and this often means sampling from multiple source populations (Godefroid et al., 2011). The capacity for evolutionary adaptation is critical in light of global climate and environmental changes, and the presence of genetic variation may facilitate many species' adaptation to climate change, even over relatively short timeframes (Weeks et al., 2011). In what would be considered a genetically successful restoration, an introduced population would maintain levels of genetic diversity similar to that of wild populations (Menges, 2008; Ramp et al., 2006). In order to accomplish this, sufficient numbers of individuals must be sampled from one or more source populations and newly introduced populations must expand sufficiently within a few generations (Weeks et al., 2011). However, practitioners must find a balance between maximizing genetic variation in introduced populations and minimizing the risk of outbreeding depression that leads to decreased local adaptation (Vergeer et al., 2004). The prevailing attitude among restoration practitioners and ecologists has been a 'better safe than sorry' approach of favoring local populations for source transplant material, but some argue that these risks are overstated and have unduly restricted the use of translocation as a much needed restoration strategy (Weeks et al., 2011).

Although molecular markers used in population genetic studies represent neutral variation that does not necessarily correspond to adaptive variation, they can be directly applicable to restoration in detecting genetic risk factors such as strong founder effects (Hufford and Mazer, 2003). If translocation source populations are already the result of recent genetic bottlenecks, introduced populations based on a limited number of transplanted individuals may exhibit severe founder effects (Menges, 2008).

Here I used random amplified polymorphic DNA (RAPD) markers to assess the genetic variation within and among populations of the endangered, endemic Hawaiian fern *Marsilea*

villosa Kaulf. with the goal of informing conservation managers on the best practices for translocation and restoration efforts.

Marsilea villosa has only seven surviving populations on the islands of O‘ahu and Moloka‘i (Bruegmann, 1996; W. Garnett, Rare Plant Species Recovery, Moloka‘i, HI, personal communication; personal observations). Species of *Marsilea* are unusual among ferns in being heterosporous, producing sporocarps (i.e., highly modified leaves with a thick drought-resistant walls that contain sporangia and spores), and requiring flood and drought to complete their sexual life cycle (e.g., Palmer, 2003). *Marsilea villosa* produces photosynthetic leaves when rain is abundant enough to keep soil moist and produces sporocarps towards the end of the rainy season. Standing water (the following year or later) is required for sporocarps to germinate, initiating sexual reproduction. During the dry season, the leaves die and rhizomes are dormant. Sporocarps may be viable for up to a century, as found in the closely related species *Marsilea oligospora* Goodd. (Johnson, 1985). When conditions are wet enough for leaf production but not for sexual reproduction, which may last several years, *M. villosa* grows vegetatively by spreading rhizomes. Production of long-lived sporocarps and vegetative growth likely contribute to the ability of *M. villosa* to recover from stressful conditions, such as a drought of a year or more, as long as flooding occurs in subsequent rainy seasons (personal observations). This combination of characters makes *M. villosa* an ideal candidate for restoration efforts; however, nothing is known about the population genetics of this species.

Population genetics were studied for *Marsilea strigosa* Willd., an endangered species from the Mediterranean basin, and it was found to have high differentiation among populations, suggesting highly restricted gene flow and reproduction predominantly through selfing (Vitalis et al., 2002). Similar patterns of differentiation were found in the endangered vernal pool species *Limnanthes floccosa* ssp. *californica* Arroyo (Sloop et al., 2011), suggesting that higher differentiation among populations may be typical of plant species found in ephemeral pool habitats. However, Ramp Neale et al. (2008) found that the majority of genetic variation was distributed within populations of the vernal pool endemic *Lasthenia conjugens* E. Greene. Thus, even when habitat specificity may suggest certain genetic patterns, it is important to examine genetic structure of any species targeted for restoration, particularly endangered species.

The extant populations of *M. villosa* include locations in a variety of habitats and represent historical and outplanted populations, providing the potential for variation among

