2019

INTRASPECIFIC VARIATION IN DEHYDRATION TOLERANCE: INSIGHTS FROM THE TROPICAL PLANT *MARCHANTIA INFLEXA*

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Digital Object Identifier: https://doi.org/10.13023/etd.2019.190

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INTRASPECIFIC VARIATION IN DEHYDRATION TOLERANCE: INSIGHTS FROM THE TROPICAL PLANT *MARCHANTIA INFLEXA*

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences at the University of Kentucky

By
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2019

ABSTRACT OF DISSERTATION

INTRASPECIFIC VARIATION IN DEHYDRATION TOLERANCE: INSIGHTS FROM THE TROPICAL PLANT *MARCHANTIA INFLEXA*

Plants are threatened by global change, increasing variability in weather patterns, and associated abiotic stress. Consequently, there is an urgent need to enhance our ability to predict plant community dynamics, shifts in species distributions, and physiological responses to environmental challenges. By building a fundamental understanding of plant stress tolerance, it may be possibly to protect the ecological services, economic industries, and communities that depend on plants. Dehydration tolerance (DhT) is an important mechanism of water stress tolerance with promising translational applications. Here, I take advantage natural variation in DhT to gain a deeper insight into this complex trait. In addition, I address questions related to the causes and consequences of sexual dimorphisms in DhT. Understanding sexual dimorphisms in stress tolerance is critical because these dimorphisms can drive spatial segregation of the sexes, biased sex ratios, and may ultimately reduce sexual reproduction and population persistence.

This work takes an integrated approach, addressing DhT on multiple scales from ecology, to physiology, to genomics in the tropical liverwort *Marchantia inflexa*. Initially, I tested for correlations between DhT and environmental dryness, sex differences in DhT, and genetic vs. plastic contributions to DhT variability. I found that patterns of variation in DhT are associated with environmental variability, including complex sexual dimorphisms, and derive from a combination of plasticity and genetic differences in DhT. Subsequently, I leveraged the variability in DhT to identify candidate DhT enhancing genes. In *M. inflexa* intraspecific differences in DhT are impacted by baseline variability among plants, as well as unique gene expression responses initiated during drying. In parallel, I assembled a draft genome assembly for *M. inflexa*, which was employed to investigate questions of sex chromosome evolution and sexual dimorphism in DhT. Finally, the bacteriome of *M. inflexa* was characterized and found to be extremely diverse and variable.

Collectively, this work adds to a growing understanding of DhT and highlights the importance of sampling approaches that seek to comprehensively describe variability

in DhT. I detected complex patterns of variability in DhT among populations and the sexes of *M. inflexa*, which were used to gain insight into the genetic intricacies of DhT.

KEYWORDS: intraspecific variation, dehydration tolerance, *Marchantia inflexa*, genomics, eco-physiology

Rose A. Marks

April 25th, 2019

INTRASPECIFIC VARIATION IN DEHYDRATION TOLERANCE: INSIGHTS FROM THE TROPICAL PLANT *MARCHANTIA INFLEXA*

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ACKNOWLEDGEMENTS

To say that it takes a village would be an understatement; it took my whole world. Although my path has been winding and sometimes circuitous, I have been headed towards this for a long time. For that reason, it is impossible to thank everyone who contributed to this collective work, but I must acknowledge the foundational sources of support and guidance.

First and foremost, I want to express my utmost gratitude for my advisor and mentor Dr. Nicholas McLetchie. I almost certainly would not have survived my PhD without his kind, unwavering, and often humorous support. Not only has Nicholas been a great source of scientific, academic, and professional wisdom, but he has taught me invaluable lessons about life and mangoes. As corny as it sounds, I really couldn't have asked for anything more. Next, I would like to thank the rest of my graduate committee - Drs. Jeramiah Smith, Carol Baskin, Arthur Hunt and outside examiner Dr. Michael Goodin - all of whom have been incredibly generous with their time, space, and expertise. The bulk of the molecular and bioinformatics work described here would not have been possible without the generosity of Drs. Jeramiah Smith and Arthur Hunt, both of whom opened up their labs to me, tolerated my constant interruptions and questions, and helped troubleshoot numerous problems. In fact, I spent so much time in the Smith lab, that it became something of a second home. I'd like to thank my colleagues and lab mates (particularly Jonathan Moore and Brittany Slabach) for the friendship, insight, and comradery. I'd like to thank the Biology Department more broadly for providing a supportive and inclusive environment to work in, and for the numerous conversations (both scientific and not) that motivated me to keep learning, try harder, and stay humble.

Finally, I'd like to acknowledge the talented undergraduate students that participated in this work and taught me invaluable lessons about mentorship, teaching, and friendship: Hellen Rogers, James Burton, Olivia Boughey, Brennan Pike, and Caylen Railey. Without the dedicated effort of these students it is very likely that I would still be collecting data.

I want to thank my family for everything; not only for their support throughout this process, but for raising me to value education and diversity, to question everything, and to trust myself. I'd like to thank both my patents for letting me have a childhood, make my own mistakes, and for the occasional reality check. I'd like to thank my mom for encouraging me to dream, my dad for teaching me not to be afraid to try, my sister and brother for letting me be different.

Next, I'd like to thank my tribe of friends. I am especially grateful for Jane Fineberg who seems to have an endless supply of wisdom and kindness; Tara Eliot with whom I have been pushing the boundaries since ~1993; Esther Sullivan who is often mistaken for (and feels like) another sister. I'd like to thank those who were brave enough to tell me the truth, even when I didn't want to hear it: Scott Hotaling for being a constant source of honesty, insight, and perspective; Cate Nakimeria for showing me what strength and resiliency look like; and Pierce Birdsong for encouraging me to stand up for myself. I'd like to thank Elo Ibekwe for reminding me that you can have it all. I want to thank my many climbing and travel partners (in particular Kyler Pallister, Katie Cook, Ben Yates, Sarah Rhomberg, Scott Graves, Michelle Ellington, Jeoffery Teague, Tiffany Chochran, and Bethany Fenton) without whom I would have graduated a lot sooner! The adventures, endless jokes, and catches that we shared provide the backdrop to this work.

I have benefited enormously from conversations and correspondences with other biologists, collogues, and collaborators at and beyond the University of Kentucky. In particular, I would like to thank Dr. Quentin Cronk for his involvement in the genome sequencing project, Dr. John Bowman for sharing his insight on bryophyte genomics, Drs. Jill Farrant, Mel Oliver, Robert VanBuren, Lloyd Stark, and John Moore for sharing their knowledge of desiccation tolerance with me, and Drs. Lisa Rew and Barb Keith for getting me started in undergraduate research ten years ago in 2009.

This work would not have been possible without critical access to sites and facilities provided by the University of Kentucky, College of Agriculture, Food and Environment (greenhouse space); the Wildlife Section, Forestry Division, Ministry of Agriculture Land and Marine Resources of Trinidad and Tobago (collection and export permits); the Water and Sewage Authority (access to field research sites), and Andrea and Darryl McLetchie (logistical support while in Trinidad). Finally, this work was made possible through the generous support and funding provided by the University of Kentucky Department of Biology Ribble Endowment (research funding and a graduate fellowship), the American Bryological and Lichenological Society (Anderson and Crum Bryology Research Award and travel funding), the American Botanical Society (a fellowship and travel funding), the Kentucky Science and Engineering Foundation (research and development grant that funded chapter 4), the Natural Sciences and Engineering Research Council of Canada Discovery Grants Program (genome sequencing), and the Karri Casner Environmental Sciences Fellowship (research funding).

It is impossible to fully express my gratitude for all those who made it possible for me to pursue this path. Thank you for supporting me. It mattered, it still matters, and I hope that one day we'll look back and know that we made a difference.

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CHAPTER ONE

INTRODUCTION

Background

Plant communities are threatened by changing land use practices, a growing human population, and environmental fluctuations due to climate change. Both natural and agricultural systems are challenged by decreasing arable land, increasing consumption, and abiotic stresses associated with climate change (Osakabe *et al.* 2014). Consequently, there is an urgent need to develop a theoretical framework to predict plant population dynamics, physiological adaptations, and molecular responses to environmental challenges. Drought in particular, is a major threat to plant productivity (Osakabe *et al.* 2014), and many climate change models predict an increasing frequency of drought across the globe in the coming years (Dai 2013). Importantly, drought stress may disproportionally impact communities that rely on subsistence food production systems with limited mechanization and low-tech irrigation systems (Mongi *et al.* 2010; Trenberth 2011). Therefore, we must not only develop strategies to mitigate drought induced losses but should focus on those that require minimal inputs and are economically sustainable. Further, these efforts should include building a fundamental understanding of naturally evolved stress tolerance mechanisms, the diversity of which represents a valuable resource that can be leveraged to improve plant stress tolerance.

Desiccation tolerance (DT, also desiccation tolerant) is a rare trait that enables tissues to survive extreme drying (down to or below an absolute water content of -100 MPa) (Bewley 1979). DT is highly adaptive for plants that occupy environments with limited, sporadic, and unpredictable water availability and provides a promising avenue

for research aimed at minimizing drought induced losses. The occurrence and distribution of DT plants has important implications for environmental management. Changes in weather patterns and water availability due to climate change are likely to lead to range shifts for both DT and desiccation sensitive (DS) species, changes in community composition, and potential biases in population sex ratios due to sex-specific DT with possible consequences for local productivity. Many stress tolerant plants are slow growing, relatively small, perennial, and have reduced palatability (Theory and Grime 1977). Despite the possible reduction in productivity of communities dominated by DT plants, a DT rich assemblage could increase ecological stability in areas with extreme, persistent, or frequent drought by increasing water holding capacity and minimizing soil erosion (Watkins *et al.* 2007). Characterization of the ecology of DT plants will offer insight into species range shifts, ecological productivity, and stability.

DT organisms persist in dry periods by inducing protective mechanisms that combat the mechanical, metabolic, and oxidative stresses of drying. Ultimately these mechanisms allows them to enter a state of quiescence in which nearly all metabolic activity ceases (Hoekstra *et al.* 2001; Moore *et al.* 2009; Dinakar and Bartels 2013b). Perhaps the most striking observation that can be drawn from research on DT is that the phenotype is very complex. DT depends on multiple physiological pathways, which are initiated in a sophisticated sequence of events. Briefly, to combat the mechanical stress of drying, small non-reducing sugars (Bianchi *et al.* 1993; Ingram and Bartels 1996) and space filling proteins (Illing *et al.* 2005; Liu *et al.* 2009) accumulate in DT cells via changes in metabolism and gene expression. These compounds help to stabilize sub-cellular structures and fill space to combat the mechanical stress caused by shrinkage. In

some species, increased cell wall flexibility can further reduce mechanical strain on drying cells (Jones and McQueen-Mason 2004; Moore, Farrant, *et al.* 2008; Holzinger and Karsten 2013). Oxidative stress is another major source of damage under dry conditions (Dinakar and Bartels 2013a). Normal metabolic processes are disrupted in water limited conditions and reactive metabolic intermediates can accumulate (Oliver *et al.* 2010). At low levels, these reactive oxygen species (ROS) can act as signaling molecules, but at higher levels they cause substantial damage to membranes, enzymes, and nucleic acids (Oliver *et al.* 2010). Consequently, most DT tissues deploy numerous antioxidants to combat damaging ROS (Dinakar and Bartels 2013a). Other subtle changes during drying in DT plants prevent protein aggregation, light induced damage, and regulate photosynthetic processes. Upon rehydration, DT plants mobilize recovery mechanisms of damage repair, turnover, and finally the resumption of normal metabolism (Dinakar *et al.* 2012).

DT organisms offer an exciting opportunity to broaden our understanding of water stress, with potential translational utility, but there are still major gaps in our understanding of DT that must be overcome in order to move towards applied solutions in agricultural and natural systems. I sought to address three specific knowledge gaps here. Initially, I aimed to characterize intraspecific variability in DT. Very few studies have focused on quantifying intraspecific variability in DT, yet substantial variability in DT may exist among populations and individuals. If such differences exist and are heritable this would an important source of genetic variability, which could be used to increase DT via breeding and bioengineering approaches. Further, understanding the patterns of variability in DT will provide critical insight into the adaptive potential of

plant populations and will enhance our ability to predict ecological consequences of climate change. The second knowledge gap that I aimed to fill was the limited understanding of less extreme cases of dehydration tolerance. DT plants can survive drying to < -100MPa, and dehydration tolerant (DhT) plants can survive drying to < -10MPa but not -100MPa (Marks *et al.* 2016). Few studies have focused on DhT plants but building a better understanding of DhT will provide insight into the differences among DS, DhT, and DT plants. My final aim was to characterize sex differences in DhT. Due to their sessile nature, dioecious plants may face challenges related to mate availability (Bierzychudek and Eckhart 1988). Specifically, differences in male and female reproductive biology can lead to the development of secondary sexual dimorphisms, which may reduce co-occurrence or reproductive synchronization of the sexes. Secondary sexual dimorphisms in abiotic and biotic stress tolerance may lead to local scarcity of a sex, biased sex ratios, or spatial segregation of the sexes (Juvany and Munné-Bosch 2015), all of which can reduce sexual reproduction. The sex-specific ecology of DhT (and DT) plants is still poorly understood, but has important implications for population sex ratios, reproduction, and population persistence. Consequently, I aimed to elucidate the mechanisms and consequences of sex differences in DhT.

In general, bryophytes (mosses, liverworts, and hornworts) provide informative systems with which to investigate DT. Because bryophytes lack vascular tissue and other complex water management systems, they are prone to drying. Unlike tracheophytes, which have stomata and sophisticated water management systems, most bryophytes have thin tissues with limited structural features for water retention, which causes them to

rapidly equilibrate to the relative humidity (RH) of the surrounding air (Proctor *et al.* 2007). Consequently, a high proportion of bryophytes exhibit DT.

To characterize intraspecific variation in DhT, I conducted multiple studies, integrating field ecology, eco-physiology, and genomic approaches (Figure 1.1). I used the tropical liverwort *Marchantia inflexa* (Nees & Mont) for this work because it is DhT and exhibits substantial intraspecific variability in related traits (McLetchie and Puterbaugh 2000; Groen *et al.* 2010ab; Brzyski *et al.* 2014) Further, *M. inflexa* is dioecious, which provides an important opportunity to investigate sexual dimorphisms and sex chromosome evolution, both of which are fundamental questions in biology. The dominant life stage of *M. inflexa* is haploid and sex is chromosomally determined with a male V or female U chromosome. See figure 1.1 for a graphical representation of *M. inflexa's* life cycle. *Marchantia inflexa*'s native habitat is in low light, high humidity sites along tropical streams and rivers, but it can also colonize more exposed and disturbed sites, such as road cuts within forests (Brzyski *et al.* 2014). I leveraged the unique biology of *M. inflexa* to test for differences in DhT among the sexes and populations, to describe gene expression responses to dehydration, identify sex-linked genes associated with DhT, investigate sex chromosome evolution, and characterize associated microbial communities.

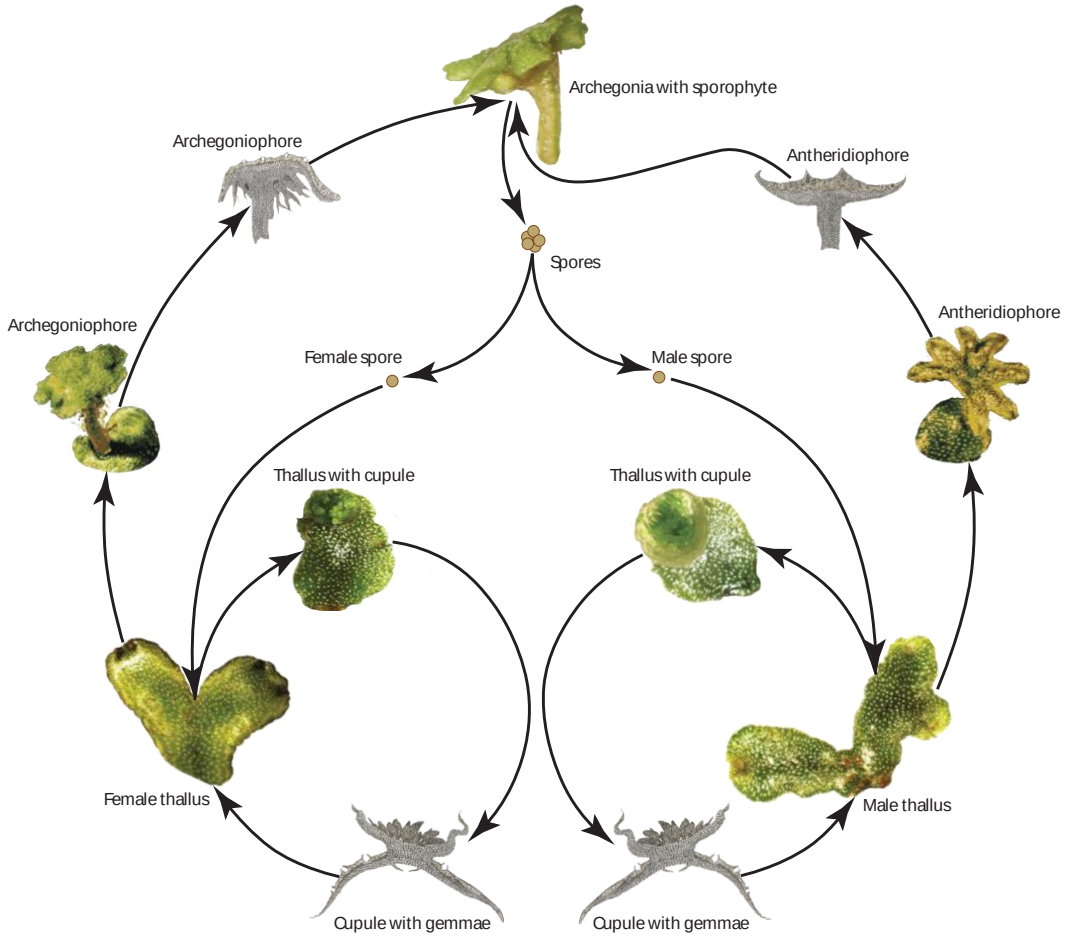

Figure 1.1. The life cycle of *Marchantia inflexa* is dominated by the haploid gametophyte, which grows as a dichotomously branching thallus with dorci-ventral organization. Plants are unisexual and can reproduce either sexually via spores or asexually via gemmae.

Overview

This work is comprised of five separate, but related studies that increase our understanding of intraspecific variability and sexual dimorphism in DhT. Initially, I characterized DhT in *M. inflexa* through a series of progressively intense drying treatments (chapter 2). I dehydrated vegetative tissues of male and female plants from two natural populations in Trinidad, Republic of Trinidad and Tobago and quantified their recovery. Both common garden and field collected tissues were subjected to drying

treatments at multiple intensities and recovery was monitored by chlorophyll florescence. Verification studies were conducted to confirm the severity of dehydration, the rate of drying, and associated changes in photosynthetic rates. I verified my expectation that *M. inflexa* is DhT and found that females exhibit higher DhT than males. Field collected samples showed differences in DhT corresponding to the environmental conditions at their collection site, but when cultured in a common garden plants did not differ in DhT suggesting plasticity in DhT. This work provided a foundation for subsequent studies and indicated that variation in DhT exists in *M. inflexa*.

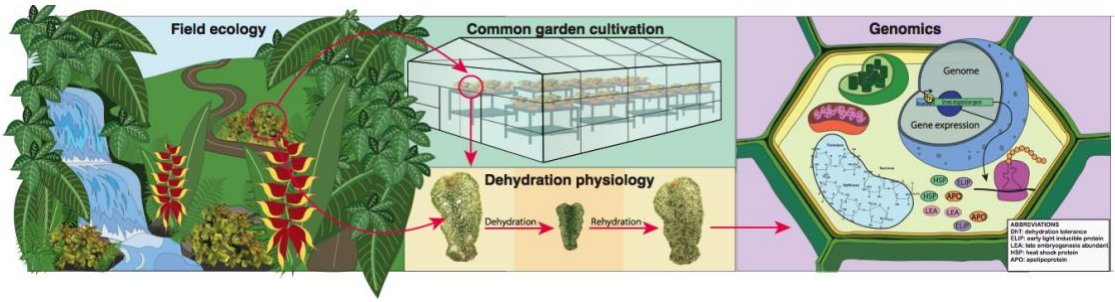

Figure 1.2. This schematic depicts the multilevel approach to studying DhT and highlights some of the methods used. Ultimately, this work sought to characterize DhT on multiple levels, spanning from field ecology, to physiology, to genomics. Field studies were employed to quantify environmental variation, population differences in DhT, the impact of differences in DhT on population sex ratios, and community level interactions. Common garden experiments were used to test for genetic differences in DhT, quantify the extent of dehydration that *M. inflexa* can tolerate, and test for sex and developmental differences in DhT. Genomic and transcriptomic approaches were utilized to identify novel genes and beneficial alleles impacting DhT, and to characterize gene expression during dehydration and rehydration.

To gain a deeper understanding into DhT variability in *M. inflexa* I conducted a more extensive characterization of population differences in DhT (chapter 3). I explicitly tested if DhT was correlated with environmental exposure, if variation in DhT was genetically determined, and if male and female plants had contrasting DhT phenotypes. To do so, I collected plants from five natural populations, spanning an environmental

gradient in the tropical forests of northern Trinidad, Republic of Trinidad and Tobago. I measured DhT immediately after collection, and again after one year of cultivation in a common garden. I found that DhT varied significantly among populations paralleling the environmental gradient. Additionally, I showed that population differences in DhT were maintained in the common garden, suggesting that underlying genetic differences contribute to DhT variability. Interestingly, I detected population-specific sex differences in DhT (in contradiction to the findings of chapter 2). Males were more DhT than females in exposed sites, but females were more DhT than males in less exposed sites. This fluctuating sexual dimorphism in DhT was driven primarily by male variation while females exhibited a consistent DhT phenotype across sites, suggesting that patterns of sexual dimorphism in DhT are complex and possibly transient.

Next, I aimed to characterize the molecular biology of DhT in *M. inflexa* (Figure 1.1). To do so I measured gene expression during dehydration and rehydration in *M. inflexa* to gain insight into the timing and nature of cellular changes during dehydration (chapter 4). I took advantage of intraspecific variation in DhT to target variation contributing to differences in relative DhT in *M. inflexa*. My analyses detect a characteristic accumulation of late embryogenesis abundant (LEA) proteins, substantial modifications in carbohydrate metabolism, and changes in lipid transport during the dehydration – rehydration process. I speculate that low expression of other genes (i.e early light inducible proteins (ELIPs) and heat shock proteins (HSPs)) during the dehydration – rehydration process and seemingly low levels of temporal organization may reduce the overall tolerance of *M. inflexa* relative to highly DT lineages. Analyses of sex and tissue specific gene expression suggests that baseline variation in signaling

pathways and cell wall characteristics may impact the relative tolerance of samples. Taken together these findings indicate that multiple mechanisms of enhancing DhT exist in *M. inflexa* and are driven primarily by baseline differences among samples.

Subsequently, I sought to develop foundational genomic resources to facilitate genetic inference in *M. inflexa*. Consequently, I sequenced and assembled a draft genome for *M. inflexa*, which adds to a growing body of genomic resources for bryophytes and provides an important perspective on the evolution and diversification of land plants (chapter 5). I specifically addressed questions related to sex chromosome evolution, sexual dimorphisms, and the genomic underpinnings of DhT. For assembly, I leveraged the recently published genome of the related liverwort, *M. polymorpha,* to improve scaffolding and annotation, aid in the identification of sex-linked sequences, and quantify patterns of sequence differentiation within *Marchantia*. I found that genes on sex chromosomes are under greater diversifying selection than autosomal and organellar genes. Interestingly, this difference is driven primarily by divergence of male-specific genes, while divergence of other sex-linked genes is similar to autosomal genes (parallel to male variability driving populations differences in DhT dimorphism (chapter 3)). Through analysis of sex-specific read coverage, I identified and validated genetic sex markers for *M. inflexa*, which will enable diagnosis of sex for non-reproductive individuals. To investigate genomic patterns of DhT I capitalized on a difference in DhT between genetic lines, which allowed me to identify seven dehydration tolerance genes with substantially higher coverage in a tolerant female relative to less tolerant male, suggesting that dehydration tolerance is facilitated by increased copy number of multiple

genes. Interestingly, two DhT genes with differences in copy number among genetic lines appear to be sex-linked, providing a possible explanation for sex differences in DhT.

Finally, in an effort to scale back up to species interactions, community ecology, and ecosystem function, I considered the role of plant microbe interactions in *M. inflexa* (chapter 6). *Marchantia inflexa* (like many other bryophytes) is known to have a robust microbiome, but the relationship between microbiome composition and DhT is not well studied. Initially, I sought to characterize the diversity and variability of the *M. inflexa* microbiome, with ultimate aim of linking this to variability in DhT. I hypothesized that variation in the environment and sex of a host plant would impact the composition and diversity of associated microbial communities. To test this hypothesis, I characterized the bacteriome of *M. inflexa*, in both males and females across multiple habitats by targeted sequencing of the bacterial 16S rRNA gene. I found that the bacteriome of *M. inflexa* is abundant and diverse, showing some similarities with other non-vascular plant lineages. I detected a habitat specific component of the bacteriome, as well as sex differences under common garden conditions. On the basis of known microbial functions, my analyses suggest that the taxonomic assemblages of bacteria in particular sites may serve functional roles; allowing plants to better acclimate to their local environment, and that sex differences in the bacteriome may correspond to subtle differences in the physiology and morphology of the sexes. My initial characterization of variation in bacteriome composition of this *M. inflexa* provides valuable information for better understanding the patterns of plant-microbe interactions, and downstream work seeks to link this variation to DhT.

Collectively this work adds to a growing understanding of plant stress tolerance and highlights the importance of considering natural variation. I found that DhT is highly variable and that both genetic differences and plasticity contribute to this variability. When combined with complex patterns of sexual dimorphism in DhT, the challenge of predicting population and species responses to climate change is exacerbated. However, with additional studies that seek to comprehensively measure natural variation in DhT and other stress tolerance traits, it may be possible to improve management of both natural and agricultural systems.

CHAPTER TWO

SEX DIFFERENCES AND PLASTICITY IN DEHYDRATION TOLERANCE: INSIGHT FROM A TROPICAL LIVERWORT

Reproduced with minor edits from: Marks, R. A., Burton, J. F. & McLetchie, D. N. Sex differences and plasticity in dehydration tolerance: insight from a tropical liverwort. *Annals of Botany*. **118**, 347–56 (2016).

Introduction

In a warming and increasingly variable climate adaptations to cope with water scarcity are particularly relevant, and characterization of these adaptations may improve management of natural and agricultural resources. Some plant species have the ability to resurrect from near complete dehydration and apparent death. This capacity is known as desiccation tolerance, an extreme case of dehydration tolerance (DhT, also dehydration tolerant). A variety of research has been conducted on the natural history (Alpert 2000), physiology (Bewley 1979), mechanisms (Le and McQueen-Mason 2006; Gechev *et al.* 2012), and prevalence (Wood 2007) of DhT with particular focus on highly desiccation tolerant species.