populations (Table 4.1). There are four populations on O‘ahu. Lualualei Naval Base is a historical population on the western side of the island and contains at least five distinct subpopulations, which range in size and in habitat from open fields to kiawe forest (*Prosopis pallida* [Humb. & Bonpl. ex Willd.] Kunth). Koko Head is a natural population on the eastern side of the island, and it served as the propagule source for two outplanted populations in the same region. Makapu‘u was outplanted in the 1960’s, and Hanauma Bay was outplanted in 2002 (Alan Hong, personal communication). There are three populations on Moloka‘i, including Kamaka‘ipo on the southwestern tip of the island, and Ka‘a and Kaeo in the northwestern region. The latter two each have several subpopulations. Given that water is the primary dispersal agent for spores (Palmer, 2002), populations and some subpopulations are assumed to be relatively isolated; however, movement of resistant sporocarps via wetland bird species (internally or externally) is presumed to be the mode of long-distance dispersal to Hawai‘i for the ancestor of *M. villosa* (Carlquist, 1976) and of long-distance dispersal among most *Marsilea* species in general (Johnson, 1986). Malone and Proctor (1965) demonstrated that intact sporocarps of *Marsilea vestita* Hook. & Grev., the probable sister species to *M. villosa* (Nagalingum et al., 2007), could pass through digestive tracts of various wetland bird species including mallard ducks (*Anas platyrhynchos* L.). On the naval base at Lualualei, a managed pond serves as a wildlife refuge for endangered Hawaiian water birds, including Hawaiian moorhen (*Gallinula chloropus sandvicensis* Streets), Hawaiian coot (*Fulica alai* Peale), and Hawaiian stilt (*Himantopus mexicanus knudseni* Stejneger), as well as the common mallard-Hawaiian duck hybrid (*A. platyrhynchos* x *A. wyvilliana* P. L. Sclater). Of these, Hawaiian stilts had previously been observed outside of the managed pond in other flooded areas, but not specifically within the *M. villosa* subpopulations (Hawaii Natural Heritage Program, 2004). In Jan and Feb 2011, within two separate *M. villosa* subpopulations (Table 4.1), I observed the mallard-Hawaiian duck hybrid whose range includes all of O‘ahu, but not Moloka‘i (Fowler et al., 2009). Thus, there is at least the potential for gene flow via bird dispersal, though I speculate that migration may be limited increasingly with distance. Other studies have found relatively high percentages of genetic differentiation among islands in endangered plant species, such as *Anagyris latifolia* Brouss. ex Willd. (Fabaceae) in the Canary Islands (Gonzalez-Perez et al., 2009), and in clonally growing species such as *Zostera muelleri* Irmisch ex Asch. (Zosteraceae) in New Zealand (Jones et al., 2008). I therefore hypothesized that there would be structure to the genetic variation of *M.*

villosa populations such that the partitioning of genetic variation would be higher among populations than within populations, and higher between the two islands than within populations. Here I present the results of the first study of genetic variation within and among populations of *M. villosa* and the implications of these results for restoration management.

Materials and Methods

Before any samples were collected, I obtained a Permit for Threatened and Endangered Species (P-121) from the Department of Land and Natural Resources, Division of Forestry and Wildlife, and was added as an authorized individual on the Pacific Naval Facilities Federal Fish and Wildlife Permit (TE096741). Leaf tissue samples were collected from 3-33 individuals at each of four populations on O‘ahu (including five subpopulations at Lualualei Naval Base) and three populations on Moloka‘i (Table 4.1). Collected leaves were immediately placed in sealed plastic bags with silica gel. Total DNA was extracted from 0.01-0.05 g of silica-dried leaves using the CTAB method of Doyle and Doyle (1987) modified by adding 3% PVP-40 and 5mM ascorbic acid.

DNA samples of one individual from each of five populations (representing both islands) were screened with each of 30 primers (kits OPA-OPB; Operon Technologies; Alameda, California, USA), of which seven were selected for analysis of all individuals. Approximately 50 ng of DNA was amplified in 15 µl polymerase chain reactions (PCR) consisting of 0.2 µM random 10-mer primer, 0.2 mM dNTP, lx *Taq* polymerase PCR buffer, 1.5 mM $MgCl_2$, 0.5% bovine serum albumin, and 1 unit *Taq* Polymerase (Promega; Madison, Wisconsin, USA). Amplifications were performed in a Mastercycler Gradient Thermocycler (Eppendorf North America; New York, New York, USA) under the following conditions: 94 ºC for 2 min, followed by 45 cycles of 94 ºC for 45 s, 35 ºC for 45 s, 72 ºC for 2 min with 0.5 ºC/s ramp rate, and a final incubation at 72 ºC for 4 min. Amplification products were electrophoresed on 1.9% agarose gels in 1x TBE (tris-borate-EDTA) buffer, for 2-4 hours at 85 mA. Gels were stained with ethidium bromide and photographed under ultraviolet light using a GelDoc-It TS Imaging System (UVP, LLC; Upland, California, USA). Putative loci were identified based on the size of bands relative to 100 bp and 1 kb ladders (Promega; Madison, Wisconsin, USA). Only markers that were well amplified and reproducible in replicate assays were scored. Since my aim was to

quantify variation among populations, only variable loci were included in the analysis, scored as present or absent.

To investigate genetic structure within and among populations, data were analyzed using the program Structure 2.3.2, which uses a Bayesian model-based clustering method to infer population structure (Pritchard et al., 2000). Using a Markov Chain Monte Carlo (MCMC) algorithm, individuals are assigned probabilistically to K populations based on allele frequencies at each locus. The number of genetic clusters (K) is inferred from the posterior probability distribution Pr(K|X), calculated from the posterior probability of the data Pr(X|K), with X representing the genotypes of individuals sampled. Independent models were run for values of K from 1 to 14. I assumed an ancestry model of no admixture and an allele frequency model of correlation among populations. An initial burn-in period of 30,000 MCMC iterations was followed by a run of 10^6 iterations for data analysis. I ran three replicates for each value of K to ensure consistency of results. To estimate the true number of clusters present in the data, I calculated ΔK based on the rate of change in the log probability of the data between successive K values (Table 4.3; Evanno et al., 2005). I used the sister program Distruct 1.1 to graphically display the results from Structure (Rosenberg, 2004). I also conducted principle coordinate analysis (PCoA) using the Gower general similarity coefficient in MVSP 3.1 (Multi-Variate Statistical Package; Kovach
Computing Services, 1987-2009). These results are presented at the population level, because it is difficult to interpret a large number of subpopulations visually in PCoA.

I examined hierarchical genetic variation using different geographical groupings as models with analysis of molecular variance (AMOVA; Excoffier et al., 1992) as implemented in Arlequin 3.5.1.3 (Excoffier et al., 2005). I tested differentiation using the seven populations as the highest hierarchical level, with subpopulations nested within some populations, and I tested island-level partitioning between O‘ahu and Moloka‘i, with populations nested within islands (Table 4.1, 4.3). Explanatory power of each hierarchical level was tested for significance at $P < 0.05$.

Results

Seven RAPD primers were examined for all sampled individuals, and from these 106 variable loci were scored. There was a range of 10-28 variable loci per primer with an average of

18 loci. Out of 212 possible alleles (i.e., presence or absence at each locus), there were a number of rare alleles (Table 4.2). Seven of these (3%) were unique to a single individual, while 26 (12%) were present in less than 5% of individuals sampled. There were four populations with unique alleles, and while three of these had only one unique allele, Lualualei had eight. There were also 14 alleles unique to two populations, and all except one were shared between Lualualei and one other population. Of these 13 alleles, six were shared with another O‘ahu population and seven were shared with a Moloka‘i population. Between islands, there were greater than eight times as many alleles unique to O'ahu (25 alleles; 12%) than to Moloka'i (3 alleles; 1%).

Replicate models run in Structure for values of K from 1 to 14 produced consistent results, with the highest log probability of the data estimated with K=4 (Table 4.3). However, using the method of Evanno et al. (2005), I found that the modal value of ΔK showed a spike occurring at K=3, which is indicative of the true number of clusters present in the data (Fig. 4.1). In this model, the five subpopulations within the Ka‘a and Kaeo populations of northwestern Moloka‘i showed 100% membership in inferred cluster 1, as did the LUA4 subpopulation of Lualualei (west O'ahu). Kamaka‘ipo (southwest Moloka‘i) and Hanauma Bay (east O‘ahu) showed 100% membership in inferred cluster 3. Koko Head and Makapu‘u (east O‘ahu), and the remaining subpopulations of Lualualei had membership in at least two of the inferred clusters, with not more than 80% membership in any single cluster, but inferred cluster 2 was present only among O‘ahu populations.

Results from the Principle Coordinates Analysis (PCoA) reinforced these patterns of genetic structure in *M. villosa*. The two axes in Fig. 4.2 together accounted for 27.3% of the total variation. The first PCoA axis separates the southwestern Moloka‘i population (Kamaka‘ipo) from the northwestern Moloka‘i populations of Ka‘a and Kaeo, which cluster together. The second PCoA axis separates Hanauma Bay from Koko Head and Makapu‘u, despite Koko Head being the source population for both Makapu‘u and Hanauma Bay (all in the eastern O‘ahu region). Kamaka‘ipo and Hanauma Bay also cluster together, while Lualualei (western O‘ahu) is found along both axes and contains individuals with genetic similarity to each of the other populations, reflecting the results of the Structure analysis.

I used analysis of molecular variation (AMOVA) to test the significance of two different hierarchical structures and their power to explain genetic variance in *M. villosa*. My two models tested for variation 1) among geographically isolated populations, among subpopulations nested

within populations, and within subpopulations and 2) between the two islands, among populations nested within islands, and within populations (Table 4.4). In both models, all three levels of variation showed strong statistical support for their explanatory power. Both models also showed the large majority (75-77%) of genetic variation distributed at the most local level, and double the amount of diversity at the intermediate level (16-18%) compared to the highest hierarchical level (7%).

Discussion

I found evidence of genetic structure among populations of *M. villosa*, supporting my hypothesis of greater structural differentiation between islands than within islands. The presence of only two of the three genetic clusters on Molokaʻi indicates that less variation is present there than on Oʻahu, and suggests that Oʻahu was likely the source for colonization to Molokaʻi. Oʻahu also shows more mixing of clusters within populations, with the exception of Hanauma Bay, which is also distinct from other eastern Oʻahu populations in PCoA. Since it is known that Hanauma Bay was outplanted only ten years ago from a limited number of individuals, it is not surprising that the population shows membership only in one cluster, indicating a founder effect.

The fact that all subpopulations within northwestern Molokaʻi fall within one cluster, either by Structure analysis or PCoA, suggests that the region also experienced a genetic bottleneck, likely due to founder effect. A similar bottleneck apparently occurred with the southwestern Molokaʻi population. However, since Kamakaʻipo shows 100% membership in a different cluster than northwestern Molokaʻi in Structure and is genetically distant from northwestern Molokaʻi in PCoA, it is likely that the two regions represent two separate colonization events from Oʻahu to Molokaʻi. Since Oʻahu is the older of the two islands, this pattern is consistent with the "progression rule," in which dispersal and colonization often occur in a west-to-east direction (Wagner & Funk, 1995). This "conveyor belt" effect also increases the likelihood of divergence between source and colonial populations on different islands (Fleischer et al., 1998).