Dehydration tolerance is rare. Most plant and animal tissue cannot withstand cellular water potentials below -5 to -10 MPa (Proctor and Pence 2002; Proctor *et al.* 2007). However, DhT tissues consistently recover from cellular water potentials below this level (Oliver *et al.* 2010), and desiccation tolerant tissues survive cellular water potentials below -100 MPa (Gaff 1971). Dehydration tolerant organisms have unique protection and repair systems that are employed to combat the myriad of drying stresses, including membrane rupture (Hoekstra and Golovina 1999), protein aggregation, and oxidative damage (Leprince *et al.* 1990; Oliver *et al.* 2005). More specifically, changes in carbohydrate metabolism, accumulation of protective proteins, increased cell wall

flexibility, and induction of antioxidant systems have been associated with tolerance (see Black and Pritchard, 2002; Lüttge et al., 2011, and citations within).

Despite being uncommon, DhT is found across many life forms, from bacteria (Billi and Potts 2002), to animals (Clegg 2005; Gusev *et al.* 2014), to plants. In plants, DhT likely evolved as a necessary adaptation for the transition from water to land by early plants (Oliver *et al.* 2000). Through evolutionary time, most plants lost DhT in their vegetative tissues, perhaps as a trade-off for more complex water storage and transport systems supporting larger growth forms (Alpert 2006; Gaff and Oliver 2013). However, species from multiple plant lineages re-evolved vegetative DhT independently (Farrant and Moore 2011). Interestingly, most dehydration sensitive plants retain DhT in their pollen, seeds, or spores, suggesting that a wider array of plants may have the genetic potential for DhT, but that it is constrained to specific tissue types and developmental stages as a product of taxon-specific life history (Walters *et al.* 2002; Grene *et al.* 2011). Although there are DhT representatives across taxonomic groups of land plants, DhT is most prominent among bryophytes, lichens, and green algae (Proctor *et al.* 2007; Wood 2007).

To date, the majority of research has focused on the extremely DhT (or desiccation tolerant) species, which has contributed valuable insight to the process and mechanisms of DhT. However, such extreme phenotypes are not applicable to many plant forms and may obscure nuanced components of the process. There has been a relative paucity of research on species that show lower levels of tolerance, although some recent studies address this (Koster *et al.* 2010; Cruz de Carvalho *et al.* 2011, 2012, 2014, 2015; Pardow and Lakatos 2013; Bader *et al.* 2013; Stark *et al.* 2013), and studies have

investigated the drought response of numerous species (Hsiao 1973; Osakabe *et al.* 2014). Bryophytes showing moderate DhT likely occupy a large ecological niche and understanding their response to abiotic stress is important for predicting species range shifts due to environmental changes.

Intraspecific variability in DhT has also been relatively underexplored, though its existence has long been acknowledged (Schonbeck and Bewley 1981). Evidence suggests that DhT is not fixed within a species and examples of plasticity in DhT (Dilks and Proctor 1976; Schonbeck and Bewley 1981; Robinson *et al.* 2000), seasonal variation (Beckett and Hoddinott, 1997; Farrant *et al.* 2009), differences between developmental stages (Koster and Leopold 1988; Stark *et al.* 2004, 2007), and sex-specific variation (Newton 1972; Stieha *et al.* 2014) exist. Characterizing plasticity in DhT may be particularly informative for understanding parameters that modulate DhT and sex differences in DhT could explain the existence of biased population sex ratios in nature.

In this study, I tested for intraspecific variation in DhT in the tropical liverwort *Marchantia inflexa.* I hypothesized that *M. inflexa* would show moderate DhT and also considerable intraspecific variability in DhT. For this study I used plants collected from populations located along a moisture availability gradient and subsequently grown in a common garden. I predicted that *M. inflexa* would show reduced DhT in plants originating from mesic habitats compared to less mesic habitats because plants originating from a moist environment would have little use for DhT. Based on previous studies (Stieha *et al.* 2014) females were expected to be more DhT than males, which would provide a potential explanation for the female biased sex ratios seen in this and many bryophyte populations.

Methods

Study organism, sampling, and growth conditions

Marchantia inflexa (Nees & Mont) is a New World liverwort with unisexual individuals, found from northern Venezuela to the southern United States (Bischler 1984). *Marchantia inflexa* exhibits genetic variation in DhT in some developmental stages (gemmae) (Stieha *et al*. 2014), and its ecology and physiology have been the subject of multiple relevant studies (McLetchie and Puterbaugh 2000; Groen *et al.* 2010ab; Brzyski *et al.* 2014; Stieha *et al.* 2014).

Plants for this study were collected from Trinidad, The Republic of Trinidad and Tobago and maintained in greenhouse conditions at The University of Kentucky, Lexington, KY, USA. Specimens were vouchered at the Missouri Botanical Garden (St. Louis, Missouri, USA, specimen numbers M092113 and M092115) and at the National Herbarium of the Republic of Trinidad and Tobago (St. Augustine, Trinidad, specimen number TRIN34616, D. N. McLetchie, collector). Species identification was verified by Alan Whittemore (New York Botanical Garden, Bronx, New York, USA). Plants were collected from populations in two distinct habitats along a moisture availability gradient: streams (native habitat) and roads (novel habitat). The streams are humid and shaded by trees, representing a high moisture habitat, while the roads are exposed and are expected to be less humid. Furthermore, the presence of a dry season (although intermittent rainfall occurs) causes the roads to experience intervals of drying.

Male and female plants were collected from randomly chosen locations (patches) along one stream site (Turure River) and along one road site (Cumaca Road). Each patch

was physically separated from the other to guarantee that individuals were genetically different, and uniqueness of each isolate was confirmed by PCR analysis (Brzyski *et al.* 2014). Eighteen genotypes were selected for use in the current study: four males from the stream site, four males from the road site, five females from the stream site, and five females from the road site.

For this study, thalli of stock plants with their meristematic region intact were transplanted onto steam-sterilized soil (collected from the North Farm, University of Kentucky, Lexington, Kentucky, USA) in 12 well plug trays (3.5 x 4 cm). At least 36 clones (three sets) of each genotype were maintained in a randomized layout in the greenhouse. Trays were placed on capillary mats that were kept wet by daily watering with deionized water and covered with a shade cloth to mimic field light conditions.

Dehydration tolerance assay and recovery

Dehydration conditions of differing intensity were generated using saturated salt solutions to modify the relative humidity (RH) in dehydration chambers. Experimental RHs, water vapor pressure deficits (VPD), and corresponding treatment levels are shown in Table 2.1. Water vapor deficit was calculated to enable comparison of treatment intensity with my field assay (Anderson, 1936). Each experimental RH was calculated based on an equilibrium RH chart (Wexler and Hasegawa, 1954) and verified using a HOBO™ humidity sensor attached to a data logger (Onset Computer Corporation, Bourne, MA, USA).

Table 2.1. Treatment conditions for laboratory dehydration assays. Temperature was maintained in a growth chamber on a 12 hour light/dark cycle. Relative humidity was verified using a HOBO™ data logger with sensors attached. All assays were 22 hours long. Vapor pressure deficit indicates the relative intensity of the combined temperature and relative humidity during the assay.

Treatment Level 1	Temperature	Relative Humidity	Vapor Pressure Deficit	Salt
Level 1	13°C	95.41%	0.08kPa	KNO_3
Level 2	13°C	85.92%	0.21kPa	KCl
Level 3	13°C	75.61%	0.37kPa	NaCl
Level 4	13°C	55.87%	0.66kPa	MgN_2O_6

Dehydration assays were conducted in dehydration chambers (air tight plastic boxes 24 x 10 x 32 cm) and were placed in a growth chamber with a 12 hour light/dark period and a constant temperature of 13°C. Healthy vegetative thallus tips (~7 mm in length) from each of the 18 genotypes were collected from the greenhouse, saturated with distilled water and placed in the growth chamber for 24 hours to ensure that each sample was fully hydrated before the assay began. After 24 hours, each thallus tip was randomly assigned to one of four treatment groups and blotted to remove external water. Each thallus tip was then placed in a 35 x 10 mm falcon™ brand Petri dish along with a single filter paper disk and 200 μL of distilled water. These Petri dishes were placed on the internal perimeter of the dehydration chamber, and a bowl of saturated salt solution (differed between treatments) was placed in the center of the box. A small battery powered fan sat on a wire mesh stand above the salt solution (Figure 2.1). Plants were dehydrated for 22 hours, after which the fan was stopped, the plants were rehydrated with distilled water, and maintained in the same growth chamber for 1 week. Treatments were replicated through time across all 18 genotypes: level 1 = three replications; level 2 = ten replications; level 3 = nine replications; level 4 = three replications.

Recovery of each individual thallus tip was analyzed by chlorophyll fluorescence after 1 week of recovery to determine the condition of photosystem II. Chlorophyll fluorescence is a common method use to assay plant recovery after dehydration stress (Krause and Weis 1984; Csintalan 1999). Maximum potential quantum yield (F_v/F_m) readings of dark-adapted leaves (20 minutes) were taken using an OS5-FL modulated chlorophyll fluorometer (Opti-Sciences, Tyngsboro, Massachusetts, USA). Recovery was also assessed by monitoring plant growth over 2 months, during which time the plants were maintained in a hydrated state in the same growth chamber.

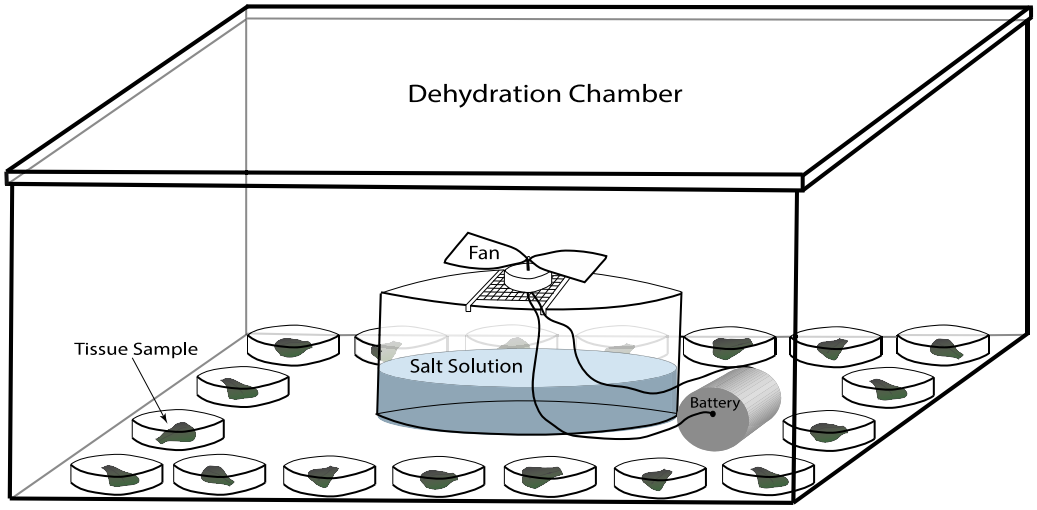

Figure 2.1. Schematic of the dehydration assay set up. Each box contained one tissue sample from each of the 18 genetic lines. Each sample was placed in a Petri dish along with a single filter paper disk and 200 μL of distilled water. The dehydration chamber was placed in a growth chamber with a constant temperature of 13°C and a 12-hour light/dark cycle.

Relative water content

The relative water content (RWC) of tissue was measured to determine the percentage of water lost by samples in each treatment level. Vegetative tissue of *M. inflexa* was collected from the greenhouse and hydrated for 24 hours in the growth chamber. Following hydration, the tissue was blotted dry to remove external water. Each

plant was then weighed to the nearest nanogram using a Chan 29 electrobalance to determine the turgid mass (M_t), randomly assigned to one of the four treatment groups, and subjected to the dehydration assay described above. However, following completion of the assay at 22 hours, the tissue was not rehydrated. Instead, samples were weighed to determine sample fresh mass (M_f), and the tissue was placed in a drying oven (80°C) for 3 days after which its dry mass (M_d) was taken. The RWC was calculated for each sample with the following formula:

$$RWC = \frac{(M_f - M_d)}{(M_t - M_d)} \times 100$$

Field study

A study was conducted to investigate corresponding patterns of DhT at the field sites on Trinidad, The Republic of Trinidad and Tobago, during March 2015. Vegetative thallus tips (~7 mm in length) were collected from plants growing in two habitat types (stream and road). The samples were collected from moist substrate and appeared hydrated when collected. Sampled thallus tips were saturated in stream water for 24 hours, blotted dry, and then placed randomly into one of two dehydration treatment levels (Table 2.2). Three trials were conducted with ten thallus tips in each. Half of the thallus tips in each trial were from the stream habitat and half were from the road habitat. The assay was conducted following the same protocol as the laboratory dehydration assay (described above) with a few minor changes. First, because there was no access to a temperature regulated growth chamber in Trinidad the dehydration assay was conducted under a raised building to avoid direct sunlight. Ambient temperatures ranged from 26°C (night) to 30°C (day). The corresponding VPDs and RHs for these treatments are listed in

Table 2.2. The VPD was needed to compare the relative intensity of field and laboratory treatments because it accounts for the effect of temperature (Anderson, 1936). The thallus tips were allowed to dehydrate until they were noticeably dry as indicated by visual changes in thallus curvature, after which they were rehydrated with stream water. Recovery was assessed by chlorophyll fluorescence 1-week post rehydration.

Table 2.2. Treatment conditions for dehydration assays conducted in the field. The temperature and humidity were confirmed using a HOBO™ data logger and sensors. The temperature varied with the ambient air temperature in Trinidad, and the assay was conducted in a location to avoid direct sunlight. Vapor pressure deficit indicates the combined impact of temperature and relative humidity during the assay.

Treatment Level	Temperature	Relative Humidity	Vapor Pressure Deficit	Salt
Field Level 1	26°C to 30°C	83.63%	0.50 to 0.64 kPa	KCl
Field Level 2	26°C to 30°C	75.09%	0.80 to 1.07 kPa	NaCl

Verification of equilibration and rate of drying

To confirm that samples had equilibrated to the RH in the dehydration chambers after 22 hours, vegetative thalli were collected from a single randomly selected genotype (1F), subjected to the dehydration assay described above, and changes in RWC over time were documented. Samples were weighed before treatment and then randomly assigned to one of the four dehydration treatment levels. To minimize disturbance of the samples and potential changes to treatment conditions, samples were not weighed for the first 16 hours of the dehydration assay (except in treatment level 4 because these samples dried much faster than the others). Samples were then weighed at 16, 18, 20, 21, and 22 hours using the Chan 29 electrobalance. When sample mass did not decline for three consecutive measurements, I considered tissues to be equilibrated to the RH in the chamber. If the samples were not equilibrated to experimental RH at 22 hours the assay was extended for an additional hour. After completion, samples were oven dried and

weighed as described above. The RWC for each sample was calculated based on the initial turgid mass (M_t), the mass at each time point (M_f), and the final dry mass (M_d) using the equation for RWC.

Physiological changes

Because the RHs used in this study were higher than those typical for bryophyte dehydration studies, I tested the physiological consequence of these treatments by measuring changes in photosynthesis and respiration. I focused this effort on changes in gas exchange during dehydration at 75% RH. The higher experimental RHs (85 and 95%) were not used because plants retained high RWC at equilibrium with these RHs, and the lowest RH (55%) was not used because all *M. inflexa* samples died from this treatment.

Thallus tips (~7 mm long) were collected from greenhouse-grown specimens of one randomly selected male (8X) and female (2X) and placed in distilled water. Sample area was measured using ImageJ (Schneider *et al.* 2012). Prior to placement in the gas exchange chamber, the thallus tip was blotted to remove external water and 20 μL of distilled water was added to make the thallus tip adhere to the plastic sheet used to support it in the gas exchange chamber.

Gas exchange was measured with an open-flow system (LI-6400, Li-Cor, Lincoln, Nebraska, USA) equipped with a fluorescence attachment (6400-40, Li-Cor). Air temperature was allowed to vary with the ambient laboratory temperature (22 - 24°C), and RH ranged from 72- 80%. Thus, the VPD varied from 0.53 - 0.84 MPa. Airflow rate was set at 100 μmol s^{-1}, and CO_2 was set at 450 μmol mol^{-1}. Infrared gas analyzers (sample and reference) were matched prior to the assays.

To obtain baseline CO_2 assimilation rates (μmol CO_2 m^{-2} s^{-1}), I first took three readings 15 minutes after equilibrium at a light intensity of 150 μmol m^{-2} s^{-1}. To measure respiration, light intensity was set at 0 μmol m^{-2} s^{-1} and three readings were taken after 15 minutes. In *M. inflexa*, gas exchange rates usually equilibrate in 5 - 6 minutes after a change in light intensity (unpublished data). After taking baseline measurements, each plant was immediately subjected to the assay to measure either changes in photosynthesis or respiration, but never both because these assays damage tissues. For photosynthesis, plants were assayed using the AUTO program of the LI-6400, at a light intensity of 150 μmol m^{-2} s^{-1} at 20-minute intervals for 5 hours, then assayed at 0 μmol m^{-2} s^{-1} to confirm that photosynthesis had ceased (i.e., no change in gas exchange rates between 150 and 0 μmol m^{-2} s^{-1}). For respiration, plants were assayed using the AUTO program of the LI-6400 at a light intensity of 0 μmol m^{-2} s^{-1} at 30-minute intervals for 7.5 hours, then assayed at 150 μmol m^{-2} s^{-1} to confirm that photosynthesis was not contributing the gas exchange rates (i.e., no increase in carbon fixation from 0 to 150 μmol m^{-2} s^{-1}). The difference between input and output air mmol H_2O mol^{-1} at each time point was used as a measure of the dehydration state of the tissue, where decreasing positive values indicated that the tissue was still drying and zero indicated an equilibrium dehydrated state. The interval and duration of these assays were based on trials showing that plants in the same light intensity took 3 to 4 hours, and those in the dark took 6 to 7 hours to equilibrate to the RH.

Statistical analyses

All statistical analyses were done in JMP®, Version 10. SAS Institute Inc., Cary, NC.

Dehydration tolerance assay: To account for genotypic variation I computed $\Delta F_v/F_m$ (initial F_v/F_m – recovery $F_v/F_m = \Delta F_v/F_m$) for each genotype using 5 thallus tips per genotype. Larger values indicate less recovery. Differences in recovery across treatment levels were analyzed using a mixed linear model with $\Delta F_v/F_m$ as the dependent variable. The fixed effects tested were treatment level, sex, habitat, and the sex by habitat interaction. Genotype (nested within sex) and trial (replications) were random effects. Post hoc comparisons were made with a Tukey HSD test.

Because of strong treatment differences (high recovery for levels 1 and 2, and no recovery for level 4), level 3 was selected for further use in elucidating differences in responses to dehydration between sexes and habitats. That is, responses were most variable at this treatment level. The dependent variable analyzed was $\Delta F_v/F_m$. The fixed effects tested were, sex, habitat, and the sex by habitat interaction. Genotype (nested within sex) and trial (replications) were random effects. Post hoc comparisons were made with a Tukey HSD test.

Relative water content: Differences in RWC among treatment levels were tested for significance using a mixed linear model. The dependent variable was RWC, and the fixed effect tested was treatment level. Genotype (nested within sex) and trial (replications) were random effects. Post hoc comparisons were made with a Tukey HSD test.

Field study: Differences between habitat types in ability to recover from dehydration were analyzed exclusively for field treatment level 1 using the mixed linear model. Field treatment level 2 was not used because samples did not recover from this treatment. I did not take initial F_v/F_m readings for these samples, so recovery was assessed using only the recovery F_v/F_m values. The dependent variable was F_v/F_m, and the fixed effect analyzed was habitat. Plant ID and trial (replications) were random effects. The sex of these plants was unknown, and therefore sex effects were not investigated.

Verification of equilibration and rate of drying: Differences in the rate of change in RWC were investigated by repeated measures MANOVA with a full factorial design, repeated through time. I considered no change in RWC over three time points to be an indication of equilibration to treatment RH. Thus, the final three time points were analyzed separately to ascertain if the RWC had stopped changing using repeated measures MANOVA.

Physiological changes: The difference between initial and final photosynthetic rate, as well as the verification of photosynthetic cessation, were investigated using 95% confidence intervals calculated for both mean initial and mean final photosynthetic rate across trials and genotypes. Respiration rates were too low to detect a change over time. Thus, respiration levels were not analyzed further.

The response variable of photosynthetic rate was examined using a mixed linear model in which photosynthetic rate was the dependent variable, time and genotype were fixed effects, and trial (replications) was a random effect. The response variable of

dehydration state was examined by a similar mixed linear model where dehydration state was the dependent variable, time and genotype were fixed effects, and trial (replications) was a random effect. The relationship between time to stop photosynthesis and the time to reach dehydration equilibrium was investigated using the mixed linear model where time was the dependent variable, process (photosynthesis or dehydration equilibrium) and genotype were fixed effects, and trial (replications) was a random effect.

Results

Dehydration tolerance assay and recovery

Treatment intensity: Treatment level had a significant effect on recovery ($F_{3,21} = 21.39$, $P < 0.001$). Samples in level 1 were the least damaged and those in level 4 were the most damaged by dehydration (Figure 2.2). Estimation of survival by long-term observation and mortality tracking over 2 months was consistent with the F_v/F_m data. All samples survived the level 1 treatment, and 96% of plants survived level 2 treatment. Sixty-three percent of plants survived level 3 treatment, but growth of these plants was substantially reduced. All plants died in the level 4 treatment.

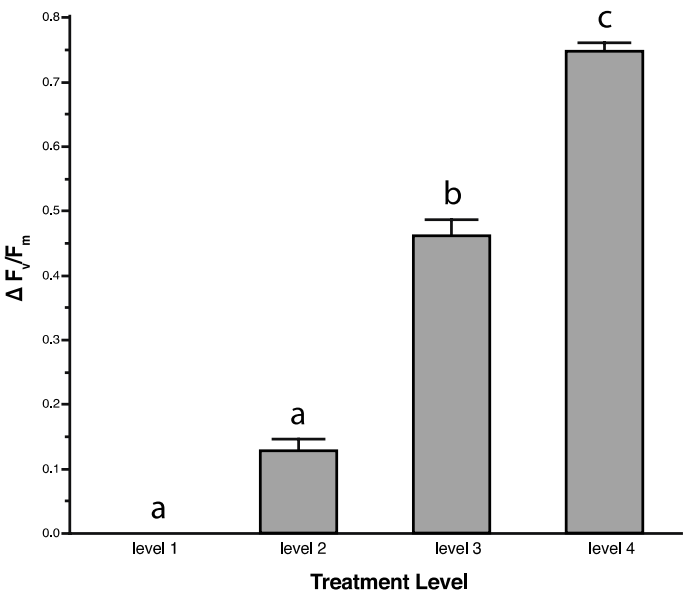

Figure 2.2. $\Delta F_v/F_m$ of samples for each of the four levels of dehydration treatment. Recovery was measured after 1 week. Sample sizes used to calculate survival were: level 1 = three; level 2 = ten; level 3 = nine; level 4 = three. Higher $\Delta F_v/F_m$ indicates more damage.

Sex and habitat patterns: Analysis of recovery from treatment level 3 revealed a sex effect ($F_{1,13.37} = 7.60$, $P = 0.016$) and a sex by habitat interaction ($F_{1,13.37} = 4.94$, $P = 0.044$). Females were less damaged by dehydration than males (Figure 2.3a). More specifically, females were less damaged than males from the stream populations, but this sex difference was not evident in plants from road populations (Figure 2.4). No overall population difference was found between individuals from stream or road habitats (Figure 2.3b).

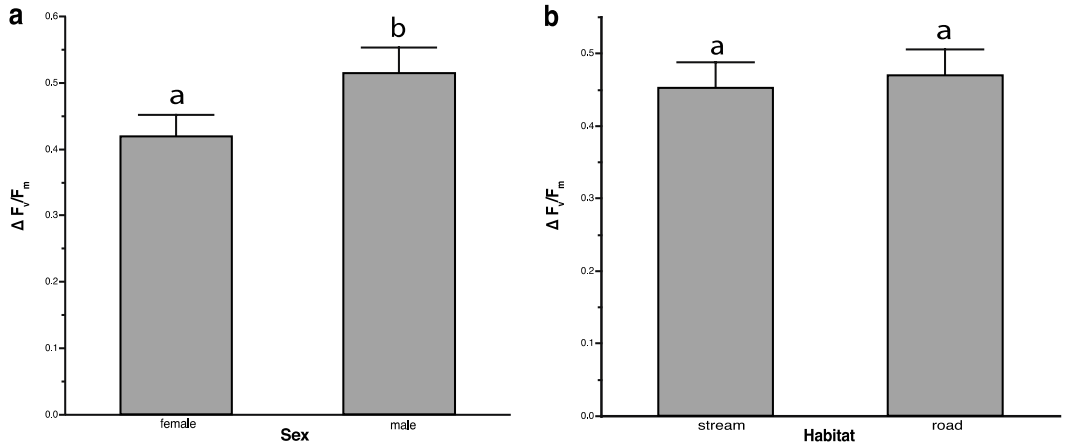

Figure 2.3. (a) Difference in damage incurred from dehydration at level 3 between the sexes is significant with females being less damaged than males (P = 0.016). (b) Difference in damage incurred for plants from wet vs. dry source habitats at level 3 is not significant. Recovery was measured after 1 week. There were nine trials.

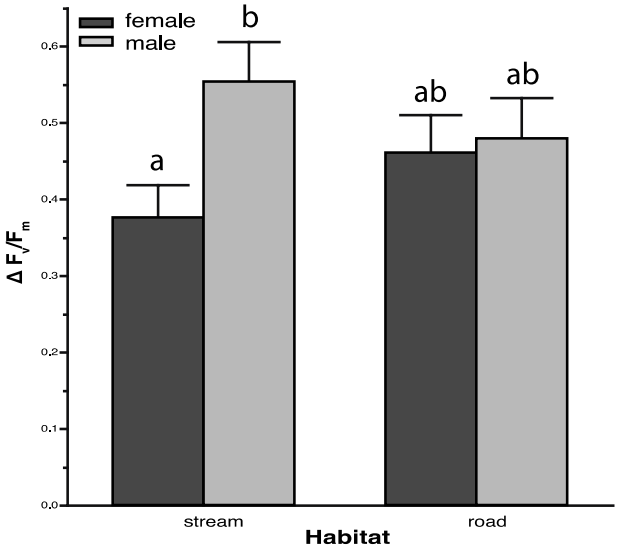

Figure 2.4. Females from the stream were less damaged than males from the stream, whereas females and males from the road did not differ from one another. This interaction is statistically significant at treatment level 3 (P = 0.044). Recovery was measured after 1 week. There were nine trials.

Relative water content

Differences in mean RWC across treatment level were significant ($F_{3,195}$ = 278.25, P < 0.001; Figure 2.5). Tissue in the level 1 treatment maintained full saturation. Tissue in the level 2 treatment lost some cellular water (RWC = 79.9% ± 4.4%), and that in the

level 3 treatment lost a substantial amount of cellular water (RWC = 19.7% ± 2.9%). Tissue in the level 4 treatment lost almost all cellular water.