Lualualei subpopulation LUA4 also falls within a single genetic cluster, but the mechanisms behind this distinction are less clear. The subpopulation was only recently discovered in Jan 2011, and despite its large size and relative proximity to the LUA3 subpopulation and the naval base access road (within 100 m of each), it is unknown whether this

subpopulation is actually new or simply had not been observed before. It is deeper into kiawe forest relative to the access road and surrounded by tall guinea grass (*Megathyrsus maximus* [Jacq.] B. K. Simon & S. W. L. Jacobs), and may have been overlooked, particularly since surveys of the base do not happen on a regular basis and their timing may not always coincide with optimal years for *M. villosa* growth. For many species, the sheer size of the subpopulation would preclude the possibility of it being newly established, but as *M. villosa* has a high rate of vegetative growth (Bruegmann, 1986), I cannot rule out that possibility. Given the variation present among the other O‘ahu populations and subpopulations (excluding Hanauma Bay), these results provide some support for the hypothesis that LUA4 was established recently, and if so, its genetic structure is likely another example of founder effect. If I reject that hypothesis, the proximity of LUA4 to LUA3 suggests at least the possibility of gene flow via water or bird dispersal, making such a distinctive genetic structure seem unlikely. A final possibility is that these results may be due to sampling effects, in which case increasing the sample size may reveal more variation.

I did not find support for my hypotheses of greater variation among than within populations, or a higher degree of distinction between islands in the AMOVAs; rather the percentage of variation was increasingly higher with each lower level in the hierarchy. My results were in contrast to studies that found greater partitioning of variation among populations in other ephemeral pool plants, such as *M. strigosa* (Vitalis et al., 2002) and *Limnanthes floccosa* ssp. *californica* Arroyo. (Limnantheaceae; Sloop et al., 2011). However, the vernal pool endemic *Lasthenia conjugens* E. Greene (Asteraceae) had 82-84% of variation within populations (Ramp-Neale et al., 2008), which is similar to the pattern observed in *M. villosa*. Additionally, ~75% of variation at the most local level in *M. villosa* is comparable to average within-population variation of seed plants that are long-lived perennial (75%), endemic (74%), outcrossing (73%), water dispersed (75%), or ingestion dispersed (73%), based on 160 studies using RAPD analysis (Nybom, 2004). This degree of variation within subpopulations is likely explained by relatively small geographical distances between subpopulations (< 2 km), where dispersal may occur more often than between populations. The maintenance of moderately high levels of variation within populations (as well as subpopulations) is likely the result of the interplay between rare to occasional dispersal, and extensive clonal growth. Although the percentage of variation between island populations (7.27%) was lower than I hypothesized, it is still relatively high compared to

that found in some other island-dwelling species such as *Digitalis minor* L. (Scrophulariaceae) in the Balearic Islands (1.57%; Sales et al., 2007), *Pinus canariensis* C. Sm. (Pinaceae) in the Canary Islands (0%; Gomez et al., 2003), and *Cladophoropsis membranacea* (Hofman Bang ex C. Agardh) Borgesen (Chlorophyta) in the Canary Islands (5.62%; Van der Strate et al., 2003), and also in species with island-like distributions such as the lakeshore dwelling *Ranunculus reptans* L. (Ranunculaceae) in Switzerland (5.89%; Fischer et al., 2000). The relatively high variation between island populations of *M. villosa* may reflect the decreased potential for dispersal between islands, particularly if the mallard-Hawaiian duck hybrid is the dispersal vector, since Molokaʻi is not within its range (Fowler et al., 2009). The lower level of variance than I expected between islands is also consistent with my hypotheses of founder effects that I inferred from the analysis of genetic structure, showing support for the hypothesis that Molokaʻi populations were colonized by migrants from Oʻahu populations.

Patterns of dispersal and colonization among the Hawaiian islands can also be inferred from the distribution of rare alleles (Ranker et al., 2000). The difference in frequency of island-specific alleles (12% on Oʻahu and 1% on Molokaʻi) lends further support of Oʻahu as the source for colonization and the occurrence of founder effects on Molokaʻi. Eight of the 11 alleles that were unique to one population were unique to Lualualei, which suggests that it may have been the ancestral population for all other extant populations. Of the alleles that were unique to just two populations, 1-3 alleles were shared between Lualualei and each of the other six populations, lending further support for Lualualei as the source for all subsequent colonizations. This hypothesis is also strengthened by the PCoA, in which Lualualei samples can be found clustering with each of the other populations or regional clusters.

Based on previous work on *M. villosa* conservation and restoration ecology (Chapter 2, 3), I have recommended reintroduction of *M. villosa* through outplanting and management of new populations as a restoration technique likely to increase the stability of this endangered species. Translocations can also serve as common-garden experiments that can be used to evaluate local adaptation (Menges, 2008), particularly if the genetic composition of source plants is accounted for. *Marsilea villosa* would be an excellent candidate for this type of *in situ* restoration experiment. I recommend at least two different practices for sampling of *M. villosa* plants, rhizomes, or sporocarps for reintroduction efforts. For translocations on Oʻahu, propagules should be sampled from all populations growing in similar environments in order to

capture as much genetic diversity as possible, as well as any rare alleles that may exist in one or few populations, such as those listed in Table 4.1. Since Lualualei is the largest population and also shows evidence of being the source for all other populations, Lualualei should be well sampled for reintroduction efforts on Oʻahu.