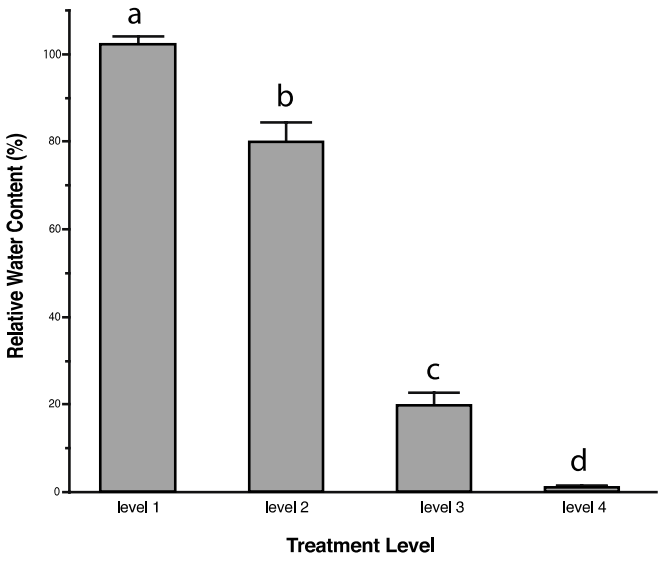

Figure 2.5. Mean RWC of *M. inflexa* tissue after treatment at each of the four dehydration intensities (Table 2.1). Sample size for each treatment group was three replicated dehydration chambers, each containing 18 individuals.

Field study

Plants sampled from the dry road habitat recovered more completely from dehydration treatment than those collected from the moist stream habitat ($F_{1,26} = 4.49$, $P = 0.044$, Figure 2.6).

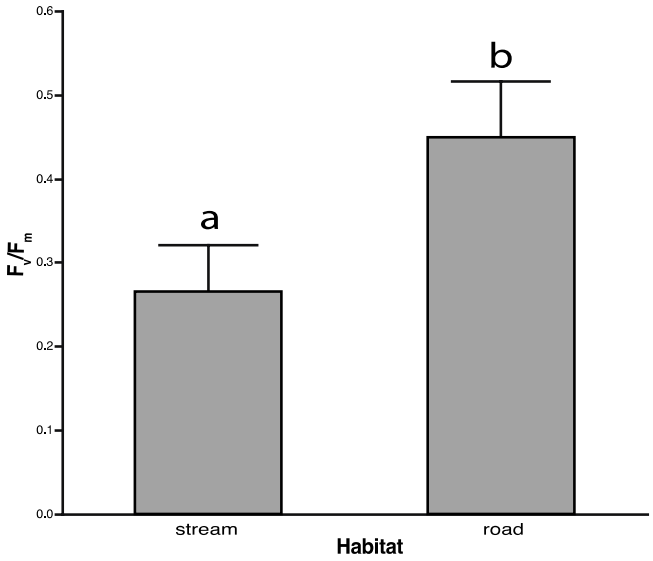

Figure 2.6. The differences in recovery F_v/F_m of plants collected directly from stream or road sites and subjected to dehydration conditions was significant (P = 0.0438). Recovery was measured after 1 week. There were three trials with ten thallus tips in each.

Verification of equilibration and rate of drying

There was a significant effect of time in all treatment levels ($F_{5,3}$ = 529.77, P < 0.001) as well as a significant time by treatment interaction (Wilks' Lambda $F_{15,8.68}$ = 17.69, P < 0.001), indicating that the rate of drying differed across treatments (Figure 2.7). Samples in both treatment levels 3 and 4 equilibrated to the treatment RH well within the timeframe of my assay, as evidenced by a lack of change in RWC during the last 4 hours of the assay for treatment level 3 and the last 12 hours for level 4. Samples in treatment levels 1 and 2 took the entire 22 hours to equilibrate. During the final 3 hours of the assay there was no significant time effect on RWC at any treatment level (level 1: $F_{2,1}$ = 96.53, P = 0.072; level 2: $F_{2,1}$ = 3.36, P = 0.36 ; level 3: $F_{2,1}$ = 0.38 P = 0.75; level 4: $F_{2,1}$ = 0.55, P = 0.4), suggesting that tissues were equilibrated to treatment conditions during this final time period.

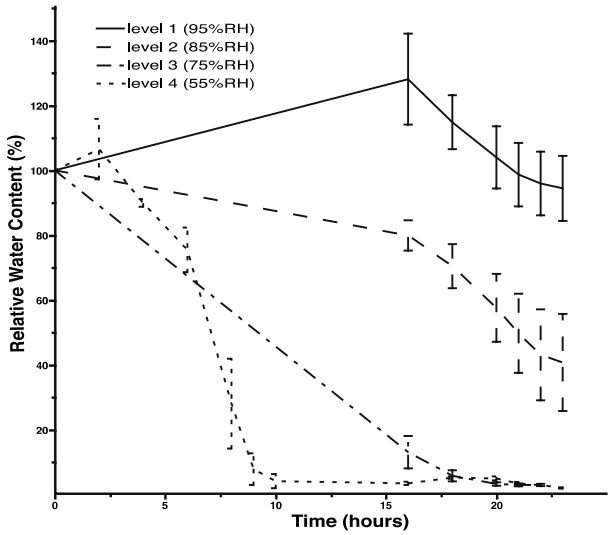

Figure 2.7. Drying rate as change in RWC over time for the four treatment levels. Three samples of a single genotype (1F) were used in each treatment level. RWC was calculated using fully hydrated sample mass, mass measured over the course of the assay, and the final oven dry mass. The slight initial increase in RWC seen in level 1 and level 4 treatments is a result of the samples picking up external water prior to drying.

Physiological changes

Tissue samples in the light equilibrated to ~75% RH after ~3 hours as indicated by the lack of difference in mmol H_2O mol^{-1} of input and output air (Figure 2.8a). Samples stopped assimilating CO_2 after ~3 hours (Figure 2.8b).

Neither initial ($F_{1,4} = 0.85$, $P = 0.408$) nor final ($F_{1,4} = 3.37$, $P = 0.14$) photosynthetic rates were significantly different between genotypes, but there was a significant change in photosynthetic rate during the process of equilibration to ~75% RH (Figure 2.8b). The mean initial photosynthetic rate was 5.48 µmol $CO_2m^{-2}s^{-1}$ (95% confidence interval: 5.10 - 5.86 µmol $CO_2m^{-2}s^{-1}$). The mean final photosynthetic rate was -0.02 µmol $CO_2m^{-2}s^{-1}$ (95% confidence interval: -0.19 - 0.16 µmol $CO_2m^{-2}s^{-1}$). Because the confidence intervals do not overlap, I conclude that the initial and final photosynthetic rates are significantly different from one another. Furthermore, the 95% confidence interval for the final photosynthetic rate includes 0, suggesting that photosynthesis was

likely shutdown at this point. I found that initial respiration levels were too low to detect a change over time.

There was no significant difference detected in the time to dehydrate to equilibrium at ~75% RH and the time to cease photosynthesis ($F_{1,5} = 0.72$, $P = 0.435$), nor any genotype effect ($F_{1,4} = 0.14$, $P = 0.729$), indicating that both processes happen simultaneously, and consistently across genotypes.

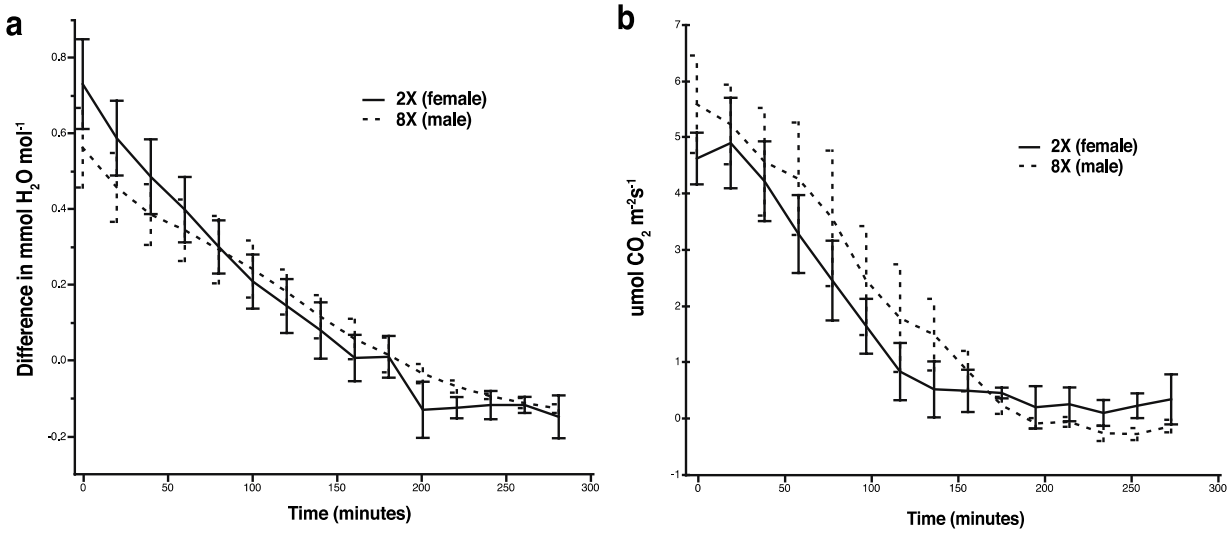

Figure 2.8. (a) The dehydration state of samples over time was measured as difference in mmol H_2O mol^{-1} input to mmol H_2O mol^{-1} output. (b) The change in photosynthetic rate was measured over the same time period. Photosynthetic rates were measured as µmol $CO_2m^{-2}s^{-1}$ and decreased to 0. between 2 and 3 hours of drying at ~75% RH. There was no difference between genotypes in their dehydration state or photosynthetic rate (n = 6).

Discussion

These results indicate that *M. inflexa* displays a moderate degree of DhT, which confirms my expectation and extends existing knowledge of the diversity of DhT phenotypes within bryophytes. *Marchantia inflexa* also exhibits a sex difference in DhT, with females showing a higher degree of DhT than males. This sex differences may be caused by differences in male and female reproductive strategies, which in turn may

explain biased population sex ratios. The sex difference is particularly intriguing because it is only observed for plants originating from the moist stream habitat. I did not find evidence of genetic adaptation to dry habitats, but I did detect plasticity in DhT as plants appeared to acclimate to different environments.

Recovery

No *M. inflexa* samples were able to recover from the most intense dehydration treatment (level 4), implying that *M. inflexa* is not highly DhT (or desiccation tolerant), compared to other species that can easily recover from this degree of dehydration (Proctor *et al*. 2007). However, many samples recovered from the level 3 treatment, and this treatment did dehydrate samples considerably as indicated by the relatively low cellular water content and the cessation of photosynthesis at equilibration. Moderate DhT provides an important link between desiccation sensitive crops and the highly desiccation tolerant model organisms and may provide valuable insight into the acquisition of DhT.

Marchantia inflexa females exhibited a higher degree of DhT than males (Figure 2.3a), which suggests contrasting male and female reproductive strategies or functions. This contradicts the typical expectation that females of dioecious plants should be less stress tolerant due to higher resource investment in reproduction (Juvany and Munné-Bosch 2015). However, there is mixed evidence on the direction of sex specific stress tolerance differences in dioecious plants. While most studies indicate that males have higher water stress tolerance than females (Juvany and Munné-Bosch 2015), multiple examples of higher tolerance in females have been observed as well (Ward *et al.* 2002; Sánchez-Vilas and Retuerto 2009; Melanie *et al.* 2013). Further complicating matters,

some studies have found that sex-linked differences in stress tolerance depend on life stage and environmental conditions (Rakocevic *et al.* 2009; Juvany and Munné-Bosch 2015). Notably, higher DhT has been observed in females relative to males in some other bryophyte species (Newton 1972). In *M. inflexa* (McLetchie 1992; Stark *et al.* 2000) males likely allocate more resources to pre-fertilization sexual investment than females, possibly at the expense of traits related to survival. Although females have higher post fertilization reproductive investment, this is rarely realized because fertilization rates are low. Additionally, females may experience stronger selection for self-preservation than males because they must persist during offspring maturation. Although plants were in a non-reproductive stage in this study, the underlying differences in male and female reproductive strategies could still come into play. For instance, *M. inflexa* males consistently reach sexual maturity more readily than females (Brzyski *et al.* 2014) and this precocious trait might trade-off with DhT. While this could explain the higher degree of DhT in females compared to males, resolving it will require more systematic investigation of sex specific trade-offs in DhT and sexual reproduction.

I predicted that plants collected from dry habitats (roadsides) would show higher recovery from dehydration than those collected from moist habitats (streams). However, I found no difference in the DhT of these two groups when plants were grown in a common garden. This suggests that there are no adaptive differences in DhT between plants from these divergent habitat types (Figure 2.3b). However, the road habitats have been recently colonized and thus it is not particularly surprising that I did not detect evidence of DhT adaptation to this environment. However, when plants were collected directly from these two habitat types (field study) and subjected to a dehydration

treatment, samples from dry habitats had higher DhT than plants from moist habitats (Figure 2.6). This pattern fits my initial prediction, but contradicts the laboratory study, and suggests a strong potential for acclimation. In other words, it implies that DhT in *M. inflexa* is a plastic trait. The ability of *M. inflexa* to acclimate to dry conditions corroborates pervious work on protoplasts showing that slight decreases in water potential can lead to increased DhT during subsequent drying events and multiple studies demonstrating hardening in vegetative tissue (Schonbeck and Bewley 1981; Beckett 1999; Walters *et al.* 2002; Beckett *et al.* 2005; Cruz de Carvalho *et al.* 2011). I conclude that genetic adaptations are not responsible for the habitat difference in DhT, but rather that acclimation to dry conditions can increase DhT in *M. inflexa*.

The sex by habitat interaction detected in the common garden study was driven by plants derived from the stream habitat (Figure 2.4). Females from the stream showed higher DhT than males, whereas plants from roadsides had similar responses across the sexes. While this pattern is complex, it is consistent with previous studies on *M. inflexa* showing habitat by sex interactions for other life history traits. For example, the pattern of habitat specific sexually dimorphic DhT is similar to what Brzyski *et al.* 2014 found for growth rate. In that study there was no overall habitat difference for growth rate, but stream collected plants had a sexually dimorphic growth rate and road collected plants did not have a dimorphic growth rate. One possible explanation for this pattern is that because the streams populations are native and well-established there has been more time for selection to generate the dimorphic patterns observed. Conversely, the roads are novel habitats and there has been less time for selection to act on secondary sexual dimorphisms. In addition, the road populations engage in sexual reproduction much more

frequently than those along the streams (Brzyski *et al.* 2014), which could lead to more mixing of male and females traits. Coupled with reduced evolutionary time, this mixing may explain the more homogenous DhT of the sexes in road versus stream populations.

Verification

In bryophyte dehydration studies, equilibration to different RHs is often used as a measure of dehydration stress (Proctor *et al.* 2007; Wood 2007). Thus, it was necessary for me to demonstrate that the length of these assays was sufficient for tissue to equilibrate to each treatment RH. In doing so, I showed that the rate of drying varied across treatments (Figure 2.7). Samples dehydrated at the lower RHs not only dried more completely, but also dried faster, which very likely influenced their survival. Multiple studies have shown that drying rate strongly effects survival and recovery from dehydration, with faster drying typically being more damaging for intact vegetative tissues (Farrant 1999; Cruz de Carvalho *et al.* 2012, 2015), although in many seeds slow drying is more damaging (Pammenter *et al.* 1998).

Most of the work on bryophyte DhT has been done using equilibration to lower RHs than were used in my study (Wood 2007). The standard RH used for dehydration treatment is ~50% RH, which has been very informative in studying highly desiccation tolerant species, but for understanding moderate levels of DhT I find it useful to use less intense dehydration treatments. Although other studies have used similarly intense treatments (Pardow and Lakatos 2013), there may be some concern as to the physiological effect of my treatments. To address this concern, I demonstrated that photosynthetic cessation is synchronized with equilibration to ~75% RH in *M. inflexa* (Figure 2.8). Although the cessation of photosynthesis does not imply that all metabolic

activity has ceased, it is a central plant process, and it's shutdown indicates that substantial physiological changes are associated with this level of dehydration (Dilks and Proctor 1979; Deltoro *et al.* 1998).

Conclusions

Taken together, this study demonstrates that *M. inflexa* exhibits moderate levels of DhT and that, depending on the habitat, females are more DhT than males. By combining a common garden and field study, I also show that acclimation, not adaptation, is responsible for habitat differences in DhT in these *M. inflexa* populations. Although it has long been known that many bryophytes are highly desiccation tolerant, this study provides a rare empirical example demonstrating that some bryophytes are moderately DhT. These findings are particularly relevant in an agricultural context where greater understanding of moderate DhT could inform strategies for developing DhT crop species. Furthermore, with climate change predicted to increase not only temperatures on a global scale, but to also drive more variation in weather patterns (and more extreme events such as droughts), these results also provide an important step towards better predictions of the response of *M. inflexa* and similar species to environmental change.

CHAPTER THREE

GENETIC DIFFERENCES IN WATER STRESS TOLERANCE TRACK ENVIRONMENTAL EXPOSURE AND EXHIBIT A FLUCTUATING SEXUAL DIMORPHISM

A version of this chapter is currently under review at Oecologia

Introduction

With climate change models predicting increased variation in global weather patterns, including frequent and intense droughts across much of the world (Dai 2013), both ecological stability (Jentsch *et al.* 2007) and food security (Schlenker and Lobell 2010) are threatened. However, by drawing on natural variation in water stress tolerance, we can enhance our understanding of adaptive responses and improve predictions on the ecological consequences of drought. Many mechanisms of water stress tolerance exist within land plants, spanning diverse life histories, morphologies, and physiologies. This diversity represents a valuable repository of information that can be mined to gain insight into the ramifications of water shortage. By characterizing intraspecific variation in water stress tolerance expectations on adaptive potential, population dynamics, and species persistence can be improved.

Dehydration tolerance (DhT, also dehydration tolerant) is a highly effective strategy for coping with limited water availability. DhT is a less extreme version of desiccation tolerance (DT, also desiccation tolerant) (Gaff 1971; Oliver *et al.* 2010; Marks *et al.* 2016). Extant DT and DhT plants are phylogenetically diverse but tend to occupy similar ecological niches, suggesting that environmental selection has been a strong driving force in the retention and re-evolution of DT and DhT (Alpert 2005; Le and McQueen-Mason 2006). Although, plants spanning the entire spectrum from

desiccation sensitivity to DT have been identified, the extent of intraspecific variation in DT and DhT is unclear, and whether this variation is driven by genetic differences among populations has rarely been studied. While comparative studies among species can distinguish general characteristics of DT and DhT, studies of intraspecific variation in DT and DhT will inform predictions on species range shifts, population resilience, and provide a resource for investigating the molecular mechanisms underlying DT and DhT. The limited studies on intraspecific variation in DT and DhT demonstrate that DT and DhT vary in response to environmental differences (i.e. seasonally (Farrant *et al.* 2009), among populations (Oliver *et al.* 1993; Farrant and Kruger 2001), and under specific culturing methods (Stark *et al.* 2014, 2016)), or developmentally (i.e. among life stages (Stark *et al.* 2007) and the sexes (Marks *et al.* 2016)). However, few studies focus on detecting genetic differences in DT and DhT within or across populations.

In addition to abiotic stress, dioecious plants face the threat of distorted population sex ratios, which can lead to mate scarcity, and in extreme cases a reduction or loss of sexual reproduction. Sex differences in stress tolerance are likely an important driving force of population sex ratio distortions (Retuerto *et al.* 2018), and consequently, detecting sex-specific responses to stress will enhance our understanding of population dynamics in dioecious species. Broadly speaking, sexual dimorphisms in both plants and animals can arise due to distinct selective pressures on males and females (Shine 1989; Badyaev and Hill 2003). Contrasting selection among the sexes often leads to unidirectional dimorphisms, (i.e. colorful male plumage in birds), but variable sexual dimorphisms have also been documented in numerous animal taxa (Alexander *et al.* 1979; Berry and Shine 1980; Woolbright 1983; Badyaev *et al.* 2000). Although

seemingly unlikely, complete reversals of sexual dimorphisms have been observed (Kahlke *et al.* 2000). This suggests that sexual dimorphisms are not fixed, and can in fact, fluctuate across space. The focal species, *Marchantia inflexa*, exhibits numerous sexual dimorphisms, many of which fluctuate among populations (Brzyski *et al.* 2014; Marks *et al.* 2016). Interestingly, in the above examples, the fluctuation is driven by male variability, which I speculate is related to more variable sexual selection on males leading to rapid male diversification. Understanding the patterns of fluctuating sexual dimorphisms will improve predictions on local sex ratios, reproductive potential, and population persistence in dioecious plants.

Here, I investigated variation in DhT in the tropical liverwort *M. inflexa* to provide insight into the evolutionary and ecological dynamics of DhT. My primary aims were to characterize intraspecific variability in DhT across environments, test for genetic differences in DhT, investigate if a fluctuating sexual dimorphism in DhT exists, and to test if variability in DhT is linked to population sex ratios. Building on previous studies that measured DhT in plants from a limited number of populations (chapter 2), I expanded the scope of my investigation to cover additional sites, intentionally targeting natural, relatively undisturbed streams. I collected plants from five populations, spanning an environmental gradient in the tropical forests of northern Trinidad, Republic of Trinidad and Tobago. I measured DhT immediately after collection, and again after one year of cultivation in a common garden. Data on recovery from dehydration were analyzed to explicitly test the following four hypotheses: 1) plants collected from more exposed (drier) sites will exhibit higher DhT than plants collected from less exposed sites; 2) measurable variation in DhT will be retained under common conditions due to

underlying genetic differences among populations; 3) the degree of sexual dimorphism in DhT will fluctuate among populations, as a consequence of male variability; and 4) population sex ratios will be biased in favor of the sex that is more DhT in that population.

Methods

Study organism and sample collection

Marchantia inflexa (Nees & Mont) is a New World liverwort with unisexual individuals that is distributed from northern Venezuela to the southern United States (Bischler 1984). The dominant life stage of *M. inflexa* is the haploid gametophyte, which grows as a bifurcating thallus. Sex is chromosomally determined with one sex (U/V) chromosome and eight autosomes. Sexual reproduction produces spores, and asexual reproduction produces specialized asexual propagules (gemmae) (Figure 3.1a) or occurs via thallus fragmentation. *Marchantia inflexa*'s native habitat is low light, high humidity sites along tropical streams and rivers, but it can also colonize more exposed and disturbed sites, such as road cuts within forests (Brzyski *et al*. 2014). *Marchantia inflexa* can recover from intermittent drying events, and prior studies found that females recover more consistently than males (Stieha *et al*. 2014; Marks *et al.* 2016).

Marchantia inflexa plants for the current study were collected from five sites on the island of Trinidad, Republic of Trinidad and Tobago in May 2016. All sites were located along separate streams in the moist lowland tropical forests of northern Trinidad (Table 3.1). Forty-eight samples (comprised of a bifurcated thallus tip 1cm long) (Figure 3.1b) were collected from each site between 20 - 27th May, 2016. Plants were sampled

haphazardly along a roughly linear transect following the stream, with the restriction that no two samples were within 1 M of each other. This sampling scheme was based on prior studies showing that the probability of collecting duplicate genotypes drops to zero at distances > 0.4M (Brzyski *et al.* 2018). Upon collection, isolates were placed directly into 24 well plates, hydrated with stream water, and transported back to the William Beebe Tropical Research Station where they were kept fully hydrated under low light conditions for 24 hours prior to experimental treatments. A total of 240 isolates were collected.

Table 3.1. The name and location of collection sites in Trinidad, Republic of Trinidad and Tobago, collection date, and the number of male and female isolates collected at each site. Sites are ordered from most to least exposed. Isolate sex was determined after collection, via direct observation, induction of sex organ development, and the use of DNA sex markers.

Collection site	***Coordinates***	***Number ♂***	***Number ♀***
North Oropuche	10°40’08”N 61°08’44”W	20	26
Rio Seco	10°43’51”N 61°02’04”W	3	39
Quare	10°40’37”N 61°11’40”W	8	37
West Turure	10°41’00”N 61°10’04”W	32	15
East Turure	10°41’04”N 61°09’39”W	10	38

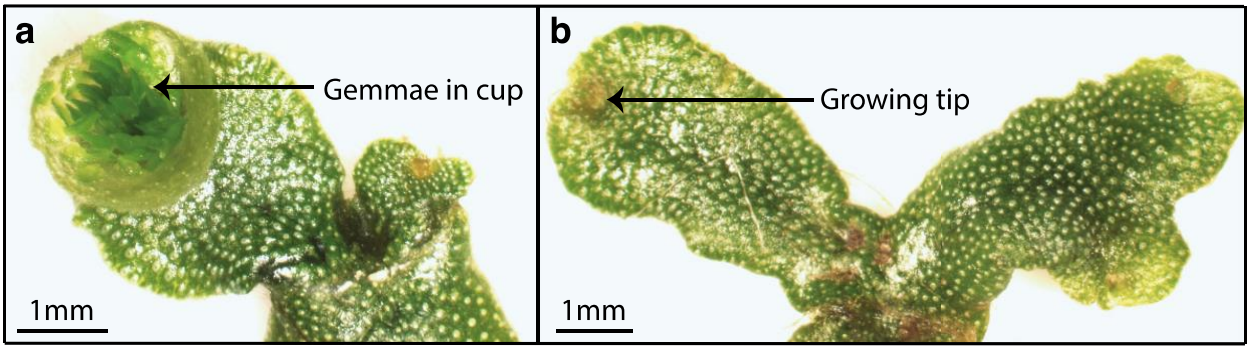

Figure 3.1. (a) *Marchantia inflexa* gemmae cup with gemmae, and (b) thallus tissue exhibiting a main bifurcation (and a secondary early stage bifurcation on the right branch). All samples collected at field sites consisted of a bifurcated thallus. One bifurcation was subjected to field dehydration treatment, and the other transported to the University of Kentucky, Lexington, KY, USA where gemmae production was induced, clones were propagated, and maintained in a common garden.

Field site characterization

Environmental differences among collection sites were characterized via canopy openness, which is a measure of exposure and has been linked to plant response in other studies (Fuselier and McLetchie 2004; Groen *et al.* 2010). Hemispherical canopy photos (taken using a Nikon CoolPix 4500 camera with a 180º lens attached) were used to quantify canopy openness. Photographs were taken at each site at the beginning, middle, and end of the sampling area. Canopy photographs were characterized with WinSCANOPY™ (Reagent Instruments, Québec, Canada) following the manufactures instructions to quantify canopy openness.