For translocations on Molokaʻi, I recommend sampling only from local populations, since sampling from Oʻahu may introduce genetic variation that is not adaptive to environmental conditions on Molokaʻi that differ from Oʻahu. For example, all Molokaʻi populations experience lower mean annual rainfall than those on Oʻahu (Giambelluca et al., 2011). I also suggest carefully taking into account the habitat characteristics at reintroduction sites on Molokaʻi. While Kaʻa may be considered a true seasonal wetland, Kamakaʻipo is even drier than northwestern Molokaʻi and is most likely a remnant of a larger population that has been influenced by development, and Kaeo is different from any other *M. villosa* population in that its natural drainage topography precludes standing floods in most areas. It would be an interesting experiment to sample from all Molokaʻi populations to produce a common garden experiment at a new location on the island. Further, since there is evidence that all Molokaʻi populations may have origins in Lualualei, it could be worth experimenting with translocations from Lualualei to Molokaʻi. The benefits of augmenting the genetic variation present on Molokaʻi may outweigh the risks of genetic mixing, particularly if reintroduction efforts are conscientiously monitored after outplanting (Weeks et al., 2011). However, if resources are limited to the most critical management efforts, I would recommend sampling from Molokaʻi populations or subpopulations that occur in environments most similar to the target area for restoration.

In conclusion, our insight into the genetic diversity of *Marsilea villosa* will directly inform conservation and restoration management in Hawai'i. The differences between *M. villosa* population genetics and those of *M. strigosa* (Vitalis et al., 2002) suggest that further study of any of the other ~60 *Marsilea* species will be important, especially for those that are rare or endangered. This study also contributes to a small body of literature on genetics of ephemeral pool plants, which tend to be endangered, vary considerably, and merit further research. Additionally, I offer hypotheses on biogeographical patterns for a species previously unstudied in the Hawaiian Islands.

TABLES

Table 4.1 Summary of populations and subpopulations sampled. Mean annual rainfall is taken from the closest weather station available in the Rainfall Atlas of Hawai‘i (Giambelluca et al., 2011).

Island	Island region	Population/Subpopulation	N	Approx. area (m^2)	Mean annual rainfall (mm)	Habitat characteristics and other notes
O‘ahu	west	Lualualei Naval Base				
		Lua1[a]	11	2500[b]	619.1	large depression/ditch on roadside
		Lua2	6	250	619.1	small depression on roadside
		Lua3[a]	6	350	576.5	small seasonal wetland in kiawe forest
		Lua4	5	20000[c]	576.5	large seasonal wetland in kiawe forest
		A441B	5	10	576.5	depression near radio transmission antenna
	east	Koko Head	6	100[d]	724.0	volcanic crater, source for outplantings
	east	Makapu‘u	5	< 200	692.8	abandoned jeep trail, outplanted 1960s
	east	Hanauma Bay	5	10	724.0	small depression in lawn, outplanted 2002
Moloka‘i	southwest	Kamaka‘ipo	3	20	360.0	small depression on roadside
	northwest	Ka‘a				large seasonal wetland in open area
		Kaa1	5	15000	513.1	main subpopulation
		Kaa2	1	1	513.1	small outlier
		Kaa3	1	1	513.1	small outlier
	northwest	Kaeo				rocky slopes along natural drainage area
		Kae1	5	200	513.1	main subpopulation
		Kae2	3	5	513.1	small subpopulation

[a]Subpopulations with observed mallard-Hawaiian duck hybrids. [b]Subpopulation expanded 200-300 m^2 in 2009, following major flooding event. [c]Subpopulation discovered in 2011 after major flooding event. [d]Population previously as large as ~5000m^2, currently in severe decline. Served as source population for outplantings prior to decline.

Table 4.2 Rare alleles among *M. villosa* populations. Alleles are defined as presence or absence of a RAPD marker; N = 106 loci. O = Oʻahu, M = Molokaʻi.

Type of rare allele	# Alleles	Frequency
Unique to 1 individual	7	0.033
Present in < 5% of individuals	26	0.123
Unique to 1 population	11	0.052
Lualualei (O)	8	0.038
Makapuʻu (O)	1	0.005
Hanauma Bay (O)	1	0.005
Kaʻa (M)	1	0.005
Unique to 2 populations	14	0.066
Lualualei (O) + Koko Head (O)	3	0.014
Lualualei (O) + Makapuʻu (O)	1	0.005
Lualualei (O) + Hanauma Bay (O)	2	0.009
Lualualei (O) + Kamakaʻipo (M)	3	0.014
Lualualei (O) + Kaʻa (M)	3	0.014
Lualualei (O) + Kaeo (M)	1	0.005
Koko Head (O) + Kaeo (M)	1	0.005
Unique to 1 island	28	0.132
Oʻahu	25	0.118
Molokaʻi	3	0.014

Table 4.3 Structure results and estimation of the true number of genetic clusters present in data (K) using ΔK, an ad hoc statistic based on the rate of change in the log probability of the data between successive K values. Rep = replicate model run.