Field dehydration treatment

Plants were assayed for DhT following the protocol outlined in Marks *et al.* 2016, with a few minor changes. Following collection, plants were held in fully hydrated, low light conditions at 25.8 °C ± 0.02 for 24 hours before dehydration treatment. To initiate dehydration treatment, a randomized set of 40 out of the 48 samples from each site were selected (the remaining eight plants were used to estimate the initial status of plants for each site). One side of the bifurcation (a thallus tip) was removed and placed in a dehydration chamber. Dehydration chambers consisted of airtight plastic containers with tissue samples in individual Petri dishes placed around the interior perimeter of the chamber. Each chamber contained a total of 20 samples (10 plants each from two sites). All samples were dehydrated to equilibrium with 85% relative humidity (maintained with a saturated KCl solution (Greenspan 1977)), and the entire dehydration treatment was conducted under low light at 25.8 °C ± 0.02. Plants were allowed to dehydrate until

visibly dry (~105 hours), after which they were rehydrated. Recovery was assessed 2 weeks after rehydration by quantifying maximum potential quantum yield (F_v/F_m) of dark-adapted tissues (Krause and Weis 1984) with an OS5-FL modulated chlorophyll fluorometer (Opti-Sciences, Tyngsboro, Massachusetts, USA). A total of 197 plants were assayed.

Confirmation that plants reached equilibrium with 85% relative humidity after 105 hours was determined by measuring the change in mass of a subset of samples throughout the dehydration assay until no additional decrease in mass was observed. Mass measurements were also used to calculate the water content (WC) (see Equation 1) for a subset of plants from (North Oropuche and Quare streams) to estimate the hydration status of samples at their most dehydrated point. The precision of the scale at the field research station did not allow me to detect changes in mass of a single sample, so mass measurements were made on four groups of 10 tips. Sample fresh weight (FW) was measured at the most dehydrated condition (~105 hours after assay initiation), and sample dry weight (DW) was measured after drying samples for 3 days in an 80 °C drying oven at the University of Kentucky Lexington, KY, USA. The mean WC of samples after field dehydration treatment was 28.32 ± 2.23 g H_2O g^{-1} dry weight.

$$\mathrm{WC} = 100 * \left(\frac{(\mathrm{FW}-\mathrm{DW})}{(\mathrm{DW})}\right) \qquad \text{Eqn 1}$$

Common garden cultivation

The remaining bifurcation (thallus tip) from each isolate was transported to the University of Kentucky, Lexington, KY, USA and placed in a growth chamber at 16 °C

with a 12-hour light/dark cycle to induce gemmae (asexual propagule) production. After ~4 months, samples were removed from the growth chamber and visually inspected for evidence of gemmae production. Gemmae were used for propagation with the intention of removing field effects. Clones of each isolate were planted on steam sterilized local soil in 59 ml pots covered with neutral density acetate lids and grown in a climate-controlled greenhouse. Plants were watered daily and covered with a shade cloth to mimic field light conditions.

Common garden dehydration treatment

After plants reached sufficient size (8-12 months) they were subjected to dehydration treatment. These dehydration treatments were designed to replicate field treatments as closely as possible. Paralleling field work, thallus tips were collected from each isolate, placed directly into dH_2O, allowed to fully hydrate for 24 hours, and then placed into dehydration conditions identical to those used in the field. The only difference in treatment conditions was a reduction in ambient temperatures from 25.8 ± 0.02 to 20.5 ± 0.01 °C, which slowed drying processes slightly. This change in temperature corresponds to a reduction in vapor pressure deficit from 0.504 kPa in the field to 0.351 kPa in the common garden. Samples were dehydrated until visibly dry (~120 hours) after which they were rehydrated, and recovery was quantified 2 weeks after rehydration by measuring maximum potential quantum yield (F_v/F_m) readings of dark-adapted tissues with an OS5-FL modulated chlorophyll fluorometer (Opti-Sciences, Tyngsboro, Massachusetts, USA). More samples (n=412) were processed in common garden assays

relative to the field assays because I had access to sufficient tissue to permit replication of isolates.

To estimate dehydration intensity in the laboratory I measured the WC of a subset of dehydrated samples. Mass measurements were made on a total of 57 samples at their most dehydrated condition and again after three days in an 80 °C drying oven using a Chan 29 electrobalance, and WC was calculated using Equation 1. The mean WC of samples after laboratory dehydration treatment was 72.27 ± 7.23 g H_2O g^{-1} dry weight.

Sex expression

To identify the sex of each isolate I observed natural sex organ development in the common garden. This allowed me to determine the sex of the majority of my samples, however some plants did not produce sex organs under greenhouse conditions. For these specimens, I induced sex organ development by exposing plants to constant far-red light at 16 °C in a growth chamber. Plants that still failed to produce sex organs (n=7), were sexed using DNA sex markers for *M. inflexa,* as described in chapter 5.

Statistical analyses

Statistical analyses were conducted in JMP® version 12 (SAS Institute Inc. Cary, NC, USA). Analyses of environmental differences used a mixed effects linear model to test the fixed effect of site on canopy openness. The location of photographs (nested within site) was included in the model as random effects.

To address the four central hypotheses of this study, the primary response variable tested was $\Delta F_v/F_m$ (initial F_v/F_m - recovery F_v/F_m), with smaller $\Delta F_v/F_m$ indicating higher

DhT. Initial F_v/F_m values were measured on a subset of plants (n=8) from each site (either directly after field collection, or after cultivation in the common garden). These initial measures were used to generate a mean initial F_v/F_m for each site and growth condition, from which experimental values were subtracted to generate $\Delta F_v/F_m$. I did not take initial F_v/F_m measures on experimental samples to minimize handling of tissues, which could alter dehydration responses.

I used a mixed effects linear model to test the fixed effects of sex, site, growth condition, and all second and third order interaction effects on the response variable $\Delta F_v/F_m$. To account for different levels of replication in greenhouse and field studies, plant ID (nested within site) was included in the model as a random effect. Dehydration chamber (nested within growth condition) was also included as a random effect. Post hoc comparisons among the sexes, sites, and growth conditions were made with Tukey's Honest Significant Difference (HSD) tests.

To characterize the relationship between environment and DhT (hypothesis one) I considered the effect of site on $\Delta F_v/F_m$ and tested for a correlation between mean canopy openness and mean $\Delta F_v/F_m$ across sites. To test for genetic differences in DhT (hypothesis two), I considered only the common garden samples. I used a mixed effects linear model to test the fixed effect of site on $\Delta F_v/F_m$ in the common garden plants. To test for sex differences in DhT (hypothesis three) I looked at the effects of sex and interactions including sex in the complete data set (field and common garden plants). To address the second aspect of hypothesis three (that any fluctuation in DhT dimorphism is driven by male variability) I computed mean $\Delta F_v/F_m$ for each sex across all sites, and then tested if the variance in site means was sex-specific using a 2-sided *F*-Test. To address

hypothesis four, site-specific sex ratios (♂/(♂+♀) were calculated using the data from Table 3.1. I used the Goodness-of-fit test to examine sex ratios within each population (with the expected sex ratio of 0.5 (1♂:1♀)) and a heterogeneity test to identify significant differences among populations. Subsequently, the difference in DhT among the sexes (♂-♀ mean $\Delta F_v/F_m$) was computed for each site. I predicted that sites with significant sex differences in $\Delta F_v/F_m$ would have sex ratios significantly different from 0.5 and in favor of the sex with higher DhT, and that sites with no significant sex difference in $\Delta F_v/F_m$ would have sex ratios not significantly different from 0.5. I compared the observed data to these expectations and considered the proportion of cases in which my prediction was met.

Results

A total of 240 isolates was collected from five field sites. Of these, 197 were assayed for DhT in the field. Two hundred and twenty-eight isolates survived throughout the study, 201 of which were assayed for DhT after being cultured in a common garden (samples were replicated at least once for total of n=412 samples). The study included 155 female isolates and 73 male isolates.

The effect of site on DhT

Canopy openness ranged from 9.4% at North Oropuche to 4.5% at East Turure, with significant differences among sites ($F_{4,10} = 23.55$, $P < 0.0001$) (Figure 3.2). Recovery from dehydration ($\Delta F_v/F_m$) also varied significantly among sites ($F_{4,116} = 2.76$ $P = 0.031$) (Figure 3.3), and this effect was in the same direction as environmental

differences. Mean $\Delta F_v/F_m$ was positively correlated with canopy openness (R squared = 0.81, $F_4 = 12.9$, $P = 0.037$). Plants from the site with the most open canopy (North Oropuche) showed the least damage from dehydration, whereas plants from the site with the most closed canopy (East Turure) exhibited the most damage from dehydration.

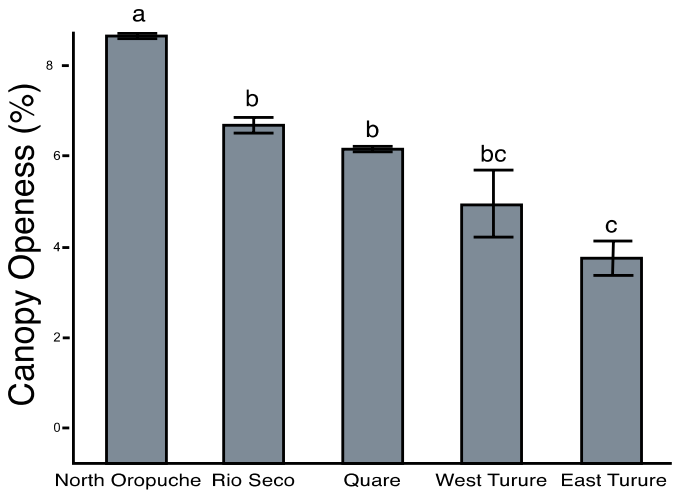

Figure 3.2. Sites (in order of most to least exposed) where *Marchantia inflexa* occurred in Trinidad, Republic of Trinidad and Tobago differed in canopy openness. Bars are standard error about the mean.

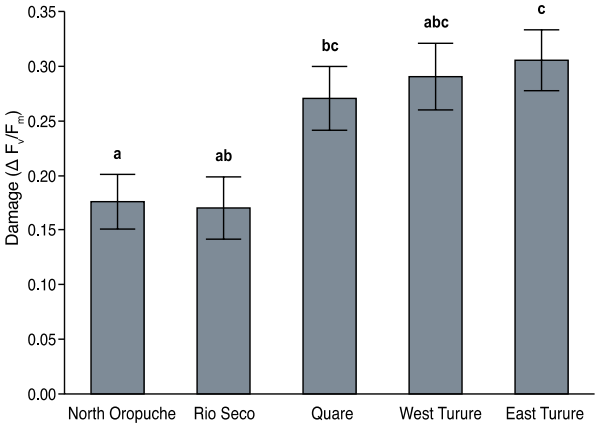

Figure 3.3. Recovery from dehydration of *Marchantia inflexa* plants collected from five streams (ordered from most to least exposed) in Trinidad, Republic of Trinidad and Tobago. Recovery from dehydration ($\Delta F_v/F_m$) was assessed by measuring maximum potential quantum yield (F_v/F_m) of dark-adapted tissues before and after dehydration treatment. Both field collected and common garden specimens are included in these analyses. Number of isolates for each site: North Oropuche (46); Rio Seco (42); Quare (45); West Turure (47); East Turure (48). Bars are standard error about the mean.

Genetic differences in DhT

To test if variability in DhT is driven by genetic differences, I considered plants grown in the common garden. There was a significant effect of site on $\Delta F_v/F_m$ ($F_{4,140}$ = 3.6 P = 0.008) (Figure 3.4). $\Delta F_v/F_m$ ranged from 0.09 ± 0.03 at Rio Saco to 0.29 ± 0.04 at East Turure.

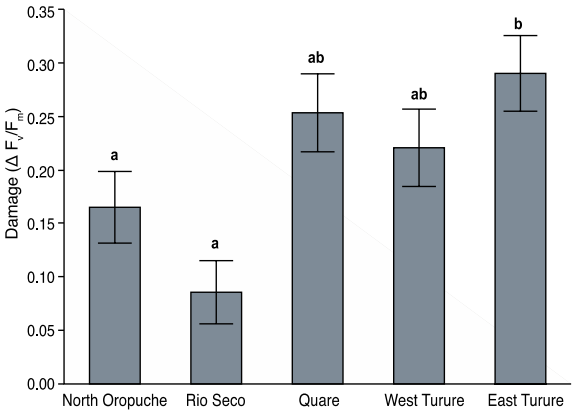

Figure 3.4. Relative damage due to dehydration ($\Delta F_v/F_m$) of *Marchantia inflexa* plants grown in a common garden. Collection sites are ordered from most to least exposed. Plants collected from the least exposed sites were more damaged by dehydration compared to plants from drier sites, even under common growth conditions. Number of isolates included in analyses: North Oropuche (40); Rio Seco (32); Quare (42); West Turure (42); East Turure (45). Bars are standard error about the mean.

Sex-specific DhT

There was a significant interaction between site and sex on $\Delta F_v/F_m$ ($F_{4,265}$ = 3.30 P = 0.012) (Figure 3.5). Specifically, males were significantly less damaged by dehydration than females from North Oropuche, the sexes did not differ in damage from Rio Seco, Quare, and West Turure, but females were significantly less damaged than males from East Turure. Further, the variance in mean $\Delta F_v/F_m$ across sites was significantly higher in males than females ($F_{4,4}$ = 10.1 P = 0.046). Mean ♀ $\Delta F_v/F_m$ ranged from 0.18 to 0.27, while mean ♂ $\Delta F_v/F_m$ ranged from 0.11 to 0.44 (Figure 3.5).

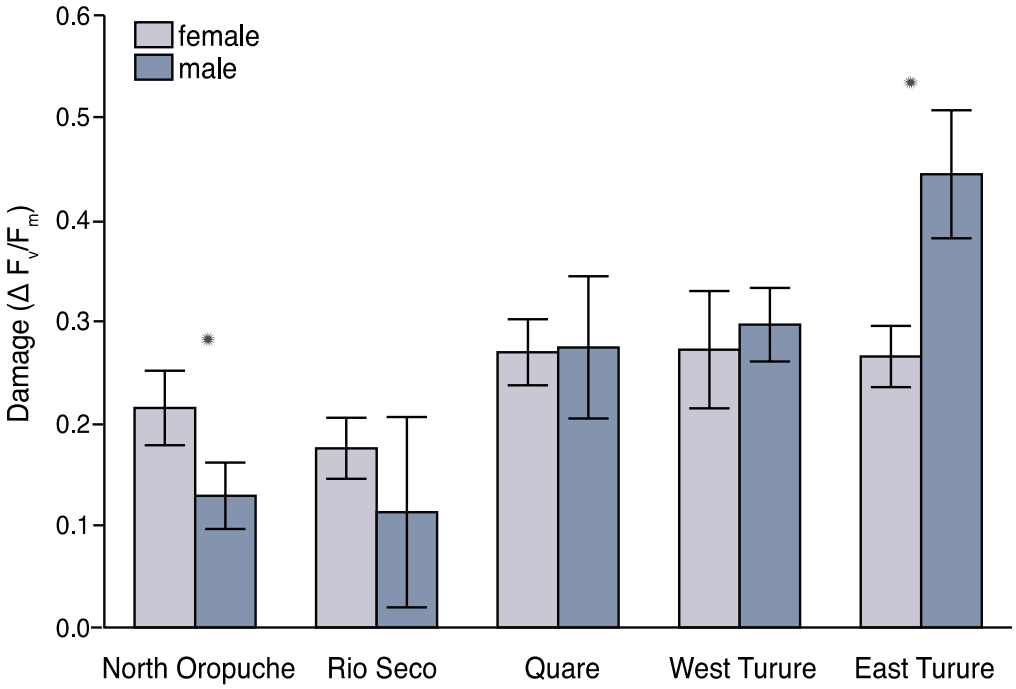

Figure 3.5. Relative damage due to dehydration ($\Delta F_v/F_m$) in *Marchantia inflexa* males and females fluctuated among the sites. Sites are ordered from most to least exposed. Males were significantly less damaged than females from North Oropuche, the sexes did not differ from Rio Seco, Quare, and West Turure, and females were significantly less damaged than males from East Turure. Both field and common garden data are included in the analysis. Number of isolates at each site: North Oropuche (♀=26, ♂=20); Rio Seco (♀=39, ♂=3); Quare (♀=37, ♂=8); West Turure (♀=15, ♂=32); East Turure (♀=38, ♂=10). Bars are standard error about the mean.

Population sex ratios

Overall populations sex ratios were heterogeneous, ranging from 0.058 to 0.64 (G_4 = 45.43, $P < 0.0001$). The sex ratio at North Oropuche did not differ from 0.5, Rio Seco, Quare, and East Turure were significantly female biased ($P < 0.0001$), while the sex ratio at West Turure had a tendency to be male biased ($P = 0.051$) (Table 3.2). Despite the fluctuating sexual dimorphism in DhT, my prediction that sex ratios would be biased favor the sex with higher DhT was met in only one out of the five populations (Table 3.2).

Table 3.2. The difference in DhT between males and females (♂-♀ mean $\Delta F_v/F_m$), and the population sex ratio (♂/(♂+♀) for each site was computed. Sites with significant sex differences in DhT and population sex ratios significantly different from 0.5 are indicated with an *. Based on the hypothesis that population sex ratios would be biased in favor of the sex with higher DhT, I predicted that the sex ratio would be male biased at North Oropuche, not significantly different from 0.5 at Rio Seco, Quare, and West Turure, and female biased at East Turure. This prediction was met in only one out of the five cases (East Turure), suggesting that DhT does not predict sex ratio.

Collection site	***Sex difference in DhT***	***Predicted sex ratio***	***Observed sex ratio***
North Oropuche	-0.09*	> 0.5	0.43
Rio Seco	-0.06	0.5	0.07*
Quare	0.00	0.5	0.18*
West Turure	0.02	0.5	0.68*
East Turure	0.44*	< 0.5	0.21*

Discussion

My results indicate that *M. inflexa* harbors substantial intraspecific variation in DhT. As expected, I found that DhT is positively associated with environmental exposure in plants collected from five natural sites in the tropical forests of northern Trinidad, Republic of Trinidad and Tobago. This pattern is driven by underlying genetic differences among populations, as indicated by retention of population-specific DhT differences in common growth conditions. More interestingly, I found that population differences in DhT are sex-specific. In less exposed sites females have higher DhT than males, but in more exposed sites males have higher DhT than females. Although this fluctuating sexual dimorphism in DhT is intriguing and suggests that the sexes are responding to environmental gradients differently, it does not scale up to population sex ratios.

My analyses detected significant differences among sites in DhT. These differences in DhT are highly correlated with canopy openness (exposure), providing evidence of local adaptation in DhT. Although not entirely surprising, the observation

that plants collected from the most exposed sites have higher DhT than plants from the least exposed sites indicates that plants respond to contrasting selective pressures in their local environment. Others have shown that variation in DT corresponds with environmental changes (Dilks and Proctor 1976; Beckett and Hoddinott 1997; Stark *et al.* 2007; Farrant *et al.* 2009), but most studies do not explicitly test for genetic differences among plants. This study adds to this body of work by establishing a correlation between genetic differences in DhT and environmental exposure in *M. inflexa*. Previous work suggested that plasticity in DhT may also play a role in this system ((Marks *et al.* 2016). However, data from the current study indicate that differences in DhT in *M. inflexa* are due to genetic differences driven by spatially variable selection. It is worth noting that absolute differences in DhT among common garden plants were slightly reduced relative to field plants. Therefore, I speculate that genetic differences in DhT are enhanced by plasticity, but additional studies will be needed to formally test this hypothesis.

I found that DhT is sexually dimorphic, but that sex differences in DhT fluctuate among populations, with females exhibiting higher DhT than males in less exposed sites and males exhibiting higher DhT than females in more exposed sites. Prior work in *M. inflexa* showed that females had higher DhT than males at East Turure (Marks *et al.* 2016), and that finding is confirmed in the current study. However, here I found that males at North Oropuche exhibit higher DhT than females, suggesting that sex differences in DhT are more complex than previously appreciated. As mentioned, DhT is not the first trait to display a fluctuating sexual dimorphism in *M. inflexa* (Groen, Stieha, Crowley, and David Nicholas McLetchie 2010; Brzyski *et al.* 2014), and other dioecious plants exhibit sexually dimorphic traits that fluctuate in response to environmental

conditions (Eppley 2006; Dudley and Galen 2007; Strømme *et al.* 2018). I speculate that this fluctuating dimorphism is driven by contrasting selective pressures on males and females, and possibly exacerbated by intra-sexual competition. Differences in reproductive resource allocation between males and females, may also contribute to patterns of fluctuating sexual dimorphisms. Higher reproductive costs for females compared to males can lead to sex-specific tradeoffs between reproduction and other plant functions (Dawson and Geber 1999; Dudley and Galen 2007; Retuerto *et al.* 2018), and this can be accentuated under stressful conditions. In the context of the current study, I speculate that *M. inflexa* males may experience little to no selection for DhT in the less exposed sites because their reproductive resource requirements are met well before the onset of drying events. Females, on the other hand, require more time in hydrated conditions to meet their elevated resource demands and are therefore exposed to selection for DhT. However, in the drier more exposed sites, selection for DhT will be extended to males and may become so extreme that females are unable to mature offspring. Over time, this stress could lead to a reduction of females in exposed sites, as has been suggested in other taxa (Freeman *et al.* 1976; Dawson and Ehleringer 1993; Eppley 2001) and an increase in local male DhT. Residual females present in these exposed habitats may result from recent colonization events and consequently, would not exhibit elevated stress tolerance. The finding that sex differences in stress tolerances do not consistently favor males or females (Retuerto *et al.* 2018; Strømme *et al.* 2018) may be due to insufficient across population studies coupled with fluctuating sexual dimorphisms.

Interestingly, the fluctuating sexual dimorphism in DhT in *M. inflexa* is driven primarily from male variability. I found that females have similar DhT across all sites,

while males display high variably in DhT across sites. I speculate that females are relatively constrained by the resource demands associated with offspring maturation and therefore exhibit low variability in DhT. In contrast, male reproductive biology is more permissive and there may be multiple viable male strategies. It is possible that high variability in males could lead to the development of contrasting male phenotypes over time. There are many examples of animal taxa where multiple male "types" have been identified (Liley 1966; Hayashi 1985; Sinervo and Lively 1996; Taborsky 1998; Zamudio and Sinervo 2000), but I am unaware of studies describing multiple male types in dioecious plants (but see Moore *et al.* 2016). I speculate that similar evolutionary pressures (as in animals) could generate multiple male types in dioecious plants. My recent genomic work also points to high male variability, showing that genes on the male-specific V chromosome of *M. inflexa* are diversifying (relative to *Marchantia polymorpha*) faster than female-specific U chromosome genes (chapter 5). These studies provide preliminary evidence for the existence or development of multiple male types in *M. inflexa*.

I found no relationship between sex differences in DhT and population sex ratios, suggesting that sex-specific DhT does not predict population sex ratio. *Marchantia inflexa* population sex ratios can be highly variable (they range from 0 to 1 in Quare stream (Brzyski *et al.* 2018)) and are likely impacted by numerous ecological and stochastic factors, such as the size of the substrate (Brzyski *et al.* 2018), sex-specific life history traits (García-Ramos *et al.* 2007), and spatial distribution of populations (Stieha *et al.* 2017). Sexual dimorphisms in colonization, establishment, growth, and asexual

reproduction also likely impact population sex ratio, so the observation that sex differences in DhT do not predict population sex ratios is not entirely surprising.

In conclusion, this study builds on prior studies of DhT in *M. inflexa* and demonstrates that patterns of DhT are more complex than previously described. I show that DhT varies along an environmental gradient and that population-specific variation in DhT is genetically determined. Evidence of genetic variation in DhT among *M. inflexa* populations and the sexes suggests that local adaptation is occurring. This adaptive response may enhance population persistence and contribute to ecological stability. Prior studies found that females were more DhT than males (Stieha *et al.* 2014; Marks *et al.* 2016), but these conclusions were based primarily on a single population (Marks *et al.* 2016), or specialized tissues (Stieha *et al.* 2014). Here, I showed that sex differences in DhT vary among populations, with males exhibiting higher DhT in some sites, while females maintain higher DhT in others. This finding highlights the importance of population-specific assessments of stress tolerance and demonstrates that sexual dimorphisms can fluctuate in response to environmental conditions. Additional studies of intraspecific variation in DhT in other species will support more generalizable conclusions.

CHAPTER FOUR

THE DEHYDRATION - REHYDRATION TRANSCRIPTOME OF MARCHANTIA INFLEXA

Introduction

Climate change, increased consumption of resources by humans, and changing land use practices impose considerable abiotic and biotic challenges on plants and the ability of plants to respond and tolerate these stresses has important implications for ecosystem function, community dynamics, and agricultural productivity (Lesk *et al.* 2016). To better predict future outcomes, a comprehensive understanding of the evolution of plant stress tolerance is needed. An important step towards this end is the characterization of physiological, biochemical, and genetic mechanisms of stress tolerance, which will improve ecological predictions, natural resource management, industry practices, and agricultural optimization.

Drought, in particular, is a major threat to plant productivity and ecosystem function (Lesk *et al.* 2016). Drought is responsible for considerable economic losses annually, and it is predicted to increase due to climate change (Trenberth 2011; Dai 2013; Lesk *et al.* 2016). Consequently, there is an urgent need to characterize the mechanisms of water stress tolerance to mitigate drought induced losses. Again, turning to naturally-evolved mechanisms of tolerance offers a promising approach for gaining insight into adaptations that can be used to optimize water stress tolerance in managed systems and secure plant-based industries. Desiccation tolerance (DT, also desiccation tolerant) is an extreme form of water stress tolerance with promising translational applications. Most plants are desiccation sensitive (DS) and cannot survive drying below -5 to -10 MPa (Proctor and Pence 2002; Proctor *et al.* 2007), but DT tissues can recover from drying to

or below and absolute water content of -100 MPa (Black and Pritchard 2002). DT is common in plant seeds, spores, and pollen, but is rare in vegetative tissues (Dinakar and Bartels 2013a). DT is a highly complex trait, dependent on the synchronized orchestration of a diverse set of physiological and molecular processes, including photosynthetic regulation, metabolic adjustment, and the accumulation of protective compounds (Walters *et al.* 2002; Moore *et al.* 2009).

To progress towards applied objectives, a detailed understanding of how changes in cell wall composition, carbohydrate metabolism, accumulation of protective proteins, antioxidant systems, and signal transduction pathways impact DT is essential. Dehydration places considerable mechanical strain on cell walls and membranes because cell shrink during drying and expand upon rehydration, which can lead to membrane rupture (Dinakar *et al.* 2012). Flexible cell walls that can undergo organized folding during dehydration reduce mechanical strain by accommodating cytoplasmic shrinkage (Platt *et al.* 1997). Thus, cell wall characteristics that increase flexibility including xyloglucan and pectin modifications, elevated arabinan and arabinogalactan levels, and calcium ion redistribution (Vicre *et al.* 2004; Moore, Vicré-Gibouin, *et al.* 2008) may increase DT.

DT plants alter carbohydrate metabolism during drying to preferentially accumulate small non-reducing sugars, which are thought to protect cells by stabilizing membranes and macromolecules though vitrification (Dinakar *et al.* 2012). In particular, raffinose, trehalose, and sucrose are observed in high quantity in many DT plants during dehydration (Smirnoff 1992; Illing *et al.* 2005; Liu *et al.* 2008). Evidence of increased sucrose synthase, sucrose phosphate synthase, and glyceraldehyde phosphate

dehydrogenase in the model DT plant *Craterostigma plantagineum*, points towards a genetic mechanism for modulating sugar composition that is induced during drying (Ingram *et al.* 1997).

In addition to sugars and compatible solutes, the accumulation of numerous protective proteins (such as late embryogenesis abundant (LEAs) proteins, early light inducible proteins (ELIPs), and heat shock proteins (HSPs)) is common in DT plants. These proteins are thought to support DT by replacing water to stabilize membranes and sub-cellular organization (Dinakar *et al.* 2012), protecting against photooxidative damage (Vanburen *et al.* 2019), and acting as chaperones. LEA proteins have received considerable attention because of their characteristic accumulation during drying in DT plants (Costa *et al.* 2017). Although the precise role of LEA proteins in DT is unclear, their transition between an intrinsically disordered structure when hydrated to a α-helical structure when dehydrated may play an important role in membrane and macromolecule stabilization (Bremer *et al.* 2017; Artur *et al.* 2018). ELIPs are also induced during dehydration in DT plants and may serve an important function in preventing oxidative damage during dehydration (Zeng *et al.* 2002). Recent evidence shows that ELIPs have undergone considerable expansion in DT lineages relative to DS lineages, suggesting a central role for ELIPs in DT (Xiao *et al.* 2015; Vanburen *et al.* 2019).

Antioxidant systems also play an important role in DT. Drying in the presence of light can lead to increased production of reactive oxygen species (ROS), especially in photosynthetic tissues (Dinakar *et al.* 2012). ROS formation is inevitable even under non-stressed conditions due to the leakage of electrons onto O_2 during electron transport activities of chloroplasts and mitochondria (Choudhury *et al.* 2017). At low levels, ROS

are not damaging and may even act as important signaling molecules (Choudhury *et al.* 2017). However, ROS can accumulate to potentially damaging levels under dry conditions due to the disruption of cellular homeostasis and the resulting build-up of reactive metabolic intermediates (Sharma *et al.* 2012). DT plants have numerous mechanisms to reduce damage from ROS, including the synthesis and mobilization of protective pigments, enzymatic, and non-enzymatic antioxidants (Dinakar *et al.* 2012). Specific ROS scavenging compounds detected in DT plants include anthocyanin and carotenoid pigments; enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, aldehyde dehydrogenase, monodehydroascorbate and dehydroascorbate reductase, glutathione peroxidase and reductase; and non-enzymatic antioxidants glutathione and ascorbate (Dinakar *et al.* 2012).

Finally, signal transduction pathways play a critical role in allowing plants to perceive and respond to changes in hydration and the fine tuning of these pathways is likely essential for DT. Changes in ROS production are thought to be an initial signal of dehydration stress and subsequent stress signaling is likely coordinated by abscisic acid (ABA) (Ingram and Bartels 1996). Protein phosphorylation cascades, secondary messengers, and transcription factors in the myeloblastosis (MYB), leucine zipper, and zinc finger families are induced in response to dehydration (Deng *et al.* 2002; Ditzer and Bartels 2006; Dinakar *et al.* 2012).

Interestingly, the underlying genetic machinery involved in DT is present in many plant species (most have DT seeds, spores, or pollen), and aspects of DT are induced in DS plants during drying. For example, nearly all plants exhibit increased antioxidant activity under stress, accumulate some LEA proteins, and modulate their metabolic

profile to preferentially accumulate small sugars (Farooq *et al.* 2009). However, most plants fail to attain DT in their vegetative tissues, suggesting that slight differences in magnitude, sequence, and timing of cellular responses can confer or abolish DT. Consequently, to predict or manipulate DT I must understand not only the physiological pathways, but also the temporal orchestration of DT, and the consequences of nuanced variation across species, tissues, and developmental stages.

To pinpoint characteristics that increase DT, variability in DT should be characterized. This can, and should, be done on multiple levels. Initially, the different degrees of tolerance (from DS to DT) should be carefully defined, and the progressive changes in physiology, biochemistry, and genetic mechanisms along this spectrum should be catalogued. While studies comparing DS and DT plants exist, currently little is known about the intermediate levels of tolerance. A thorough understanding of intermediate tolerance (known as dehydration tolerance (DhT)) may allow for insight into the progressive differences between DT and DS plants. There is a small but important group of plants that exhibit DhT and can survive drying to -10 MPa (Oliver *et al.* 2010; Marks *et al.* 2016). Additional studies of DhT plants will provide valuable insight into the mechanisms of water stress tolerance. Additionally, few studies have described the variation in DT of DhT within a species (but see (chapter 3.; Oliver *et al.* 1993; Farrant and Kruger 2001; Stark *et al.* 2007; Farrant *et al.* 2009; Marks *et al.* 2016). Within species variation provides an important resource for identifying DT enhancing characteristics across a common genetic background, and by studying within species variation in DT it may be possible to tease apart the contribution of plasticity vs. genetic differences and gain insight into the adaptive potential of populations and species.

Here, I aimed to characterize gene expression during dehydration in the tropical DhT liverwort *Marchantia inflexa*. To do so, I quantified changes in gene expression throughout the dehydration – rehydration process. This allowed me to gain a broad overview of the timing and nature of cellular changes during dehydration, which I discuss in comparison with highly DT plants. In addition, I leveraged intraspecific variation in DhT to target more nuanced variation that may contribute to differences in relative DhT between the sexes and tissue types of *M. inflexa*. From theses comparisons, I targeted genes that were differentially expressed during the dehydration - rehydration process and also differentially expressed among the sexes or tissues as possible candidates for explaining differences in relative DhT.

Methods

Study organism and plant growth

Marchantia inflexa (Nees & Mont) is a new world liverwort that is distributed from northern Venezuela to the southern United States (Bischler 1984). *Marchantia inflexa* typically grows along streams in tropical forests but can also colonize more disturbed sites along roads. The dominant life stage is haploid, and plants grow as a dichotomously branching thallus with dorsiventral organization. *Marchantia inflexa* has unisexual individuals that can reproduce sexually (via spores) or asexually via vegetative fragmentation or the production of specialized asexual propagules (gemmae). *Marchantia inflexa* exhibits numerous sexual dimorphisms (growth rate, asexual reproduction (McLetchie and Puterbaugh 2000; Brzyski *et al.* 2014), response to exposure (Groen, Stieha, Crowley, and David Nicholas McLetchie 2010; Groen, Stieha, Crowley, and D.

Nicholas McLetchie 2010), substitution rates of sex-specific genes (chapter 5), and differences in DhT among the populations and sexes (chapter 3; Marks *et al.* 2016).

Plants for this study were collected in 2009 from a natural population along East Turure River (10º41'04"N 61º09'39") in Trinidad, The Republic of Trinidad and Tobago. Specimens were vouchered at the Missouri Botanical Garden (St Louis, MO, USA, specimen numbers M092113 and M092115) and at the National Herbarium of the Republic of Trinidad and Tobago (St Augustine, Trinidad, specimen number TRIN34616, D. N. McLetchie, collector). Plants were transported to the University of Kentucky, and the genetic uniqueness of each line was confirmed by microsatellite analyses (Brzyski *et al.* 2014). Multiple clones of each genotype were propagated via vegetative fragmentation, watered daily, kept under shade cloth, and maintained in greenhouse conditions for 8 years prior to this study.

Physiological characterization of sex and tissue differences in DhT

Prior studies found that females from East Turure had higher DhT than males (Marks, Pike, *et al.*; Marks *et al.* 2016). I selected the three most DhT ♀s and the three least DhT ♂s for inclusion in the current work. In addition, prior work indicated that the meristematic tissue of *M. inflexa* appeared to be more DhT than the mature thallus tissue. To test this hypothesis, I quantified the relative DhT of these two tissue types by subjecting a subset of tissue samples (n=33) to a dehydration treatment (as described below) and documenting the proportion and location of recovered vs. dead tissues using ImageJ (Schneider *et al.* 2012). Tissues were characterized 2 weeks after rehydration and survival was assessed on the basis of green vs. brown tissues. Recovery ranged from

1.5% to 97.8% survival. In all instances, the meristematic region was among the recovered portions of the sample. Tissue death was increasingly likely as distance from the meristem increased, with the most distal region recovering only 6% of the time.

Dehydration treatment, tissue collection, and RNA extraction

Plants were subjected to dehydration treatment as described in Marks *et al.* (2016) and tissues were sampled at predetermined times throughout the dehydration-rehydration process. Briefly, thallus tissues (~5 mm x 7 mm) were harvested from greenhouse cultivated plants, fully hydrated for 24 hours, and placed into desiccation chambers with an internal relative humidity (RH) of 75%. Air circulation was maintained by inserting a small fan in the chamber, and RH was verified with a RH sensor integrated into a HOBO™ humidity sensor attached to a data logger (Onset Computer Corporation, Bourne, MA, USA). Each desiccation chamber contained 18 samples (three samples from each of the six genotypes). Samples were randomized and placed into Petri dishes within in the desiccation chamber. The chamber was maintained at 14^{o}C and plants were dehydrated for 22 hours, after which they were rehydrated with dH_2O and kept at 14^{o}C for an additional 24 hours.

Plants were sampled at five timepoints during the dehydration – rehydration process: fully hydrated (baseline), partially dehydrated at 15 hours (Dh15), fully dehydrated at 22 hours (Dh22), 2 hours after rehydration (Re2), and 24 hours after rehydration (Re24). At each hydration state, tissues were removed from the desiccation chamber and dissected into meristematic vs. thallus components using a 4 mm sterile biopsy punch. Samples were immediately flash frozen in liquid nitrogen to prevent

further transcriptional changes, RNA was extracted from samples using the Triazol®Reagent according to the manufacturer's instructions, and stored at -80ºC until needed. Because this sampling approach is destructive in nature, samples were collected and processed over multiple independent dehydration assays until every biological replicate was represented at each hydration state. Dehydration assays were conducted at designated times of day to reduce off target variation due to fluctuations in light, temperature, and circadian rhythms, and biological replicates (genotypes) were randomized for all downstream processing. To verify sample recovery, two samples were removed from each dehydration chamber at Dh22 and rehydrated. These plants were tested for recovery 1 week after rehydration using F_v/F_m measured with a modulated chlorophyll fluorometer.

Library preparation, sequencing, and preassembly read processing

Sixty sequencing libraries were prepared from RNA samples (two tissues types from three ♂s and three ♀s at five different hydration states) following the protocol described in Hunt, (2015). Briefly, mRNA was isolated through poly(A) enrichment with the NEBNext® Poly(A) mRNA Magnetic Isolation Module. The resulting samples were heat fragmented at 95ºC for 2 minutes, and barcodes were integrated during first strand cDNA syntheses with random primers. A strand switching primer was used for second strand cDNA synthesis, ultimately generating stranded libraries in a reverse-forward (RF) format. Libraries were size-selected using Magbio beads for a target fragment length of ~500 base pairs (bp) and enriched by 18 PCR cycles. Library concentration was assessed with a Qubit dsDNA High Sensitivity Assay Kit, and fragment size was measured with a

Bioanalyzer High Sensitivity DNA chip. Samples were pooled in equal concentration and sequenced on four lanes of an Illumina HiSeq4000 for 150 bp paired end (PE) reads at the University of California Davis Genome Center. All samples were completely randomized during library preparation and sequencing to minimize batch effects. The resulting sequence reads were demultiplexed with Sabre v1.000, quality assessed using FastQC v0.11.2, and trimmed with Trimmomatic-0.30_2 to remove adapter sequences and low-quality reads.

Transcriptome assembly, refinement, and quality assessment

All sequencing reads were pooled and assembled with Trinity-v2.6.6 following the genome guided pipeline (Schneider *et al.* 2003). Briefly, RNAseq reads were aligned to the *M. inflexa* draft genome assembly (chapter 5) using Bowtie2-2.3.4.1. Reads that did not map to the *M. inflexa* genome were removed prior to assembly and appeared to be derived from fungal symbionts. Transcriptome assembly with Trinity-v2.6.6 specified options for genome guided assembly, RF library format, and a max intron length of 10,000 bp. The initial assembly consisted of 50,135,468 assembled bp distributed across 93,958 Trinity transcripts with an N50 of 693 bp. Because the large number of assembled transcripts suggests redundancy in the assembly, I sought to eliminate poorly supported transcripts and improve confidence in gene identity. To do so, RSEM-1.3.0 (Li and Dewey 2011) was implemented to calculate sample-specific expression for all assembled transcripts. The resulting gene expression matrix was used to remove all transcripts with less than one mapped read using the Trinity script filter_low_expr_transcripts.pl. The resulting transcriptome was used for all subsequent analyses. Finally, to estimate

assembly completeness, I quantified the percentage of Universal Single-copy Orthologs from the plant set of OrthoDB v9 of BUSCO v3 (Simão *et al.* 2015) present in this *M. inflexa* transcriptome assembly.

Functional annotation

Functional annotation of *M. inflexa* transcripts was accomplished by implementing the Trinotate annotation pipeline (Haas *et al.* 2013). Trinotate uses multiple methods for functionally annotating transcripts, including homology searches, identification of protein domains, and information from annotation databases. Initially, transdecoder (https://github.com/TransDecoder) was used to identify protein coding regions for each *M. inflexa* transcript, HMMER (Eddy 2011) was implemented to define Pfam protein domains (Finn *et al.* 2014), and BLAST+ (Altschul *et al.* 1990) was utilized for homology searches against UniProtKB/Swiss-Prot, and reference genomes (downloaded from phytozome (Goodstein *et al.* 2012)) for *Arabidopsis thaliana* (Initiative 2000) , *Physcomitrella patens* (Rensing *et al.* 2008), and *Marchantia polymorpha* (Bowman *et al.* 2017). Gene ontology (GO) terms were associated with each *M. inflexa* transcript based on BLASTX homology searches, and all annotation information was integrated with SQLite (https://www.sqlite.org) to generate a combined annotation report. If homology could be determined, *M. inflexa* transcript names were updated to reflect annotation information by appending *M. polymorpha* and UniProtKB/Swiss-Prot gene names to the *M. inflexa* Trinity transcript ID. If homology could not be determined, transcript names were not modified. To summarize general patterns of gene function, I identified the most common GO terms across the entire *M.*

inflexa transcriptome using REVIGO (Supek *et al.* 2011) and the most prominent protein classes using PANTHER (Mi *et al.* 2017).

Characterization of global gene expression and identification of differentially expressed genes

RSEM-1.3.0 (Li and Dewey 2011) was implemented to calculate sample-specific gene expression. Reproducibility among samples and biological replicates was assessed via principal component analyses (PCA) of TMM normalized gene expression values with the Trinity script PtR.pl. Gene expression data were log2 transformed prior to PCA, and genes with less than 10 total counts were excluded prior to clustering.

To test for genes that were differentially expressed over time, I implemented edgeR (Robinson *et al.* 2010) using the Trinity script run_DE_analysis.pl. Gene count data (obtained from RSEM) were normalized within edgeR. To characterize overall temporal patterns, I identified genes that were significantly up- and down-regulated at each hydration state (Dh15, Dh22, Re2, Re24) relative to baseline conditions. To characterize baseline plants, I identified genes that were significantly up- and down-regulated at baseline relative to all other times combined. The resulting differentially expressed genes (DEGs) were analyzed to characterize changes throughout the dehydration – rehydration process, and similarity among samples was assessed via hierarchical clustering analyses implemented with the Trinity script analyze_diff_expr.pl.

To test for general differences in gene expression between the sexes and tissues, I identified genes with significant differences in expression between males and females and meristem and thallus tissues across all hydration states. These DEGs represent

transcripts that show consistent sex- or tissue-specific expression throughout the dehydration – rehydration process. Clustering analyses of gene and sample relationships were applied to sex and tissue DEGs by implementing the Trinity script analyze_diff_expr.pl, allowing me to identify genes with similar expression patterns and characterize sample relationships.

Functional profiling of differentially expressed genes and analysis of gene ontology enrichment

To characterize the primary functional processes induced during the dehydration – rehydration process and identify functional differences among the sexes and tissues, I conduced GO enrichment analyses. I identified the most enriched (more abundant than expected) GO categories among dehydration – rehydration, sex, and tissue DEGs. GO terms were defined with Trinotate, as described above. Functional enrichment of GO categories was determined implementing the R package GOseq (Young *et al.* 2010) with the Trinity script analyze_diff_expr.pl and the option --examine_GO_enrichment. I identified enriched GO categories for both up- and down-regulated genes at each hydration state, between the sexes, and between tissue types. GO terms were considered significantly enriched at p-value < 0.05.

Identification of candidate DhT associated genes and targeted tests of intraspecific variation

Genes that were differentially expressed during the dehydration - rehydration process and also differentially expressed among the sexes or tissues were of particular

interest to me based on my hypotheses that differences in gene expression between the sexes and between the tissues may explain differences in relative DhT. To identify such genes, I targeted transcripts that were significantly up- or down-regulated in response to dehydration and rehydration and also significantly up- or down-regulated among the sexes or tissues. From these, I specifically identified those that were up-regulated in the more tolerant samples (females and meristems) as possible DhT enhancing genes.

Finally, to gain insight into the temporal differences in gene expression between the sexes and between the tissues, I conducted targeted comparisons to identify DEGs in males vs. females and in meristem vs. thallus tissues at each individual hydration state.

Results

Transcriptome assembly statistics

Sequencing of 60 RNAseq libraries (two tissues types from three ♂s and three ♀s at five different hydration states) resulted in 1,342,651,063 PE reads. After assembly and removal of poorly-supported transcripts, the *M. inflexa* dehydration – rehydration transcriptome consisted of 26,977 transcripts (21,704,334 assembled bases) with an N50 of 1,039. Assessment of transcriptome completeness indicated that a relatively low proportion of the expected universal single copy orthologues were present in this assembly (19.5% complete, and 9.9% fragmented), which may reflect inherent limitations of applying BUSCO to deeply-derived lineages as has been noted in prior work (Smith *et al.* 2018). Alternatively, because I did not sample a comprehensive panel of developmental stages and tissues (i.e. sporophyte, reproductive tissues, and gemmae), this transcriptome may not capture the full suite of *M. inflexa* genes.

Functional annotation

Functional annotation with Trinotate identified homologues for 21,501 of the 26,977 (79.7%) *M. inflexa* transcripts across UniProtKB/Swiss-Prot, *A. thaliana, P. patens,* and *M. polymorpha*, which is similar to annotation percentages in other non-model species (Gao *et al.* 2017; Rathi *et al.* 2019). Of these, GO terms were assigned to 16,309 transcripts. Summary of GO terms revealed that the most common GO categories (biological process) in the *M. inflexa* transcriptome were regulation of transcription, carbohydrate metabolism, cell cycle, and multicellular organism development. Other GO terms related to DT were detected, including cellular response to desiccation, lipid transport and metabolism, protein ubiquitination, and heat acclimation. The most abundant protein classes represented in the *M. inflexa* transcriptome were nucleic acid binding, hydrolase, transferase, oxidoreductase, and transporter classes.

Characterization of global gene expression and identification of differentially expressed genes

PCA analysis of global gene expression was conducted to characterize broad patterns of similarity among samples. The first two PCAs accounted for 17.21% (13.28, and 3.93% respectively) of the variance in gene expression (Figure 4.1). All baseline samples appeared to exhibit similar gene expression patterns, but other hydration states were less distinct. Dh15 and Dh22 were hardly distinguishable from one another, and Re2 and Re24 samples only showed moderate segregation along PC2 and PC1.

Additional PCAs did not provide further insight into sample relationships. There were weak patterns of association among the sexes and tissues (not shown).

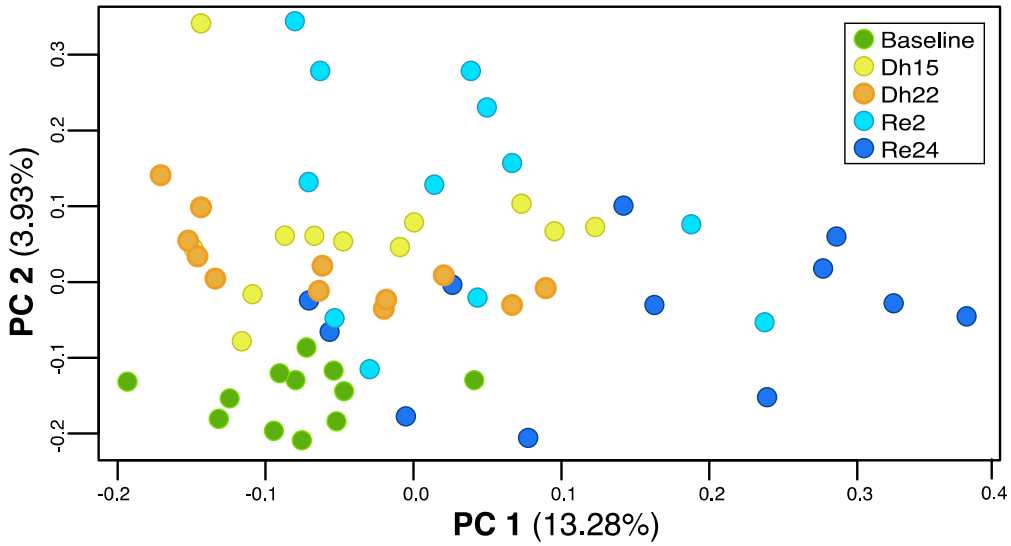

Figure 4.1. Principal component analyses of similarity in global gene expression among all samples. The hydration condition of samples is represented by color. Clustering of samples indicates similarity in overall gene expression. Negligible differentiation among samples was evident along additional PCs.

To investigate temporal differences in gene expression during the dehydration – rehydration process, I identified genes that were significantly up- or down-regulated at each hydration state relative to baseline conditions. This analysis detected a total of 438 genes with significant changes in expression (FDR p-value <0.05) in response to changing hydration status. Two-way clustering of gene and sample relationships revealed complex and variable patterns of gene expression (Figure 4.2). Similar to PCA analyses, baseline samples had the most consistent expression profiles. Re24 samples exhibited relatively consistent gene expression patterns across samples. Gene expression profiles of plants during Dh15, Dh22, and Re2 appear to be less distinct than those of baseline and Re24 plants, possibly related to a lack of temporal regulation under dehydrated conditions, or complex patterns among the sexes, tissues, and genotypes (Figure 4.2).

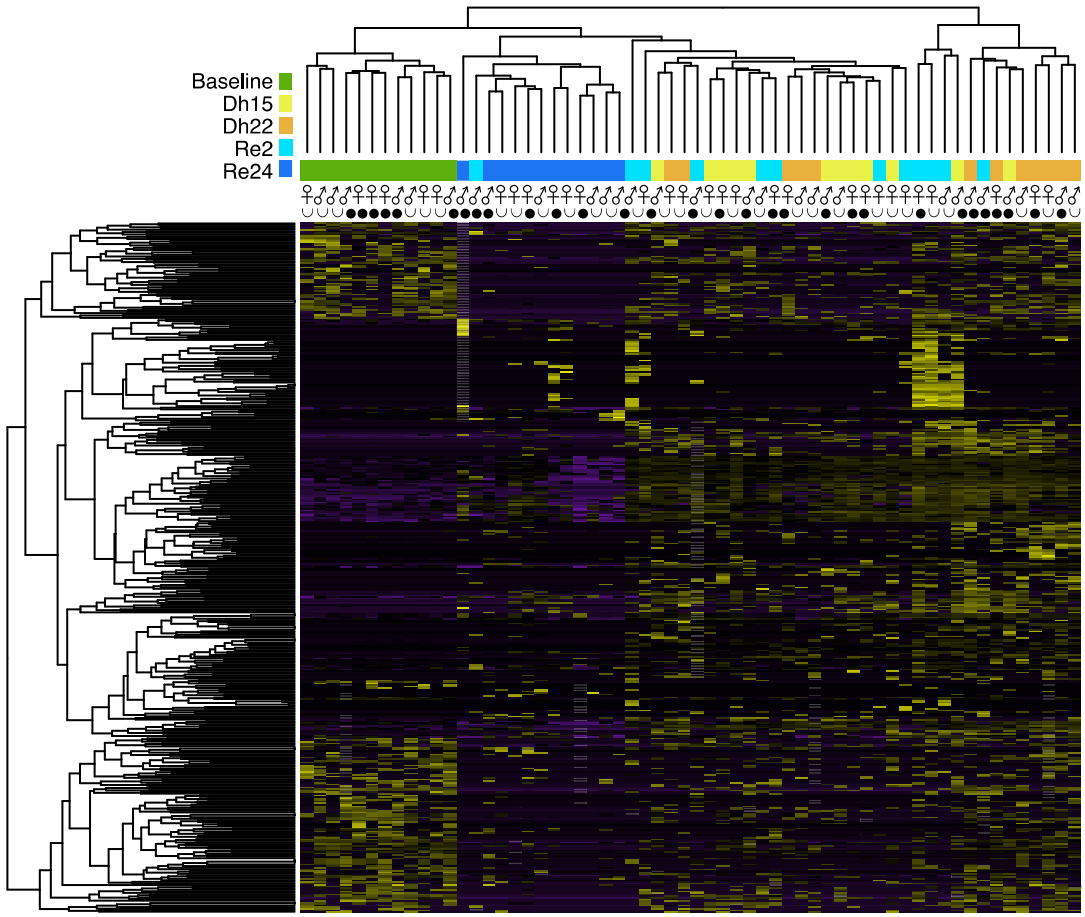

Figure 4.2. Hierarchal clustering of hydration responsive genes (differentially expressed across time (FDR P-value < 0.05)) and secondary clustering of sample relationships. Each column represents a single sample, and each row represents a single transcript. Expression values are indicated by color, with yellow indicating relatively higher expression, and purple indicating relatively lower expression. Hierarchal sample relationships are shown along the top x-axis and gene relationships are shown on the left y-axis. Colored boxes are used to indicate hydration state, male (♂) and female (♀) symbols indicate sample sex, and meristem (•) and thallus (∪) symbols indicate tissue type.

Subsequently, I investigated differences in gene expression between the sexes and between the tissues across all hydration states. These analyses identified 213 genes that were differentially expressed among the sexes (FDR p-value < 0.05), 199 of which were up-regulated in males and 14 were up-regulated in females. Based on homology with *M. polymorpha* the majority of these DEGs are putatively located on sex chromosomes. A parallel comparison of meristematic and thallus tissues identified 926 genes that were

differentially expressed among the tissues across all hydration states. Of these, 884 were consistently up-regulated in meristems and 42 were up-regulated in thallus tissues.