	Estimated ln probability of data			
K	Rep 1	Rep 2	Rep 3	ΔK
1	-2600.8	-2600.6	-2601.7	
2	-2323.2	-2323.2	-2323.4	
3	-2228.0	-2228.3	-2228.3	1054.82
4	-2210.1	-2214.9	-2214.6	29.78
5	-2300.7	-2284.3	-2295.9	11.32
6	-2372.3	-2386.8	-2395.3	2.01
7	-2396.5	-2420.8	-2406.3	5.57
8	-2383.5	-2394.5	-2453.8	1.18
9	-2466.0	-2428.5	-2418.0	3.16
10	-2491.4	-2456.9	-2492.1	2.86
11	-2457.1	-2514.4	-2508.3	1.55
12	-2521.2	-2578.3	-2567.3	1.62
13	-2528.6	-2575.8	-2546.1	2.84
14	-2623.4	-2543.7	-2633.8	1.53

Table 4.4 Hierarchical analyses of molecular variance (AMOVA) of *M. villosa* at the population level and island level; see Table 1 for divisions within hierarchical levels. SS = sum of squares; VC = variance components.

Source of variation	SS	VC	Variation	*P*	Φ statistic
Model: Population level (7 groups)					
Among populations	145.51	0.85	7.23 %	0.0029	0.0724
Among subpopulations within populations	117.01	1.87	15.95 %	<0.0001	0.1720
Within subpopulations	444.86	9.00	76.81 %	<0.0001	0.2319
Total	707.38	11.72			
Model: Island level (2 groups)					
Between islands	40.59	0.88	7.27 %	<0.0001	0.0727
Among populations within islands	221.94	2.17	18.03 %	<0.0001	0.1945
Within populations	444.86	9.00	74.69 %	<0.0001	0.2531
Total	707.39	12.05			

FIGURES

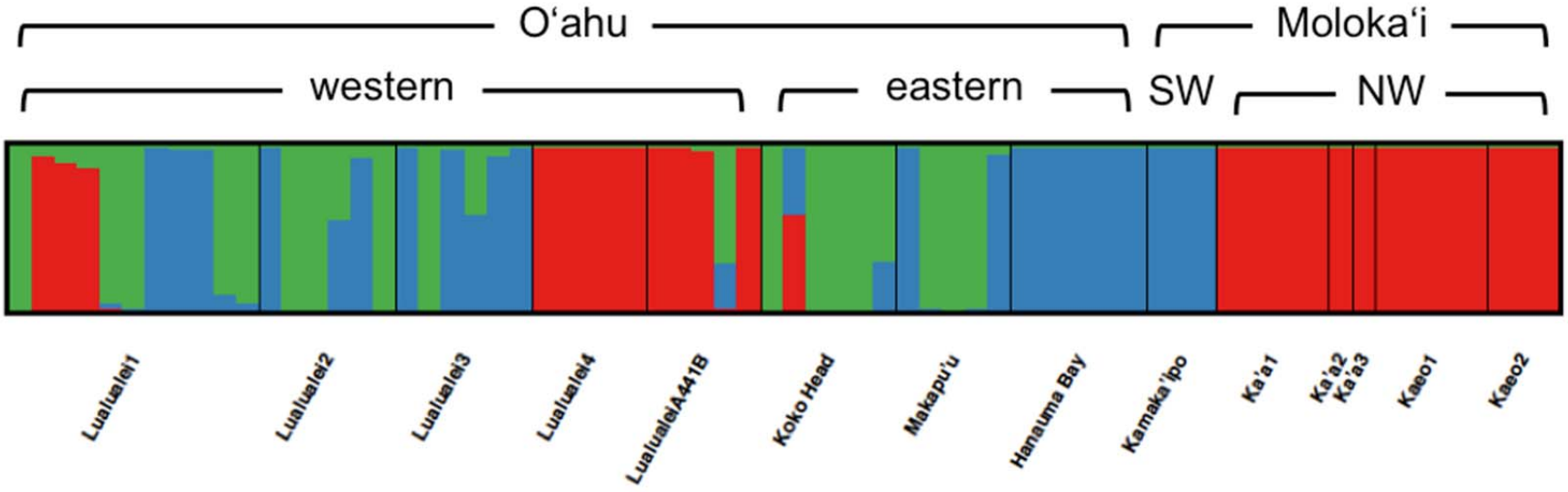

Figure 4.1 Estimated genetic structure of all *M. villosa* populations and subpopulations. Individual samples are represented by vertical bars, which are divided into colored segments that represent estimated membership fractions in each of K=3 clusters. Populations or subpopulations are separated by vertical black lines and are identified by labels below the graph. Top labels above the graph indicate islands, and labels below those indicate the region of the island in which populations or subpopulations are located.