Functional profiling of differentially expressed genes and gene ontology enrichment

To summarize functional changes during the dehydration – rehydration process in *M. inflexa*, I identified GO categories (biological process) that were significantly enriched (more abundant than expected) among time DEGs (Figure 4.3a). At baseline conditions, protein autophosphorylation, glutamine and arginine metabolic processes, detection of and response to redox state, and photosystem stoichiometry adjustments were enriched among up-regulated genes. The most enriched GO categories among down-regulated genes at baseline conditions were lipoprotein metabolism, lipid and organic substance transport, and localization processes. Interestingly, lipoprotein metabolism and lipid transport were significantly enriched among up-regulated genes at every subsequent hydration state, suggesting major changes to lipid metabolism and localization occur throughout the dehydration – rehydration process. Lysine and diaminopimelate biosynthesis were significantly down-regulated during dehydration, and metabolic processes related to pigments were down-regulated during rehydration. More transient changes include an increase in succinate metabolism, pentose and arabinose transport, as well as a reduction in lipid catabolism, aspartate and threonine biosynthesis under partial dehydration (Dh15). At full dehydration (Dh22), there was a significant enrichment of GO terms related to isoprenoid and carbohydrate metabolism among upregulated DEGs, and an enrichment of pectin, suberin, and dicarboxylic acid biosynthesis among downregulated DEGs. During early rehydration (Re2), there was an

enrichment of long-chain fatty acid metabolism, g-protein coupled, glutamate, and cannabinoid signaling among upregulated DEGs, and an enrichment of cell matrix adhesion, vascular development, and bacterial responses among down-regulated DEGs. Twenty-four hours after rehydration (Re24), I detected an enrichment of GO terms related to dormancy by abscisic acid and ribonucleoprotein complex assembly among up-regulated DEGs and an enrichment of protein metabolism, protein dephosphorylation, and convergent extension among down-regulated DEGs (Figure 4.3a). Interestingly, many of the identifiable transcripts contributing to the persistent enrichment of lipoprotein metabolism and lipid transport were putative LEA proteins and apolipoproteins.

In parallel, I identified GO categories that showed significant enrichment in males vs. females, and meristematic vs. thallus tissues. GO categories that were significantly enriched in females relative to males included dephosphorylation of RNA polymerase II C, peptidyl-serine phosphorylation and modification, and hydrogen transport. Conversely, GO categories that were significantly enriched in males relative to females included cell budding, reproduction, asexual reproduction, actin filament reorganization, and positive regulation of cell cycle process (Figure 4.3b). Comparison of tissue types revealed significant enrichment of GO categories related to cell wall organization, cell division, pectin and hydrogen peroxide catabolic process, cell cycle, and maintenance of dormancy in meristematic tissues. Thallus tissues, on the other hand, were enriched for GO categories related to oligopeptide transport, auxin-activated signaling pathway, riboflavin biosynthetic process, Golgi vesicle budding, and mRNA splicing (Figure 4.3c).

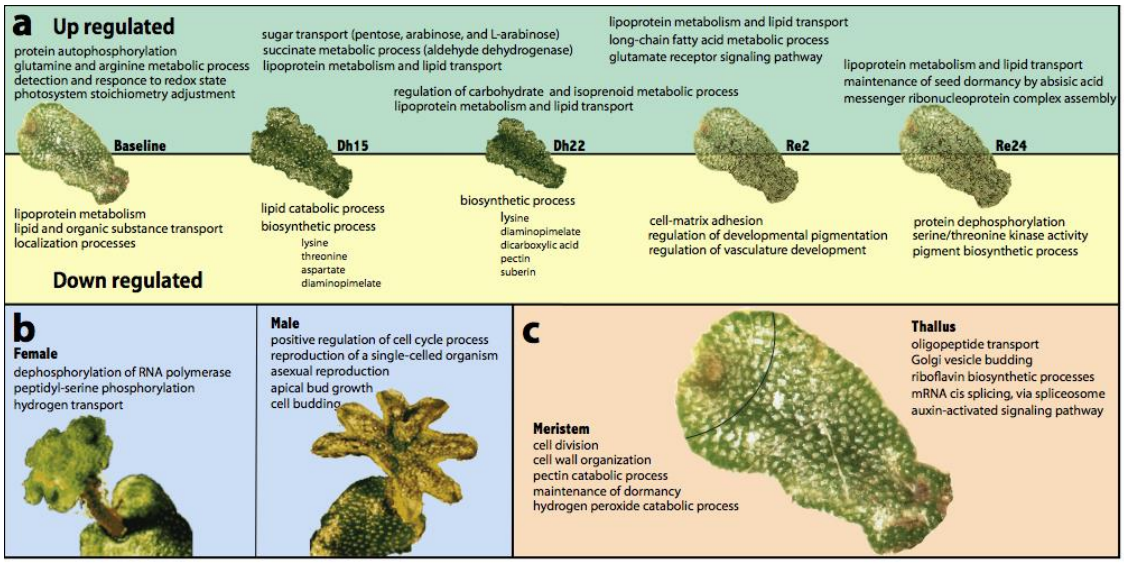

Figure 4.3. Summary of significantly enriched GO categories among (a) up- and down-regulated DEGs throughout the dehydration – rehydration process, (b) females and males, and (c) meristematic and thallus tissues.

Identification of candidate DhT associated genes and targeted tests of intraspecific variation

Next, I identified genes that were differentially expressed in response to dehydration and rehydration and also showed significant differences in expression among the sexes or tissues. Of the 438 genes that were differentially expressed during the dehydration – rehydration process, 17 were also differentially expressed between males and females, eight were differentially expressed between meristematic and thallus tissues, and 13 were shared among all three DEG groups (Figure 4.4a). Genes that were both hydration responsive and expressed in a sex-specific manner included an ATP-dependent RNA helicase, a UDP-glucose:anthocyanin 3'-O-glucosyltransferase, and syntaxin 5 (a membrane tethering protein), and 15 unidentified transcripts. Genes that were differentially expressed over time and among the tissues included a LEA-1 protein, an apolipoprotein, a dehydrin, as well as five transcripts of unidentified function. Thirteen

genes were differentially expressed in all three sets, including an apolipoprotein, a putative glucotransferase GEM-like protein, a translation initiation factor, a RAS related protein, 1-phosphatidylinositol-3-phosphate 5-kinase, alpha-dioxygenase, an elongation factor, a proteasome subunit, and five transcripts of unidentified function.

Because females and meristems exhibit increased DhT relative to males and thallus tissues, I was specifically interested in identifying transcripts that were more abundant in these samples as possible targets for enhancing DhT. Consequently, I targeted genes that were overexpressed in females, meristems and also induced during the dehydration – rehydration process (Figure 4.4b). Seven genes were differentially expressed over time and also upregulated in females, none of which had an identifiable homologue. Nineteen genes were differentially expressed in response to dehydration and rehydration and also upregulated in meristems, including two apolipoproteins, a LEA-1 protein, translation initiation and elongation factors, RAS related protein, putative glucotransferase GEM-like protein, 1-phosphatidylinositol-3-phosphate 5-kinase, Alpha-dioxygenase, and a proteasome subunit, and nine transcripts of unidentified function. No genes were shared among all three DEG groups.

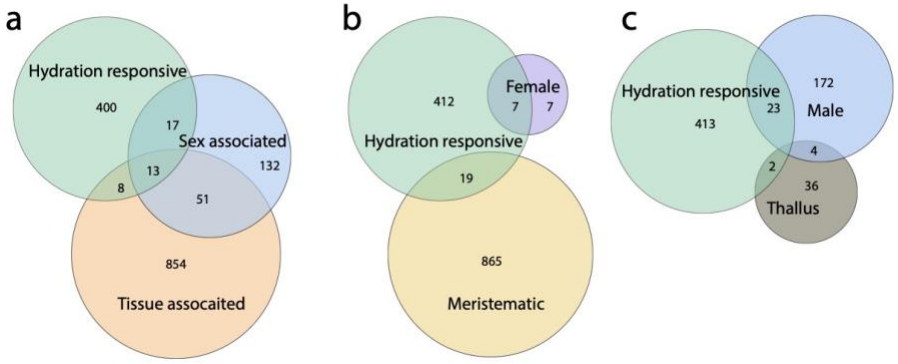

Figure 4.4. Venn diagrams of (a) genes that were responsive to dehydration and rehydration (differentially expressed over time), and also expressed in a tissue-specific and or sex-specific manner, (b) genes that were responsive to dehydration and

rehydration and also up-regulated in females or meristems, and (c) genes that were responsive to dehydration and rehydration and also up-regulated in males or thallus. Numbers of differentially expressed genes in each category are shown, and the size of the circles is proportional to the number of DEGs in that category after log2 transformation.

To gain more nuanced insight into the temporal nature of sex and tissue differences, I identified genes that were differentially expressed among the sexes and tissues at each hydration state. These analyses identify 16 DEGs among the sexes under baseline conditions, zero at Dh15, nine at Dh22, one at Re2, and zero at Re24, indicating that the most substantial differences in gene expression among the sexes exist at baseline conditions. At baseline, females exhibited elevated expression of TWIN LOV 1 (a putative serine-threonine kinase), a CDPK protein, two pre-mRNA splicing factors, a CCR4-NOT complex protein, and two uncharacterized transcripts. Males also showed elevated expression of a CCR4-NOT complex protein, in addition to a S1 related ribosomal protein, a pyruvate kinase, and six uncharacterized transcripts (Figure 4.5a). Minimal sex differences in gene expression were observed at subsequent hydration states, with the exception of full dehydration. During full dehydration, females continued to exhibit elevated expression to the female allele of CCR4-NOT complex protein, TWIN LOV 1, and one uncharacterized transcript (Figure 4.5b). Males maintained elevated expression of CCR4-NOT complex protein, and S1 related ribosomal protein, as well as a calmodulin-binding protein and three uncharacterized transcripts. One DEG (an unidentified transcript) was identified at Re2.

Analyses of differences in gene expression among the meristem and thallus state identified 19 DEGs under baseline conditions, six at Dh15, one at Dh22, and zero at Re2 and Re24. Similar to sex differences, I found that the most dramatic differences in gene expression among the tissues were evident at baseline conditions, and that subsequent

responses to dehydration and rehydration were similar in both tissue types. Under baseline conditions the meristematic tissue showed elevated expression of upstream-binding and GT-2 related transcription factors, putative uridylyltransferase (DUF2921), xyloglucan:xyloglucosyl transferase, histone H2A2, UDP-glucuronate 4-epimerase, and triacylglycerol lipase. In comparison, thallus tissues exhibited elevated expression of a NAI1 transcription factor and four uncharacterized transcripts (Figure 4.5c). During partial dehydration the meristematic tissue showed elevated transcription of katanin P60 ATPase along with 3 uncharacterized transcripts. Thallus tissues exhibited elevated transcription of only two transcripts, both of which were uncharacterized (Figure 4.5d). One DEG (an uncharacterized transcript) was identified at Dh22.

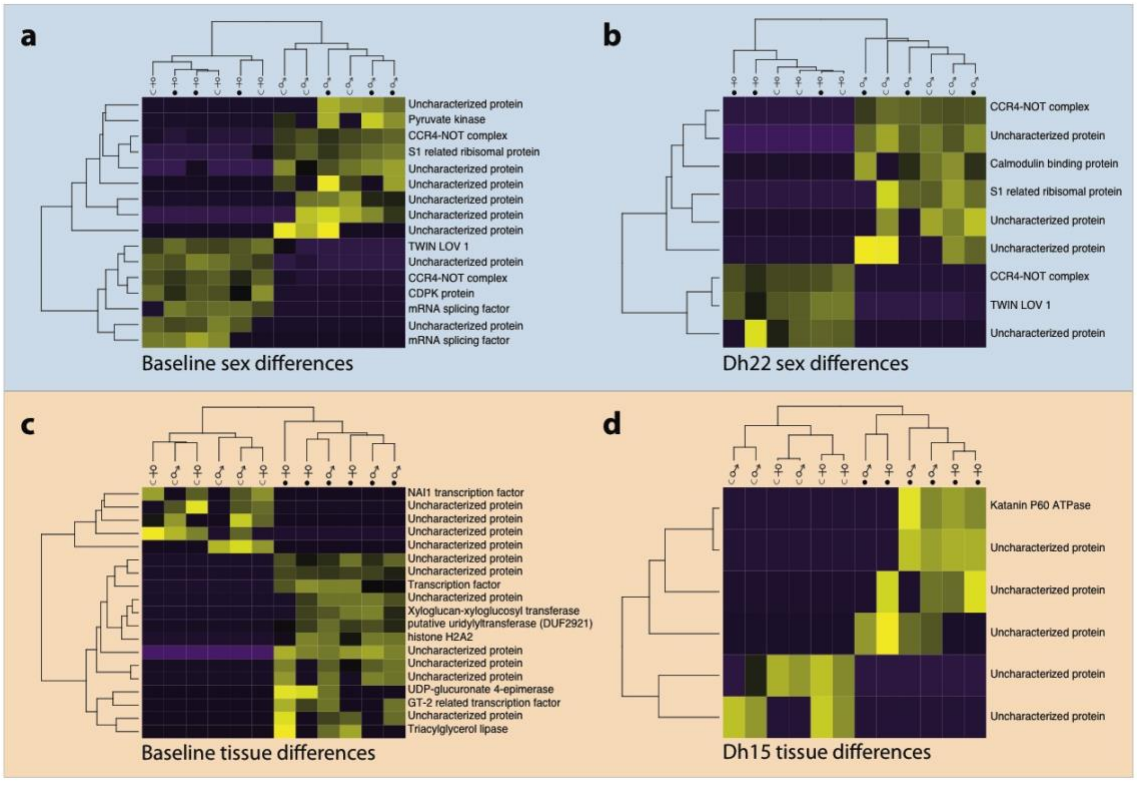

Figure 4.5. Targeted comparisons of differences in gene expression (FDR p-value cutoff of 0.05) among male and female plants at (a) baseline and (b) Dh22. There was one sex DEG (an unidentified transcript, data not shown) during Re2, zero sex DEGs at Dh15, and zero at Re24. Differences in gene expression among meristem and thallus tissues at

(c) baseline and (d) Dh15. There was one tissue DEG (an unidentified transcript, data not shown) at Dh22 and zero sex DEGs at Re2 and Re24. Expression values are represented by color, with yellow indicating relatively higher expression, and purple indicating relatively lower expression.

Discussion

My characterization of the *M. inflexa* dehydration – rehydration transcriptome is the first comprehensive description of sex differences in gene expression for a DhT plant. This assembly allows for detailed investigation of the dehydration – rehydration process in male and female plants and in two tissue types. Analyses of global gene expression in *M. inflexa* revealed strong signatures of stress responses, consistent with current models of DT. More specifically, I identified an abundance of transcripts related to carbohydrate metabolism, transcriptional regulation, lipid transport and metabolism, protein ubiquitination, and stress acclimation. Taken together, these results suggest that *M. inflexa* responds to dehydration by using many of the same central preceses observed in highly DT lineages. DT plants typically exhibit a combination of changes including morphological modifications (i.e. leaf folding and pigmentation changes); a coordinated shutdown of photosynthetic processes during drying (Dinakar *et al.* 2012); increased cell wall flexibility to alleviate mechanical stress of shrinking cells (Farrant 2000; Moore *et al.* 2006); modified carbohydrate metabolism leading to the accumulation of small non-reducing sugars (Smirnoff 1992); accumulation of various stress related proteins (ELIPs, HSPs, and LEAs) (Costa *et al.* 2017; Vanburen *et al.* 2019); increased antioxidant activity related to ROS scavenging (Dinakar and Bartels 2013a), and ABA mediated and phospholipid signaling (Dinakar *et al.* 2012).

My work suggests that the dominant physiological responses to dehydration in *M. inflexa* are similar to those of DT plants, including changes in metabolism, transport,

accumulation of protective proteins, and cell wall modifications. I detected a strong signature of altered carbohydrate metabolism during dehydration, as anticipated. Transcripts related to pentose and arabinose transport were up-regulated during early dehydration and persisted during complete dehydration. A parallel reduction in starch biosynthesis during dehydration suggests that *M. inflexa* plants may modulate existing metabolic pathways to preferentially accumulate sugars rather than starches. Changes in lipid metabolism are also evident in dehydrating *M. inflexa*. There is significant increase in the transport of lipids and reduction in lipid catabolism indicating that localization and retention of cellular lipids are important to DhT. I identified a consistent up-regulation of putative LEA proteins throughout the dehydration-rehydration process in *M. inflexa*, consistent with current models of DT, which indicate that LEA proteins play a central role in water stress responses in DT plants (Liu *et al.* 2009; Dinakar *et al.* 2012; Costa *et al.* 2017).

This work also identifies important differences between the gene expression profile of dehydrating *M. inflexa* specimens and highly DT plants. Interestingly, apolipoproteins, which facilitate the transfer of water insoluble lipids (Close 1996) and may protect against photooxidative damage through scavenging activities and membrane associations (Charron *et al.* 2008), were consistently up-regulated in dehydration and rehydrating *M. inflexa* specimens, but have rarely been implicated in DT. Although I detected an induction of LEA gene expression in *M. inflexa*, I speculate that a lack of fine scale temporal regulation of LEA expression may contribute to reduced tolerance of *M. inflexa* relative to model DT lineages. The timing, localization, and identity of specific LEA genes has been suggested to impact tolerance. In DT monocot *Xerophyta viscosa*,

LEA families 1 and 4 accumulate early during drying and are retained throughout rehydration, whereas LEA families 2 and 3 decline during dehydration and rehydration (Costa *et al.* 2017). Here, I do not detect the same level of temporal control in *M. inflexa,* as most LEA genes are induced early during dehydration and are maintained at elevated levels through rehydration. Further, both ELIPs and HSPs were conspicuously absent from dehydrating *M. inflexa* specimens. These proteins have been implicated in DT in numerous studies (Bartels *et al.* 1992; Zeng *et al.* 2002; Xiao *et al.* 2015; Yobi *et al.* 2017; Vanburen *et al.* 2019), and I speculate that the absence of ELIP and HSP expression may contribute to decreased tolerance in *M. inflexa* relative to highly DT lineages. Finally, there were numerous hydration responsive transcripts that had no characterizable function. These transcripts may impact DhT in meaningful ways, and future work should seek to characterize them.

Differences in gene expression among the sexes (independent of time) revealed higher overall expression in males, suggesting that maleness may be impacted by more genes than femaleness, or that secondary sexual dimorphisms cause males to exhibit elevated transcription. GO categories that were enriched in males were mainly related to growth and reproduction, whereas females showed enrichment of GO categories related to signal transduction and transcription. These differences provide a possible explanation for the sex difference in DhT, suggesting that overinvestment in growth and reproduction by males comes at the cost of sensing and preparing for environmental stresses.

Characterization of differences in gene expression among meristematic and thallus tissues (independent of time) identified numerous DEGs, the vast majority of which were more abundant in meristematic relative to thallus tissue. Because meristems

are the actively growing part of the plant, elevated expression is expected. Although this pattern of increased expression in the more tolerant samples (meristems) is in opposition with what was shown in the sexes (where intolerant males showed elevated expression of more genes), the specific genes up-regulated in meristems are quite different from those expressed in males. Meristems show enrichment of various cell wall related genes, suggesting that DhT is impacted by differences in cell wall characteristics. Thallus tissues, on the other hand, were enriched for genes related to auxin signaling, riboflavin biosynthesis, and transport.

Temporal analyses of sex differences revealed that substantial variability between males and females under baseline conditions. Differences in gene expression between the sexes declined once dehydration was initiated, suggesting that the sexes leverage the same basic mechanisms of DhT and that inherent differences among the sexes may drive differences in DhT. However, some sex DEGs were detected at full dehydration, most of which are male-specific. These DEGs are evidence of continued activity during the dehydration process, possibly representing a failure to effectively shut down metabolic and cellular processes, which could reduce DhT (Dinakar *et al.* 2012). Many of the genes with significant differences in expression among the sexes are not linked directly to DhT but may impact other traits that either tradeoff with or enhance DhT. Recovery mechanisms appear to be similar among the sexes (lack of DEGs detected during rehydration), suggesting consistency in rehydration responses.

Temporal analyses of tissue-specific expression revealed a rapid decline in differences among tissues during drying. Similar to sex differences, the majority of differences in gene expression among the tissues appear to be derived from baseline

differences. Again, this suggests that inherent differences among these tissues confer increased tolerance to the meristematic tissue. Interestingly, differences in expression among the tissues persist for less time than sex differences. By full dehydration there is only one DEG among the tissues, and none are detected at rehydration time points. I speculate that differences in cell wall composition may contribute to survival differences among the meristem and thallus tissues, but characterization of unannotated transcripts may reveal alternate patterns.

In general, I found evidence indicating that DhT in *M. inflexa* shares many mechanistic components with model DT plants. I detected a strong and persistent accumulation of putative LEA proteins, transcripts related to carbohydrate metabolism, and cell wall modifications, all of which are thought to be central components of DT (Dinakar *et al.* 2012). However, I also detected noteworthy differences between *M. inflexa* and other DT plants, including a conspicuous absence of ELIP and HSP expression. Taken together, it seems that the general response to drying is similar in DhT and DT plants, and that nuanced variation may underlie differences in relative tolerance among DT and DhT plants. Interestingly, many DT plants undergo a characteristic shift away from damage prevention and metabolic adjustments towards protective measures when hydration drops RWC < 40% (Zhang and Bartels 2018). Although RWC in *M. inflexa* at both Dh15 and Dh22 is < 40% (Marks *et al.* 2016), I find that the gene expression response of *M. inflexa* is still centered around metabolic adjustments. Consequently, speculate that an inability to effectively shift more energy into protection may contribute to the reduced tolerance of *M. inflexa* relative to highly DT plants.

By taking advantage of the intraspecific variation in DhT of *M. inflexa* (among the sexes and tissues) I was able to target nuanced variation possibly contributing to differences in relative tolerance. My analyses suggest that increased cell wall flexibility may contribute to elevated DhT in meristematic tissues, while differences in stress signaling and physiological regulation could drive sex differences in DhT. The observation that both the tissue and sex differences in DhT appear to be derived from baseline differences in gene expression suggests that the inherent properties of meristematic cells and femaleness can increase DhT. Interestingly, no dehydration induced DEGs were shared between females and meristems, indicating that increased tolerance in these two groups is driven by separate mechanisms. I should note that tissue differences are stronger than sex differences in DhT. In fact, some populations of *M. inflexa* have been identified where males are more DhT than females (chapter 3), but the mechanism of shifting sexual dimorphism in DhT is not clear.

In conclusion, I present a dehydration – rehydration transcriptome for the DhT liverwort *M. inflexa*. My analyses detect a characteristic accumulation of LEA proteins, substantial modifications to carbohydrate metabolic processes, and changes in lipid transport during the dehydration – rehydration process. I speculate that the absence of specific transcripts (ELIPs and HSPs) during the dehydration – rehydration process may reduce the overall tolerance of *M. inflexa* relative to highly DT lineages. Analyses of sex-specific and tissue-specific gene expression suggest that baseline variation in metabolism and cell wall characteristics may impact differences in the relative tolerance of plants. Taken together these findings indicate that multiple mechanisms of enhancing DhT exist in *M. inflexa* and are driven primarily by inherent differences among samples. These

analyses provide an overview of gene expression in dehydrating and rehydrating *M. inflexa* specimens and building on this foundation with additional analyses including pathway, time course, and network analyses of co-expressed genes will provide additional insight into DhT mechanisms.

CHAPTER FIVE

GENOME OF THE TROPICAL PLANT MARCHANTIA INFLEXA: IMPLICATIONS FOR SEX CHROMOSOME EVOLUTION AND DEHYDRATION TOLERANCE

A version of this chapter is currently in review at Scientific Reports

Introduction

Bryophytes (mosses, liverworts, and hornworts) are living representatives of an early-diverging land plant lineage (Kenrick and Crane 1997; Bowman *et al.* 2017) and they provide an important landmark for comparative phylogenetics. Although the exact relationships among bryophyte lineages are currently contested, the earliest fossil evidence assigned to a bryophyte is liverwort-like with dorsiventral complex thalluls morphology and a leafless gametophyte (Wellman *et al.* 2003; Ligrone *et al.* 2012; Morris *et al.* 2018), suggesting that liverworts retain a large suite of ancestral characters not conserved in other land plants. Importantly, building a fundamental understanding of genomic patterns can be readily accomplished by working with bryophytes because of their small genomes (Leitch and Leitch 2013), many of which contain comparatively few paralogous duplications of regulatory genes (Bowman *et al.* 2017). Thus far, genome assemblies have been developed for a number of bryophyte species (including the mosses *Physcomitrella patens* (Rensing *et al.* 2008), and *Sphagnum fallax* (Shaw *et al.* 2016), the liverwort *Marchantia polymorpha* (Bowman *et al.* 2017), and the hornwort *Anthoceros agrestis* (Szövényi 2016)), and more are underway (*Takakia lepidozioides*, *Ceratodon purpureus*, *Funaria hygrometrica* (Rensing 2017) and *Syntricia caninervis*). Relatively few assemblies of bryophyte mitochondria (Oda *et al.* 1992; Terasawa *et al.* 2007; Li *et al.* 2009; Wang *et al.* 2009; Myszczyński *et al.* 2017) and chloroplasts (Kugita *et al.* 2003; Sugiura *et al.* 2003; Wolf *et al.* 2005) are available compared to other plant

lineages, yet mitochondrial genomes bryophytes tend to be less complex than those of tracheophytes, having no large repeated sequences and limited recombination (Ohyama 1996). Genome sequencing efforts of additional bryophyte taxa will provide critical insight into more recent evolutionary changes within these lineages and may help to better resolve ancestral states.

In this context, I targeted the tropical liverwort, *Marchantia inflexa* (Nees & Mont) for sequencing and assembly. *Marchantia inflexa* is a New World liverwort with that is distributed throughout Central America and the Caribbean, from northern Venezuela to the southern United States (Bischler 1984). *Marchantia inflexa* diverged from the well-studied sister species *M. polymorpha* 68-126 million years ago (Kumar *et al.* 2017). *Marchantia inflexa* is dioecious (has unisexual individuals), with eight autosomes and one female (U) or male (V) sex chromosome, and it can reproduce sexually by spores or asexually by fragmentation and the formation of gemmae (specialized asexual propagules). *Marchantia inflexa* typically grows on rock and soil surfaces along stream banks in tropical forests, but it can also colonize more exposed and disturbed sites along roads. Vegetative growth produces a dichotomously-branching thallus mat with dorsiventral organization, and the haploid gametophyte is the dominant life phase. *Marchantia inflexa* is a useful model to investigate sexual dimorphisms, population sex ratios, and stress tolerance because prior work has established that *M. inflexa* exhibits a considerable degree of sexual dimorphism (McLetchie and Puterbaugh 2000; Brzyski *et al.* 2014), variable population sex ratios (McLetchie and Puterbaugh 2000; McLetchie and García-Ramos 2017; Brzyski *et al.* 2018), and fluctuating stress tolerance (Stieha *et al.* 2014; Marks *et al.* 2016).

Bryophytes harbor a high proportion of dioecious species. Nearly half of all extant mosses, and approximately two-thirds of liverworts are dioecious (Wyatt and Anderson 1984). In many bryophytes (including *M. inflexa*), the reported sex ratio is female biased (McLetchie and García-Ramos 2017; Brzyski *et al.* 2018), and in *M. inflexa* this may be related to females' superior ability to recover from drying events (Stieha *et al.* 2014; Marks *et al.* 2016), faster growth rate (Brzyski *et al.* 2014), or the increased establishment of female gemmae (Stieha *et al.* 2014). However, true population sex ratios are largely unknown, except for the few cases where genetic sex markers have been developed and utilized (McLetchie and Collins 2001; Korpelainen *et al.* 2008; Milewicz and Sawicki 2013). Typical methods for assessing sex ratios depend on counting the number of males and females with visible sex organs and using this information to infer the underlying population sex ratio. However, this approach fails to account for plants not currently displaying sex organs and assumes that the sex ratio of vegetative plants is equivalent to that of plants with sex organs (Holá *et al.* 2014), but this assumption may not hold true in natural settings. In fact, for both *M. polymorpha* and *M. inflexa* (where sex organ development can be artificially induced) the timing of reproductive development is sex-specific (Wann 1925) and some individuals never produce sex organs (unpublished data).

The reproductive biology of bryophytes (with the haploid gametophyte being the dominant life stage) provides a unique perspective on the evolution of sex-linked genes, as the female (U) and male (V) sex chromosomes are present at the same copy number (1N) as autosomal chromosomes for the majority of the organism's life cycle and are subject to haploid selection. Sex chromosome evolution in diploid dominant systems has

received considerable research attention. However, less is known about the forces shaping sex chromosomes in haploid dominant systems (Bachtrog *et al.* 2011), and the ramifications of haploid selection on sex chromosomes may have unique consequences. For example, exposure to haploid selection should reduce the prevalence of deleterious mutations (Bull 1978; Immler and Otto 2015) and could allow beneficial mutations fix rapidly (Immler and Otto 2015). However, lack of recombination on UV sex chromosomes could lead to degeneration on UV chromosomes (Bachtrog *et al.* 2011; Immler and Otto 2015), as has been observed in XY and ZW chromosomes. Further, the smaller effective population size of sex chromosomes relative to autosomes may increase the impact of genetic drift, further influencing adaptive evolution of sex-specific genes (McDaniel *et al.* 2013). The extent to which these forces shape sex chromosome evolution in haploid-dominant systems is not well understood, but the numerous dioecious bryophyte taxa provide novel opportunities to test related questions.

Stresses caused by environmental fluctuations are accentuated in plants due to their sessile nature. Consequently, numerous tolerance mechanisms have evolved to combat environmental pressures, many of which have potential translational utility. Some of these stress tolerance traits, such as embryo retention (allowing for the development and dispersal of desiccation tolerant spores) and UV radiation, desiccation, heat, and freezing tolerance (Bowman *et al.* 2017; Rensing 2018) may have facilitated the transition from aquatic to terrestrial environments by early plants. Many extant bryophytes retain these early stress tolerance mechanisms, allowing them to occupy marginal niches (characterized by nutrient poor substrates (Turetsky 2003), toxic concentrations of metals (Shaw 1987), variable light (van der Wal *et al.* 2005) and

moisture levels (Proctor *et al.* 2007)). Consequently, bryophytes are particularly informative with respect to understanding the evolutionary history and physiological strategies of stress tolerance.

Desiccation tolerance (DT) (Proctor *et al.* 2007) in particular, has important translational utility. A number of studies have described the genomes (Xiao *et al.* 2015; VanBuren *et al.* 2015, 2018; Costa *et al.* 2017) and transcriptomes (Oliver *et al.* 2004; Rodriguez *et al.* 2010; Gechev *et al.* 2012; Gao *et al.* 2014, 2015, 2017; Ma *et al.* 2015; Yobi *et al.* 2017) of DT plants, and the amassing data provide a strong foundation on which to construct our understanding of DT. These studies have demonstrated that DT is a complex multigenic trait (Dinakar and Bartels 2013a; Gechev *et al.* 2013; Gao *et al.* 2015; Costa *et al.* 2017; Yobi *et al.* 2017), and that there are multiple means of achieving DT (Oliver *et al.* 2005). The genomic basis of DT, although not entirely described, may derive from regulatory differences in gene expression pathways (Xiao *et al.* 2015), increased copy number of anahydrobiosis-related genes (Grene *et al.* 2011), and differences in the structural organization of these genes (Costa *et al.* 2017). However, more studies are needed to resolve the specifics of DT mechanisms, and they should include work on species spanning a wide phylogenetic range and degree of tolerance levels (such as the intermediate trait of dehydration tolerance (DhT also dehydration tolerant) (Oliver *et al.* 2010; Marks *et al.* 2016)). *Marchantia inflexa* is DhT, which provides an opportunity to enhance our understanding of the evolution of this intermediate trait.

Growing genomic resources for bryophytes provide novel opportunities to conduct comparative studies within these lineages, which are particularly well suited to

addressing questions related to sex chromosome evolution, sex differences, and stress tolerance adaptations. Here, I aimed to characterize patterns of sequence divergence between the thalloid liverworts *M. inflexa* and *M. polymorpha*, define genetic sex markers, and investigate the genomic basis of intraspecific variation in DhT. My analyses indicate that the greatest sequence conservation between *M. inflexa* and *M. polymorpha* is among chloroplast genes, likely due to the conservation of plastid function across lineages. Conversely, I show that mitochondrial genes are relatively divergent, which may be related to reduced recombination of mitochondrial genomes (as observed in *M. polymorpha*) (Ohyama 1996), or variable mutation rates (Palmer *et al.* 2000). Sex-linked genes exhibit signatures of strong diversifying selection, relative to autosomal genes. Interestingly, divergence of sex-linked genes is driven primarily by male-specific (V) genes, which I speculate is related to strong selection on males to maintain species recognition. Because sperm is broadcast indiscriminately and water dispersed in *Marchantia*, pressure to maintain species recognition is expected to be particularly strong on genes related to sperm characteristics (Palumbi 1999). Although females could be subjected to similar selective pressures, my analyses indicate that selection is acting primarily on male-specific genes in *M. inflexa*. Putatively sex-specific sequences were identified and used to develop diagnostic markers for genetic sex in *M. inflexa*. Regarding DhT, I found that the highly tolerant female genotype harbors more paralogs and higher coverage of DhT related genes relative to the less tolerant male genotype. Interestingly, some of these DhT genes are putatively sex-linked, offering a possible explanation for elevated DhT in *M. inflexa* females more broadly (Stieha *et al.* 2014;

Marks *et al.* 2016). The remaining DhT genes appear to be located on autosomes and they may contribute to changing patterns of DhT (chapter3).

Results

Genome assembly and annotation

Whole-genome sequencing of *M. inflexa*, yielded 127,147,280 male reads and 133,660,960 female reads (after quality filtering). The combined male and female k-mer distribution indicated a coverage of ~24X but showed a large quantity of unique and low abundance k-mers, suggestive of contaminating organisms. In my efforts to characterize the source of these low abundance k-mers, I detected a diverse community of microbes, consistent with recent descriptions of *M. inflexa* microbial associations (Marks *et al.* 2017). After removal of putative microbial sequences, I assembled the remaining sequence reads to generate the draft assembly M_inflexa_v1.1. The resulting scaffolds were assigned to super-scaffolds by alignment with the *M. polymorpha* reference genome, allowing me to coalesce the assembly into 300 super-scaffolds. In total 7,747 *M. inflexa* scaffolds covering a total length of 81,634,927 bp were successfully mapped to the *M. polymorpha* genome. Unmapped *M. inflexa* scaffolds were appended to the supper-scaffold assembly. The resulting assembly consists of 41,556 scaffolds, covering a total of 208,839,958 bp, with and N50 of 11,144 bp and a longest scaffold length of 2,829,880 bp. The assembly M_inflexa_v1.1 has been deposited at GenBank under the accession QLSQ00000000 and will be released upon publication.

Assessment of assembly completeness (performed with BUSCO (Simão *et al.* 2015)) indicated that 54.4% (783) of the 1,440 presumptively universal single-copy

orthologs from the plant set of OrthoDB v9 were present in the *M. inflexa* genome assembly. Another 3.5% (51) orthologs were present, but fragmented. In comparison, a parallel assessment of the *M. polymorpha* v3.1 assembly found that 60.2% (867) of these same genes were complete, and 2.9% (42) were fragmented in *M. polymorpha*. Both of these estimates are rather low, suggesting that there may be inherent limitations associated with BUSCO as has been observed for other deeply-diverged lineages (Hara *et al.* 2015; Smith *et al.* 2018). Still, I found these assessments to be informative in a comparative context within *Marchantia*.

Assembly of *M. inflexa* plastids generated nearly complete mitochondrial and chloroplast sequences (Figure 5.1). The mitochondria of *M. inflexa* is 190,056 bp, and the chloroplast is 122,620 bp. The complete mitochondrial and chloroplast sequences are available at FigShare (https://doi.org/10.6084/m9.figshare.6639209.v1) and will be released upon publication.

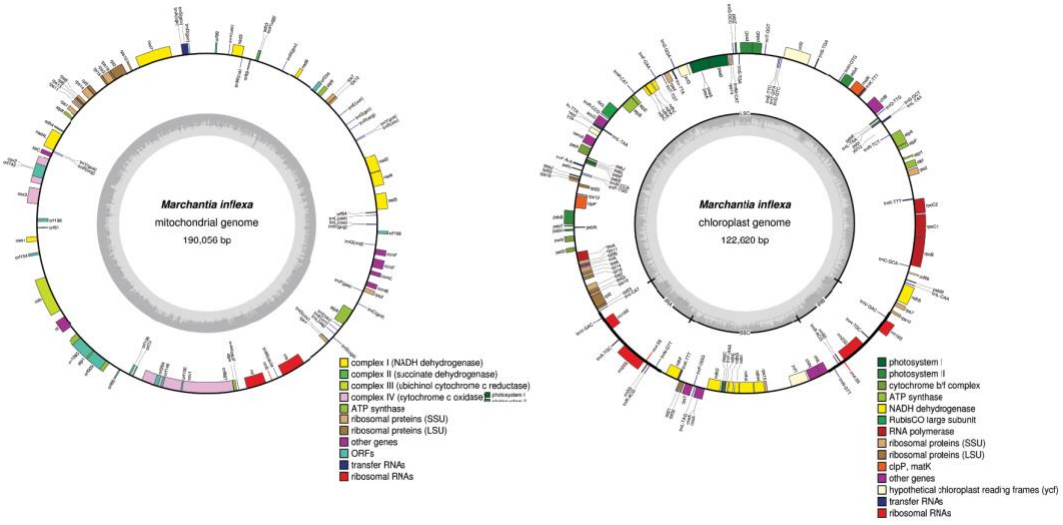

Figure 5.1. Assembled mitochondrial and chloroplast sequences of *Marchantia inflexa*. The inner circle depicts nucleotide content (G/C in dark gray and A/T in light gray). Inverted repeats (IRA and IRB) along with large (LSC) and small (SSC) single copy regions are indicated on the chloroplast inner circle. The outer circle shows annotated genes that are color coded by function. Genes located on the outside of the circle are

transcribed in a clockwise direction, and those on the inside of the circle are transcribed counterclockwise.

Annotation

Gene annotation of the *M. inflexa* draft genome utilized *de novo* gene finding in combination with the lift-over of all *M. polymorpha* annotations for orthologous genes. Lift-over annotations from *M. polymorpha* to *M. inflexa* resulted in the annotation of 10,005 orthologous proteins within the *M. inflexa* assembly. *De novo* gene finding efforts identified 13,546 predicted proteins 9,194 of which had identifiable orthologs across *M. polymorpha*, *Physcomitrella patens, Arabidopsis thaliana,* and refseq. After removal of all redundant annotations in the *de novo* and lift-over annotations the combined set of annotations consists of 11,687 predicted proteins. Not surprisingly, the highest homology was observed between *M. inflexa* and *M. polymorpha* with substantially less homology between *M. inflexa, P. patens* and *A. thaliana*, reflecting the estimated divergence times among these species (divergence time between *M. inflexa* and *M. polymorpha* is 68-126 MYA; for *M. inflexa* and *P. patens* it is 425-557 MYA; and for *M. inflexa* and *A. thaliana* it is 481-584 MYA (Kumar *et al.* 2017)).

Sequence similarity between M. inflexa and M. polymorpha

To investigate genome evolution within *Marchantia* I measured sequence divergence between *M. inflexa* and *M. polymorpha*. Initially, I compared nucleotide differentiation among coding sequences (CDS), introns, and intergenic regions to estimate the general patterns of divergence between lineages (Figure 5.2). Comparison of

orthologous CDS, introns, and intergenic sequences, revealed that (not surprisingly) intergenic sequences were the least conserved (64.5% ± 0.009%), introns were intermediate (81.8% ± 0.008%), and CDS were the most conserved (82.4% ± 0.001%) (Figure 5.2). There was a significant effect of sequence type on %ID ($F_{2,40000}$=39756, p<0.0001). Patterns of sequence divergence between *M. inflexa* and *M. polymorpha* fit general expectations that CDS should exhibit higher sequence similarity compared to introns and intergenic sequences. That being said, I observed surprisingly high sequence conservation among some introns, which I speculate is related to the relatively short length of *M. inflexa* introns, in which functional elements (such as splice sites) may be preferentially retained.

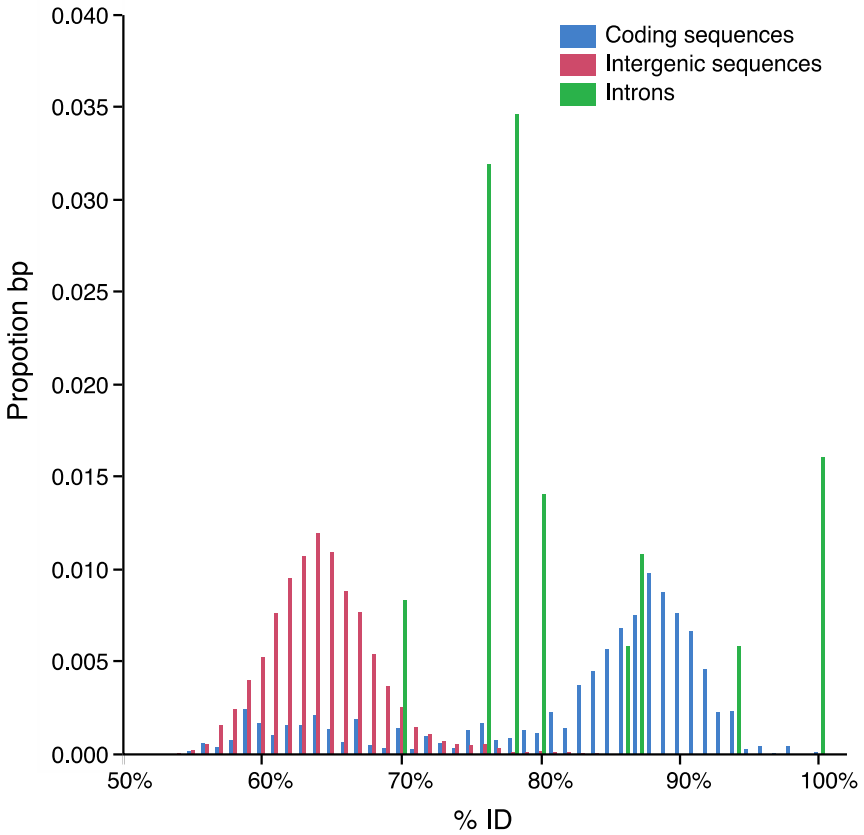

Figure 5.2. The similarity between *Marchantia inflexa* and *M. polymorpha* coding sequences (CDS), introns, and intergenic sequences is shown as proportion base pairs

(bp) at a % identity (%ID) ranging from 50-100%. As expected, CDS have higher overall similarity compared to introns and intergenic sequences. There was a significant effect of sequence type on %ID. Total bp of each sequence type is: CDS=629,624 bp; introns=1,087 bp; and intergenic=21,983,100 bp.

To assess variation in substitution rates across coding sequences, I computed the ratio of non-synonymous to synonymous mutations (dN/dS) for all orthologous CDS of *M. inflexa* and *M. polymorpha*. The resulting dN/dS values were log transformed to improve normality for statistical testing. Initially, I tested for evidence of contrasting selective pressures among autosomal, sex-linked, and organellar genes. Notably, sex linked genes and autosomes are present at 1N, whereas copy number of the chloroplast and mitochondria is variable. I computed the mean dN/dS and standard error for autosomal genes (0.24 ± 0.01 (n=4,900)), for sex-linked genes (0.48 ± 0.13 (n=53)), and for organelle genes (0.14 ± 0.03 (n=116)). I detected significant differences among groups ($F_{2,4862}$=18.54, p<0.001). Targeted contrasts revealed significant differences among sex-linked and autosomal genes (t_1=-2.38, p=0.018) and among organellar and autosomal genes (t_1=5.58, p=2.6e^{-8}). Subsequently, I tested for differences among more specific gene types, subdividing sex-linked genes into male-specific, female-specific, and male and female alleles of genes with both U and V copies. Organellar genes were subdivided into mitochondrial and chloroplast genes. Mean dN/dS of male-specific genes was 0.63 ± 0.23 (n=23), of female-specific was 0.20 ± 0.09 (n=7), of male-alleles was 0.24 ± 0.12 (n=11), and of female alleles was 0.56 ± 0.34 (n=12). Mean dN/dS of chloroplast genes was 0.03 ± 0.01 (n=74) and of mitochondria was 0.34 ± 0.06 (n=42). There was an overall effect of gene type on dN/dS ($F_{2,4862}$=20.10, p<0.001). Targeted contrasts revealed significant differences between autosomal genes and male-specific

genes (t_1=-2.88, p=0.004), chloroplast genes (t_1=10.39, p=5e^{-25}), and mitochondrial genes (t_1=-4.40, p=1.1e^{-5}) (Figure 5.3).

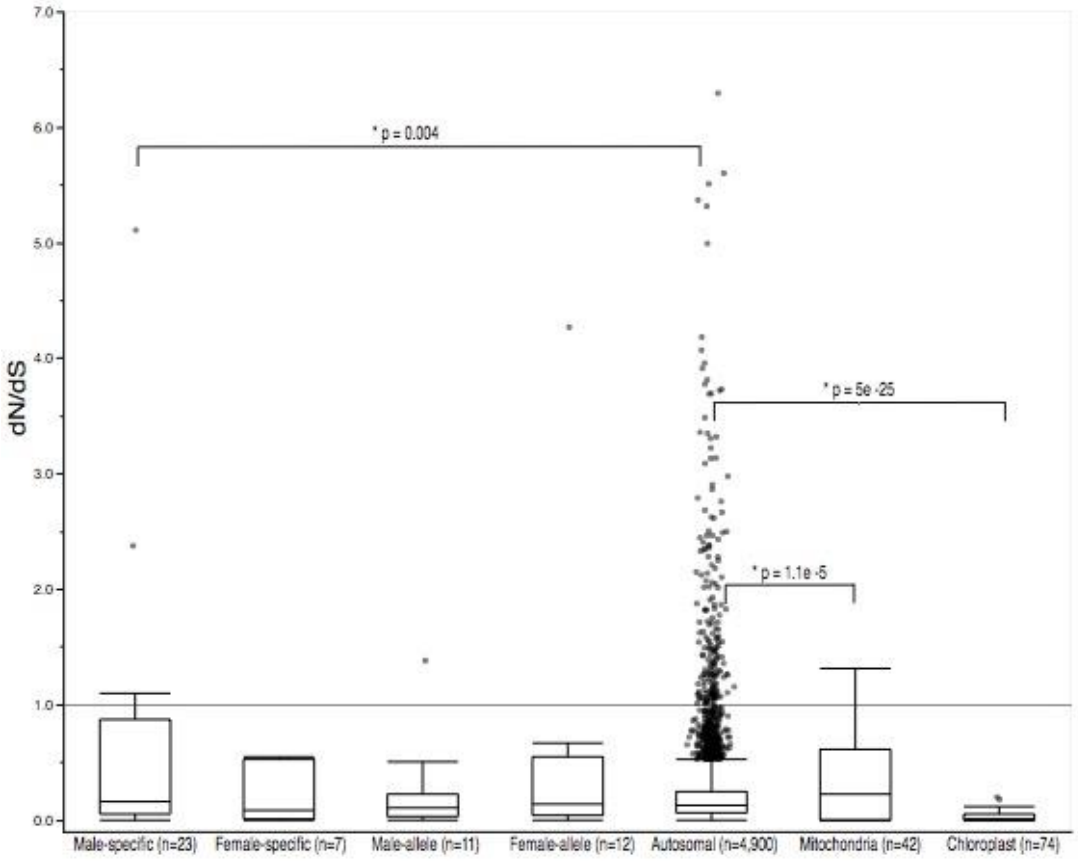

Figure 5.3. Boxplot of dN/dS values of orthologous coding sequences in *Marchantia inflexa* and *M. polymorpha*. There were significant differences among gene types in mean dN/dS. Secondary comparisons reveal significant differences in mean dN/dS among male-specific and autosomal genes, chloroplast and autosomal genes, and mitochondrial and autosomal genes. Statistical tests were performed on log transformed data to satisfy the assumptions of normality, but untransformed numbers are presented here. All dN/dS values > 1 exhibit the signature of diversifying selection.

These analyses reveal several genes and pathways that may be under diversifying selection (dN/dS > 1) in *M. inflexa* and *M. polymorpha.* Sex-linked genes with dN/dS > 1 included the female allele of CCR4-NOT transcription related complex protein (Mapoly0018s0021.1), the male allele of bHLH-MYC2 transcription factor (MapolyY_B0018.1), a male-specific phosphatidylinositol-4,5-bisphosphate 3-kinase (MapolyY_A0049.1), two male-specific genes of unknown function (MapolyY_B0032.1

and MapolyY_B0003.1). No chloroplast genes in my analyses had dN/dS > 1, but three mitochondrial open reading frames (orf) (orf 84, orf 69, rpl 10) had dN/dS > 1. Of the 243 autosomal genes with dN/dS > 1, 51 had identifiable homologs in the Uniprot database. GO analyses of these genes revealed that many were associated with the cellular components *intracellular, cytoplasm*, and *membrane*, the molecular function *catalytic activity* (followed closely by *hydrolase activity* and *transferase activity)*, and the biological processes of *metabolic process* and *cellular process*. A complete list of genes with dN/dS > 1 and associated protein names can be found in Table 5.1 at the end of this chapter.

Sex marker identification

I identified 4,468 regions (covering 2,234,000 bp) in the *M. inflexa* genome assembly with substantial differences in copy number among genetic lines through coverage analysis with DifCover (https://github.com/timnat/DifCover) (Smith *et al.* 2018). Of these, 89 were found on scaffolds also containing a predicted protein, 31 of which could be assigned to an identifiable homolog across *M. polymorpha, P. patens, A. thaliana*, and refseq databases. From this set, I identified five putatively male- and three female-specific sequences that were also orthologous to sequences on the U and V chromosomes in *M. polymorpha*. These candidate sex markers were analyzed by PCR in nine males and nine females to verify their fidelity, leading to the validation of one positive marker for each sex (Figure 5.4). Other candidate sex markers exhibited non-specific amplification and were therefore discarded. Plants used for validation were originally collected from five distinct populations, suggesting that the markers are robust

to genotypic variation. Primer sequences of the validated male and female sex markers are listed in Table 5.2.

Table 5.2. Primer sequences for validated male and female genetic sex markers for *Marchantia inflexa.*

Marker	***Left primer sequence***	***Right primer sequence***
Male marker-98683	CGTTTGATTCGTCTTCTCCAAA	AGCTCTCGTCAGAATAGTCAGG
Female marker-42793	GTCCAGTCTGTGAAGCCGTA	CCTTCTCGTAGACCAGTGCT

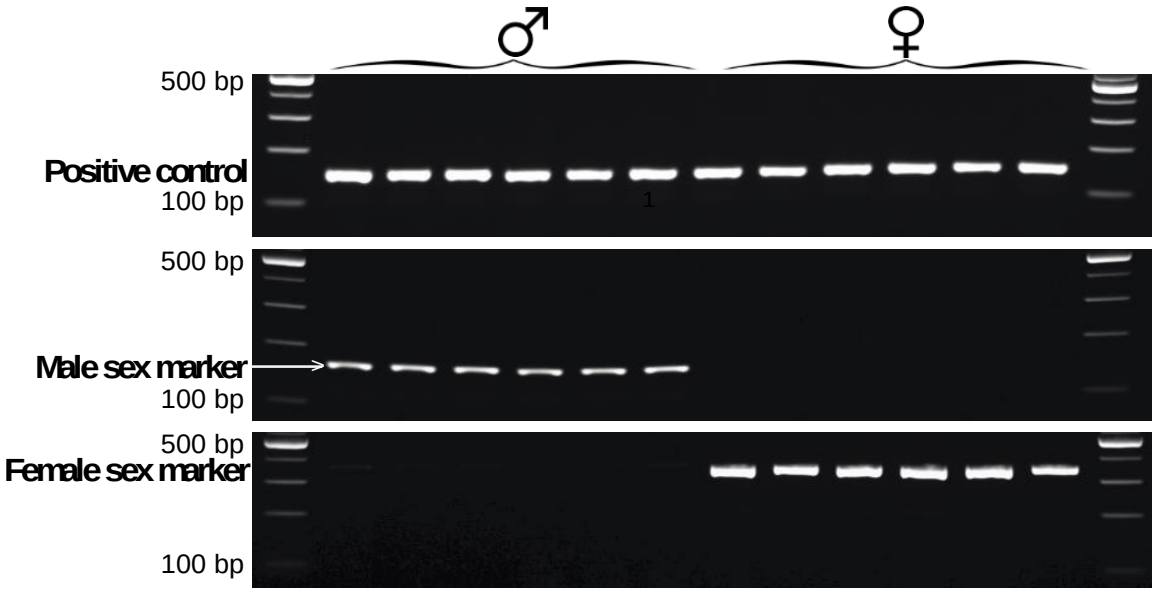

Figure 5.4. Electrophoresis pattern of sex markers and positive control (actin) amplified in six male and six female plants. Images shown here are sections cropped from two separate gels. Male and female markers are presumptive U- and V-linked sequences, respectively. Plants were originally collected from five natural populations spanning a range of environmental conditions. In total, sex markers were validated in nine male and nine female plants, but only six individuals of each sex are shown here.

Dehydration tolerance

To address specific hypotheses on DhT, I probed *M. inflexa* and *M. polymorpha* annotated proteins for orthologs to a list of 195 DT genes (compiled from publicly available mRNA sequences of genes expressed under water stress in model DT plants). Of this set of DT genes, 112 had identifiable homologs in *M. inflexa* and 141 had identifiable homologs in *M. polymorpha*. My analyses of dN/dS captured 38 of these DT

orthologs, one of which (a putative aldehyde dehydrogenase (Mapoly0030s0099.1)) had a dN/dS value > 1. The function of diversification in this gene is unclear, given the lack of evidence for any difference in DhT between these two *Marchantia* species.