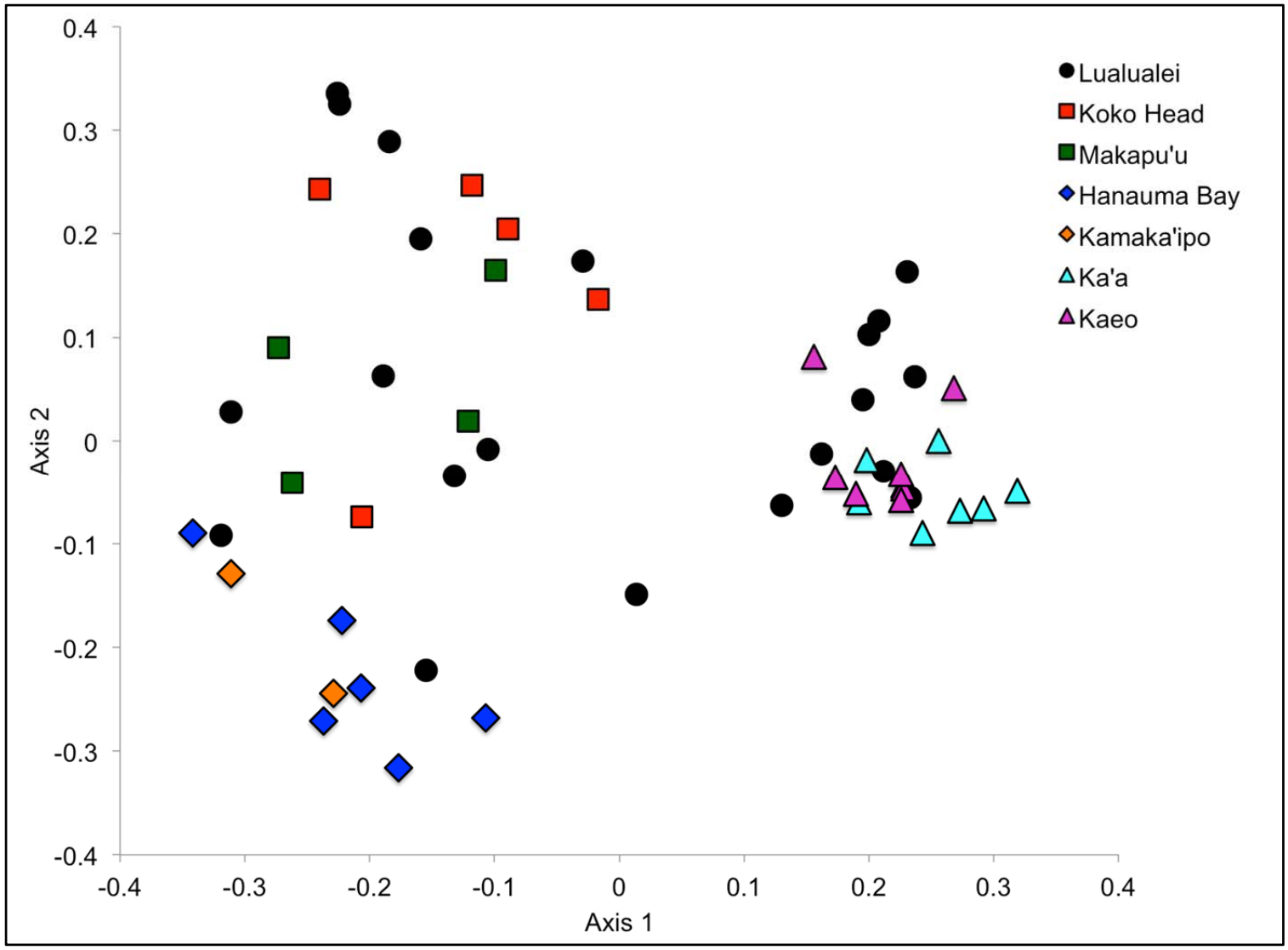

Figure 4.2 Principle coordinates analysis of RAPD data for *M. villosa*. PCoA axes 1 and 2 accounted for 27.3% of the total variation.

CHAPTER 5
CONCLUSIONS

The research presented in this dissertation provides explicit management recommendations that can be directly applied to conservation of *Marsilea villosa*, and it also contributes more broadly to our understanding of fern ecology, ephemeral pool systems, ecological interactions, and restoration of rare and endangered plant species. While some aspects of these findings confirmed my hypotheses, there were also some surprising results that will be important to the conservation of this species, which confirms the importance of conducting scientific research to inform management practices.

Summary of Findings

In the restoration experiment, I found that the combination of flooding and shade treatments promoted the greatest increase in *M. villosa* growth, and that the effects of this interaction grew stronger over time. The effects of shade were unexpected, since *M. villosa* grows more often in full sun, so these results expand the range of suitable habitats where *M. villosa* might be reintroduced. Although there were no effects of weeding over the majority of the experiment, after drought occurred, shade increased *M. villosa* growth in the absence of weeding. This effect was mirrored in the number of sporocarps produced, indicating that reproductive potential is tied to vegetative growth, and that management targeting growth of *M. villosa* should also maximize sexual reproduction.

The results of the field ecological study confirmed many of the findings of the experiment, including the importance of shade in increasing growth of *M. villosa*. This study also revealed some nuances of the flooding-shade interaction, including 1) that all plots with more than 40 cm flooding depth or any degree of canopy cover reached 100% cover of *M. villosa* by the end of the season, and 2) that in the presence of canopy cover, maximum flooding depth has no relationship with *M. villosa* cover, but in the absence of canopy cover, there is a positive relationship. Additionally, I found that non-native species had negative relationships with *M. villosa* cover, but that grass species behaved as a functional group while non-grass species effects were species-specific. Another interaction occurred where non-native grass cover had no relationship with *M. villosa* cover for plots that had flooded

within the current season, but had a negative relationship for plots that had not flooded in over two years. This research suggests that management priorities should lie with flooding and shade, which have positive synergistic effects, but that weed monitoring and targeted management may still be important.

In the population genetic study, I found that while the majority of genetic variation was found within subpopulations, there was also genetic structure that showed strong differentiation among some populations and between the two islands. Structure indicative of founder effects and strong differentiation between the two regions of Molokaʻi suggested that there were two separate colonization events from Oʻahu to Molokaʻi. The presence of rare alleles and greater genetic variation within the Lualualei population suggested that Lualualei might have been the source for all other natural populations of *M. villosa*. Based on these results, practitioners planning to reintroduce *M. villosa* should sample widely from as many populations as possible for outplanting on Oʻahu, but would be advised to sample from within Molokaʻi for outplanting on that island, especially if resources for controlled experiments and monitoring are limited.

Synthesis

One of the most exciting results of this research was that the findings of the relatively long-term field study (three years) corroborated those of the short-term experiment (less than six months), despite the unexpected result that shade can be just as important as flooding for promoting *Marsilea villosa* growth. It is gratifying to be able to recommend, with confidence, a management strategy previously not considered for this species. This also highlights the importance of conducting experiments prior to ecological management. Ideally, these experiments would be controlled, carefully designed, conducted *in situ*, and monitored over the long-term, as I recommended in Chapter 2 for potential ecosystem restoration with *M. villosa* in conjunction with other native dry forest species. However, this research shows that if resources are limited, a short-term and relatively low-cost experiment, also carefully designed, can be very informative to the both the basic biology and the practical management of an endangered species. This research also highlights the importance of studying ecological interactions, which are often overlooked, but which can explain much, if not most, of the variation in biological patterns and processes.

Just as we have recognized a need for research that increases understanding of basic ecology before engaging in ecological restoration (e.g., Drayton & Primack, 2012; Guerrant & Kaye, 2007; Hobbs & Cramer, 2008; Rout et al., 2007), there have been similar calls for knowledge of genetic variation to guide restoration through plant reintroduction (e.g., Fant et al., 2008; Fahselt, 2007; Godefroid et al., 2011; Weeks et al., 2011). This study begins to fill a gap in our knowledge of *M. villosa* genetics, and contributes to the literature on population genetics of ferns, ephemeral pool plants, and endangered species. The combination of ecological and genetic research in this dissertation provides a strong basis for a more comprehensive management plan and increases the likelihood of success in restoring *M. villosa*. Additionally, the results of the genetic study give evidence for new, testable hypotheses about the biogeography of this species, and contribute to the literature on evolution of the endemic Hawaiian flora.

This dissertation is also a contribution to small but growing bodies of literature on three separate understudied topics: fern ecology, ephemeral pool biology, and facilitation as an important component of ecological theory. The vast majority of fern species have not been evaluated for risk of extinction, yet the majority of those that have been evaluated are indeed at risk (Mehltreter, 2010). The Pteridophyte Specialist Group of the IUCN Species Survival Commission has recognized that increased study of fern ecology is essential to inform fern conservation (Jermy and Ranker, 2002). The results of this study would be especially informative to fern species with a similar biology, of which there are at least 75 worldwide (Marsileales; Hassler & Swale, 2004). Ephemeral pools are unique ecosystems with island-like distributions and high beta diversity, in which endemic and endangered species are often found, and they are useful experimental models for ecological study (De Meester et al, 2005). While the ecology of *M. villosa* is likely to be more similar to the endangered *M. strigosa* than the vernal pool endemic *Lasthenia conjugens*, its population genetics were much more similar to the latter than the former (Ramp et al., 2006; Vitalis et al., 2002). Building the literature on ephemeral pools will help to resolve these unexpected differences and lead to a better understanding of the processes at work in these ecosystems. Another ecological topic gaining recent ground is the role of facilitation in ecological theory, which has been largely ignored over the past several decades in favor of competition, environmental stress, and other challenges to survival (Bruno et al., 2003). Yet there are an increasing number of reports of

facilitation interactions in ecological studies, including those of shade effects, which have traditionally been considered for their negative impacts (Semchenko et al., 2012). My research contributes to the literature on positive interactions of shade and on facilitation as an important interaction that should be considered in ecological theory.

Finally, this dissertation is, by design, a contribution to the understanding of rare and endangered species biology, conservation, and restoration ecology. It underscores the need for basic biological research that informs management by seeking to answer questions that would best serve conservation practitioners. This research uses a synthetic approach, combining experimental and field based ecology with population genetics to provide a broader understanding of the biology of *Marsilea villosa* and a model upon which to base restoration of the more resilient endangered species in Hawai‘i and worldwide.

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