Prior studies showed that the male and female *M. inflexa* genotypes used here have reproducible differences in DhT (Marks *et al.* 2016). Consequently, I aimed to identify DT genes with substantial coverage differences among these two genotypes, presuming that they may impact relative differences in DhT. Of the 112 DT genes detected in *M. inflexa*, most had standardized coverage ratios of ~1. However, seven genes had considerably higher coverage ($\log_2$ fold change > |4|) in the highly-tolerant female and one had higher coverage in the less tolerant male (Table 5.3). Specific genes with higher coverage in the tolerant female genotype compared to the male include a CDPK protein, a major intrinsic protein PIPc, an aldehyde dehydrogenase, GRP94, heat shock proteins 70 and 101, and superoxide dismutase. The sole DT gene with higher coverage in the less-tolerant male genotype than the female is a heat shock factor 1 ortholog. Of the DT genes with coverage difference among genotypes, one (CDPK protein) was on a scaffold assigned to the putative U chromosome, and one (heat shock factor 1 ortholog) was assigned to the putative V chromosome.

Table 5.3. DhT proteins with coverage differences among the sexes in *Marchantia inflexa*. I designated a $\log_2$ fold change > |4| as the cutoff to define a significant coverage difference. Negative $\log_2$ coverage ratios indicate higher female coverage relative to males, whereas positive $\log_2$ coverage ratios indicate higher male coverage.

Gene	***Coverage ratio (M/F)***
Grp94	-16.4576
Major intrinsic protein PIPc	-5.227
Aldehyde dehydrogenase	-4.2169
Heat shock protein 101	-7.318
Superoxide dismutase	-4.095
CDPK	-9.7936
Heat shock protein 70	-16.4576
Heat shock factor 1	14.3397

Discussion

Our assembly of the *M. inflexa* genome represents a new resource for comparative studies among land plants. I capitalized on the recently-published genome of the related liverwort *M. polymorpha* (Bowman *et al.* 2017) to improve scaffolding of this assembly, estimate divergence rates among specific sequence types, and to identify sex-linked sequences that were leveraged to generate male and female genetic sex markers for *M. inflexa*. My analyses identified several genes on the autosomes, organelles, and sex chromosomes that show strong signatures of recent diversifying selection in *Marchantia*. Additionally, I identified multiple genes possibly underlying an observed genotype difference in DhT in *M. inflexa*, which point towards a complex mechanism of heightened DhT. I detected extreme differences in copy number of DhT genes across multiple loci, some of which were putatively sex-linked. Evidence of sex-linked genes underlying differences in DhT is intriguing, as prior studies indicate complex patterns of sexual dimorphism in DhT in *M. inflexa* (chapter 3; Marks *et al.* 2016).

Analyses of dN/dS ratios for genes on autosomes, sex chromosomes, and organelles in *M. inflexa* and *M. polymorpha* showed evidence of increased diversification

of sex-linked genes relative to autosomal genes, and conservation of organellar genes (particularly the chloroplast) relative to autosomes. UV sex-determination systems are expected to differ from diploid dominant (XY and ZW) sex-determination systems in multiple ways, due primarily to haploid selection (Bull 1978; Bachtrog *et al.* 2011). However, both empirical and theoretical studies on sex chromosome evolution in haploid dominant systems are limited. UV sex chromosomes exhibit suppressed recombination similar to XY and ZW sex chromosomes, which can lead to degeneration of UV chromosomes (Bull 1978; Immler and Otto 2015). However, because UV chromosomes are fully exposed to selection during the majority of the organism's life cycle, gene content is maintained, and positive adaptations may sweep through populations more rapidly than in XY or and ZW systems. My analyses indicate that rapid diversification of sex-linked genes is occurring in *Marchantia*, suggesting that exposure to haploid selection can be a diversifying force on sex chromosomes.

Interestingly, the finding that *M. inflexa* and *M. polymorpha* sex-linked genes are more divergent than autosomal genes is driven primarily by diversification of male-specific (V) genes. Diversification of female-specific (U) genes, and genes with alleles on both the U and V chromosomes is similar to diversification of autosomal genes (Figure 5.3). It has been suggested that UV chromosomes can become highly differentiated between the sexes due to sex-specific selection (Immler and Otto 2015). Males are generally thought to be under stronger sexual selection than females, and variability in male reproductive success may facilitate rapid adaptation of V-linked genes (Bachtrog *et al.* 2011). Further, because male gametes are broadcast indiscriminately, I expect strong selection on sperm characteristics to maintain species recognition in this system (Palumbi

1999). Evidence for diversification of multiple male-specific genes provides a possible mechanism for maintaining species boundaries.

Sex-linked genes with high dN/dS appear to be involved in multiple cellular processes, some of which contribute to differences in sex function in other systems. Notably, diversification of the male allele for bHLH-MYC transcription factor transcription factor (dN/dS = 1.38) is intriguing because this class of transcription factors has been implicated in floral development in *Arabidopsis thaliana* suggesting a role for this gene in reproductive processes (Riechmann and Ratcliffe 2000). Similarly, the female-allele of CCR4-NOT transcription related complex (dN/dS = 4.27) is homologous to the *rcd1*$^{+}$ gene of *Schizosaccharomyces pombe*, which is critical for nitrogen starvation induced sexual reproduction (Okazaki *et al.* 1998). Divergence of these genes within *Marchantia* points towards specific reproductive differences among these species, which may be related to selection for species-specific recognition, habitat-specific optimization, or the evolutionary dynamics of haploid selection on sex chromosomes.

My analyses define male and female-specific genetic sex markers, which will be of great utility in future studies of *M. inflexa* populations where plants are latent to express sex. The ability to sex plants via a simple PCR assay will expedite general efforts to characterize individuals and populations of *M. inflexa* and will aid in efforts to develop *M. inflexa* as a model system to investigate population dynamics and ecological genetics.

In my efforts to identify genes underlying DhT, I capitalized on a previously-identified difference in DhT in *M. inflexa* (Marks *et al.* 2016), and targeted genes that exhibited substantial coverage differences among genotypes. I identified several such

genes, which I speculate are important for increasing DhT in the more tolerant female genotype. Interestingly some of these genes are putatively sex-linked, suggesting that there may be sex-specific components to DhT in *M. inflexa*. However, the precise mechanism underlying differences in DhT is not clear, as genes with higher coverage in the more DhT genotype cover a broad spectrum of functional categories. For example, although CDPK proteins have been recognized as important hubs in plant stress signaling pathways (Schulz *et al.* 2013) with highly conserved structure (Cheng *et al.* 2002), other candidate genes appear to have diverse functions. For example, GRP94 is a stress induced presumptive molecular chaperone (Little *et al.* 1994) that may be involved in the recognition and removal of malformed and damaged proteins, heat shock protein 101 contributes to thermal stress tolerance in transgenic *A. thaliana* (Queitsch *et al.* 2000), and major intrinsic proteins are often channel proteins, such as aquaporins (Mariaux *et al.* 1998). Taken together, coverage differences in these genes point towards an explanation of elevated DhT that is impacted by a diverse array of genetic elements, enhancing DhT through multiple mechanisms. The finding that some of these DhT genes are sex-linked provides a possible explanation for the documented sex differences in DhT in *M. inflexa*.

In summary, the draft genome for *M. inflexa* adds to a growing body of genomic resources for land plants, which will enable investigation of early plant evolution and physiology. I leveraged this assembly to identify genes under diversifying selection in *Marchantia*, to develop genetic sex markers, and to target genes contributing to DhT. My analyses comprise one of the few empirical studies on haploid sex chromosome evolution and suggest that several sex-linked genes (particularly male-specific (V) genes) have undergone rapid diversification in *Marchantia*. I identified multiple sex-specific

sequences, which were used to develop genetic sex markers, and identify genes underlying differences in DhT of *M. inflexa*. Broadly, I found evidence that DhT in *M. inflexa* is likely impacted by gene dosage, where having more copies of particular genes enables a more rapid and effective cellular response.

Methods

Plant growth, DNA extraction, and sequencing

Plant specimens for genome sequencing were collected from East Turure stream (10º41'04"N 61º09'39"W) on the island of Trinidad, Republic of Trinidad and Tobago in 2009. Voucher specimens are deposited at the Missouri Botanical Garden (St. Louis, MO, USA, specimen numbers M092113 and M092115) and at the National Herbarium of the Republic of Trinidad and Tobago (St. Augustine, Trinidad, specimen number TRIN34616, D. N. McLetchie, collector). Vegetative tissue was transported to Lexington, Kentucky, USA and 36 clones (generated though vegetative propagation) of one male and one female genotype were planted on steam-sterilized soil and maintained in a randomized layout in a climate-controlled greenhouse. Plants were watered daily with distilled water and kept under shade cloth to mimic field conditions. Vegetative tissue (growing aerially with no soil contact) was collected from male and female plants after ~5 years in greenhouse conditions. Prior to DNA extraction, thalli were washed in distilled water three times to remove surface contamination. DNA was extracted following a CTAB extraction protocol modified from Doyle 1987. Sequencing libraries were constructed with 300 base pair (bp) inserts and whole genome sequencing was

conducted on an Illumina HiSeq2000 for 100 bp paired end (PE) reads at the Beaty Biodiversity Research Centre, University of British Columbia.

Genome assembly and annotation

Sequence read quality was assessed with fastQC version 3 (Andrews 2010), and filtered with Trimmomatic version 0.33 (Bolger *et al.* 2014). Male and female reads were combined to increase coverage and k-mer plots were generated with DSK version 1.1 (Rizk *et al.* 2013). Assembly was carried out using SOAP *de novo* version 2.04-r240 (Luo *et al.* 2012) with a k-mer length of 31. Reads shorter than 100 bp were not included, alignments of less than 32 bp were not considered reliable, and k-mers observed nine or fewer times were excluded from the assembly.

Following initial assembly, I plotted the length and GC content of each scaffold in JMP®, Version 12 (SAS Institute Inc.). The plot revealed two distinct clusters of well-assembled (long) scaffolds: one with a mean GC content of ~65% and one with a mean GC content of ~45% (Figure 5.5). Consequently, I probed each distinct GC cluster to identify the taxonomic source of the contributing sequence reads by aligning the 100 longest scaffolds of each GC cluster to NCBI's refseq database (Pruitt *et al.* 2005) using TBLASTX (Altschul *et al.* 1990). Taxonomic classification of the resulting alignments with Megan version 4 (Huson *et al.* 2007) revealed that scaffolds with high GC content were derived from a diverse microbial community, whereas scaffolds with low GC content were derived exclusively from plant material (Figure 5.6). Notably, other members of *Marchantia* have a GC content of ~45% (Sharma *et al.* 2014), providing additional support for the assumption that low GC content reads were derived from *M.*

inflexa. Consequently, I filtered the raw sequence data to remove all reads with a GC content > 55%. The remaining reads (although likely not entirely contamination free) represent a data set enriched for *M. inflexa* genomic information. Using only reads with a GC content < 55%, I reassembled the sequence data with the same parameters as above.

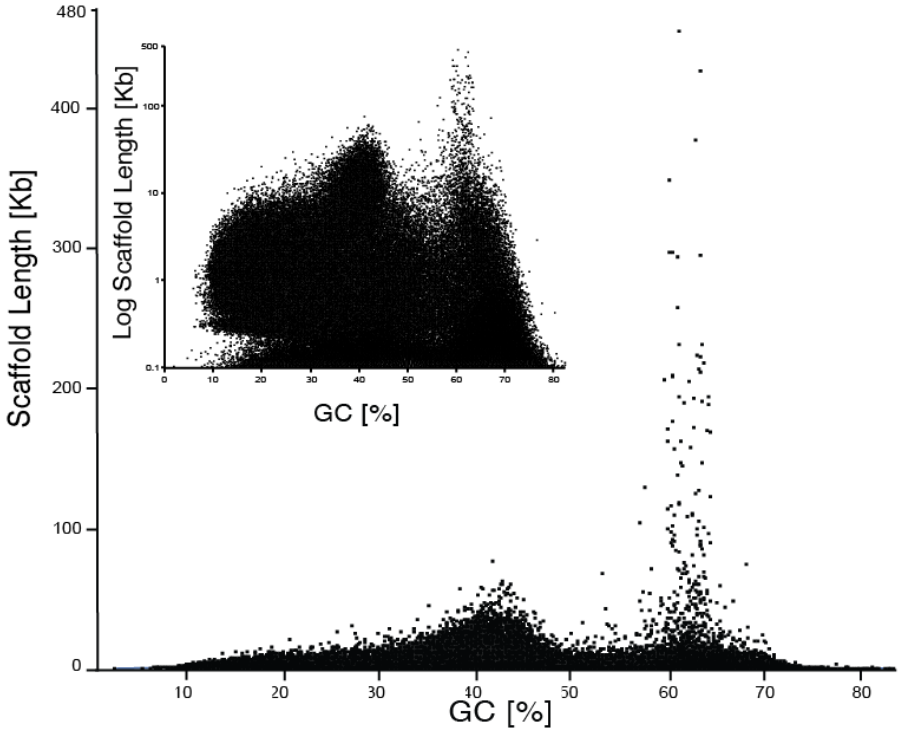

Figure 5.5. Plots of scaffold length (in kilobases) by GC content on both linear (main) and $\log_{10}$ (insert) scales show two distinct peaks of GC content. The low GC fraction consists of putative *Marchantia inflexa* scaffolds (other species in the genus have similarly low GC content(Sharma *et al.* 2014)). The high CG-content scaffolds are derived from an associated microbial community and were removed from sequence data prior to assembly of the M_inflexa_v1.1 draft genome.

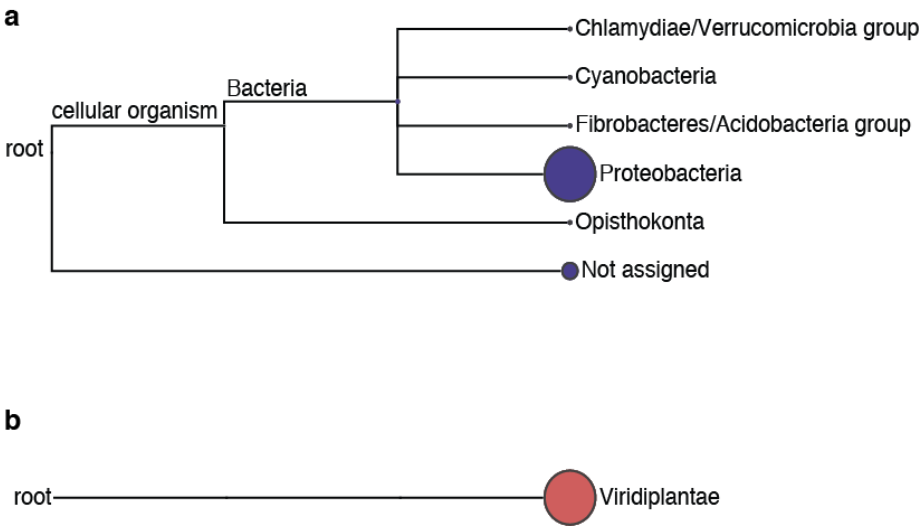

Figure 5.6. Taxonomic assignments of **(a)** high GC content scaffolds and **(b)** low GC content scaffolds. The size of the branch tip is proportional to the abundance of those taxa in the data set.

The resulting scaffolds were aligned to the *M. polymorpha* reference genome (Bowman *et al.* 2017) with BLASTN, allowing me to organize *M. inflexa* scaffolds with Chromosomer version 1.3 (Tamazian *et al.* 2016), which leverages pairwise sequence alignments and local synteny to assign orthologous regions to super-scaffolds. All *M. inflexa* scaffolds not mapped to *M. polymorpha* were appended to the super-scaffold file, and all contigs under 1000 bp were removed. Assembly statistics were computed using assemblathon_stats_2.pl script (https://github.com/lexnederbragt/denovo-assembly-tutorial/blob/master/scripts/assemblathon_stats_2.pl). To estimate assembly completeness, I quantified the percentage of Universal Single-copy Orthologs from the plant set of OrthoDB v9 of BUSCO v3 (Simão *et al.* 2015) present in the *M. inflexa* genome assembly. I conducted a parallel assessment of the *M. polymorpha* genome assembly.

To assemble the mitochondrial and chloroplast genomes of *M. inflexa*, raw reads were trimmed with Trimmomatic version 0.33 (Bolger *et al.* 2014) and error corrected

using the ErrorCorrectReads module of Allpaths-LG version 50156 (Gnerre *et al.* 2011). These were aligned to the *M. polymorpha* reference plastid and mitochondrial sequences (GI 11466673 (Ohyama *et al.* 1988) and GI 11467097 (Oda *et al.* 1992), respectively) with BWA mem version 0.7.12-r1039 (Li 2013). Reads with alignments, plus their mates, were extracted and partitioned. Each partition was assembled separately with Ray de novo version 2.3.1 (Boisvert *et al.* 2010) at k=31. Ray contigs were scaffolded against their homologous *M. polymorpha* reference sequences using Abacas version 1.3.1 (Assefa *et al.* 2009). Adjacent contig overlaps were identified using BLASTN (Altschul *et al.* 1997) and merged at the shared substring. Pilon version 1.13 (Walker *et al.* 2014) was then run for 30 iterations. Assemblies were annotated with Daisie Huang's script PLANN (https://github.com/daisieh/plann) and visualized in OrganellarGenomeDRAW (http://ogdraw.mpimp-golm.mpg.de/cgi-bin/ogdraw.pl).

I used Crossmap version 2.7 (Zhao *et al.* 2014) to transfer all *M. polymorpha* gene annotations to orthologous *M. inflexa* sequences. In addition to these lift-over annotations, *de novo* gene prediction was carried out via Maker version 2.31.8 (Cantarel *et al.* 2008) for four iterations of gene finding. The resulting predicted proteins were aligned to the *M. polymorpha* genome (Bowman *et al.* 2017), *Physcometrella patens* genome (Rensing *et al.* 2008), the *Arabidopsis thaliana* genome (Berardini *et al.* 2015), and NCBI's refseq database (Pruitt *et al.* 2005) using BLASTP (Altschul *et al.* 1990). All alignments were merged and the single best hit for each *M. inflexa* predicted protein was selected based on bitscore. The combined set of *de novo* and lift-over annotations was used for downstream analyses.

Sequence similarity between M. inflexa and M. polymorpha

To enable comparison of orthologous sequences, I aligned the entire *M. inflexa* assembly to the *M. polymorpha* assembly v3.1 with LASTZ version 1.04 (Harris 2007), and extracted orthologous CDS, introns, and intergenic sequences from both assemblies using a combination of BEDtools version 2.19.1 (Quinlan and Hall 2010) and BEDOPS version 2.4.35 (Neph *et al.* 2012). To explicitly test for differences in nucleotide differentiation among these sequence types, CDS, introns, and intergenic sequences were realigned to one another using LASTZ version 1.04. The resulting mean % identity (%ID) for each sequence type was computed and differences among sequence types were tested for significance with a mixed effects linear model in JMP®, Version 12 (SAS Institute Inc.). The fixed effect of sequence type on %ID was tested (sequence length was included in the model as a random effect).

To investigate patterns of gene divergence, I computed dN/dS for all orthologous CDS in *M. inflexa* and *M. polymorpha*. Initially, I extracted the complete CDS and translated amino acid sequence for all orthologous genes using gffread (https://github.com/gpertea/gffread). Orthologous translated CDS were aligned with Clustal Omega version 1.2.4 (Sievers *et al.* 2011), and codon aware DNA alignments were defined using PAL2NAL version 14 (Suyama *et al.* 2006), during which all gaps and internal stop codons were removed. Next, dN/dS ratios for each ortholog were calculated with the yn00 function of PAML version 4.9 (Yang 2007), which computes dN/dS using pairwise comparisons accounting for both transition/transversion bias and base/codon frequency bias (Yang and Nielsen 2000). Following filtering conventions (Villanueva-Cañas *et al.* 2013), cases in which dS = 0, dN > 2, and dN/dS > 10 were

removed from the output, and dN/dS values were log transformed to satisfy assumptions of normality for statistical testing. Differences among groups in mean dN/dS were tested for significance using a mixed effects linear model in JMP®, Version 12 (SAS Institute Inc.). Initially, the fixed effect of gene type (autosomal, sex-linked, or organellar) on dN/dS was tested (scaffold ID was included in the model as a random effect). Post hoc comparisions among gene types were made using orthogonal contrasts to explicitly compare autosomal genes to sex and organallar genes. Subesquntly, I made more detailed comparisions among specific gene types with a mixed effects linear model testing the fixed effect of specific gene type (autosomal, male-specific, female-specific, male-allele, female-allele, mitochondria, and chloroplast) on dN/dS (scaffold ID was included in the model as a random effect). Again, post hoc comparisons were made using orthogonal contrasts to specifically compare each gene type to the autosomal genes. Finally, all individual genes with dN/dS values > 1 were identified, and gene ontology (GO) terms were defined with the GORetreiver tool and summarized with the GOSlimViewer tool available at AgBase (http://agbase.msstate.edu/cgi-bin/tools.pl) (McCarthy *et al.* 2006).

Sex marker identification

Read coverage was computed using DifCover (https://github.com/timnat/DifCover) (Smith *et al.* 2018) to identify regions of the genome unique to these male and female genotypes. Briefly, I determined the genotype-specific coverage by mapping male and female sequence reads back to the draft assembly with Bowtie2 (Langmead *et al.* 2009). Coverage was calculated for 500 bp windows with BEDtools version 2.19.1 (Quinlan and Hall 2010), and DifCover was used to calculate

the $\log_2$ ratios of male:female coverage for each 500 bp window. *Marchantia inflexa* sequences that were both homologous to the *M. polymorpha* sex chromosomes and showed genotype-specific coverage were flagged as potential sex markers. PCR primers were designed with primer3 (Untergasser *et al.* 2012) for five candidate male markers and three candidate female markers.

Candidate sex markers were tested for fidelity by PCR analysis using plants from five distinct populations in Trinidad in 2016 (see Table 5.4 for sample collection info). Plants were cultivated in greenhouse conditions on steam-sterilized soil, under shade cloth, and watered daily for ~one year. When plants began to produce sex organs naturally, vegetative tissue (visibly connected to a reproductive structure to ensure accurate sex identification) was collected from nine individuals of each sex. DNA was extracted from plant tissues following a modified CTAB extraction protocol (same as above), and PCR reactions were conducted with a DNA template concentration of 0.8 ng/ul and combined forward and reverse primer concentration of 0.4 uM. Reaction conditions consisted of initial denaturation at 94 °C for 5 minutes, followed by 34 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 15 seconds, and a final extension at 72 °C for 5 minutes.

Table 5.4. Habitat type, site name, and coordinates of the locations where *Marchantia inflexa* plants were collected in Trinidad, Republic of Trinidad and Tobago. These collections were used to validate the fidelity of *M. inflexa* genetic sex markers. Plants were collected in 2016 and cultured under greenhouse conditions at the University of Kentucky, Lexington KY, USA, for 18 months prior to use.

Habitat type	***Site name***	***Coordinates***
Stream	East Turure	10°41’04”N 61°09’39”W
Stream	West Turure	10°41’00”N 61°10’04”W
Stream	Quare	10°40’37”N 61°11’40”W
Road	Guanapo	10°41’08”N 61°15’49”W
Road	Cumaca	10°41’11”N 61°09’45”W

Dehydration tolerance

To address specific hypotheses on DhT, I probed *M. inflexa* and *M. polymorpha* annotated proteins for orthologs to a list of 195 DT genes (compiled from publicly available mRNA sequences of genes expressed under water stress in model DT plants). DT orthologs were identified using BLASTX (Altschul *et al.* 1990), and the single best hits for each *M. inflexa* and *M. polymorpha* sequence were determined based on bitscore. Presuming that some DT genes would be multi-copy in *M. inflexa*, I calculated the genotype-specific coverage of each DhT ortholog using DifCover (Smith *et al.* 2018) with the aim of detecting genes contributing to the observed genotype difference in DhT in *M. inflexa* (Marks *et al.* 2016). I targeted sequences corresponding to DhT genes showing $\log_2$ coverage ratios > |4| as potential contributors to the observed difference in DhT. Finally, I computed dN/dS ratios for all DhT genes and identified those exhibiting signs of diversifying selection (dN/dS > 1).

Table 5.1. List of all genes with dN/dS > 1. The transcript ID reported here is the ID assigned to *Marchantia polymorpha* transcripts in the v3.1 assembly available on Phytozome (https://phytozome.jgi.doe.gov). Gene type refers to my categorization of genes as autosomal, sex-linked, DhT related, or mitochondrial. GO protein IDs were defined using the GOretriever tool available at AgBase (http://www.agbase.msstate.edu).

Transcript ID	***Gene Type***	***dN/dS***	***GO protein ID***
Mapoly0001s0022.1	Autosomal	1.0183	Mechanosensitive ion channel protein 1
Mapoly0003s0183.1	Autosomal	3.8104	Probable carboxylesterase 18
Mapoly0003s0246.1	Autosomal	3.8104	Polyribonucleotide nucleotide transferase 2
Mapoly0009s0052.1	Autosomal	3.8104	Shewanella-like protein phosphatase 2
Mapoly0009s0208.1	Autosomal	3.8104	Methionine aminopeptidase 2B
Mapoly0010s0113.1	Autosomal	3.8104	Ribosomal protein S19
Mapoly0019s0040.1	Autosomal	3.1317	Basic leucine zipper 61
Mapoly0022s0184.1	Autosomal	3.1317	Protein NRT1/ PTR FAMILY 8.2
Mapoly0025s0007.1	Autosomal	3.1317	kinase PAM74
Mapoly0027s0071.1	Autosomal	3.1317	UDP-glucuronate 4-epimerase 4
Mapoly0027s0188.1	Autosomal	3.1317	Inositol-pentakisphosphate 2-kinase
Mapoly0031s0063.1	Autosomal	3.1317	Pentatricopeptide repeat-containing protein At3g06920
Mapoly0031s0108.1	Autosomal	3.1317	Malate dehydrogenase [NADP]
Mapoly0031s0181.1	Autosomal	3.1317	Uncharacterized membrane protein At4g09580
Mapoly0033s0148.1	Autosomal	2.4628	Pathogenesis-related protein 5
Mapoly0035s0037.1	Autosomal	2.4628	3-oxoacyl-[acyl-carrier-protein] synthase II
Mapoly0038s0053.1	Autosomal	2.4628	Splicing factor U2af large subunit A
Mapoly0040s0067.1	Autosomal	2.4628	7-deoxyloganetin glucosyltransferase
Mapoly0040s0110.1	Autosomal	2.4628	Kinesin-like protein KLP1
Mapoly0042s0095.1	Autosomal	2.4628	Glucan endo-1,3-beta-glucosidase GII
Mapoly0043s0098.1	Autosomal	2.4628	Auxin response factor 10
Mapoly0046s0122.1	Autosomal	2.346	Bifunctional levopimaradiene synthase
Mapoly0056s0145.1	Autosomal	2.2798	kinase At1g07650
Mapoly0058s0080.1	Autosomal	2.2798	Aldose 1-epimerase
Mapoly0061s0076.1	Autosomal	2.2798	Pentatricopeptide repeat-containing protein At5g39980
Mapoly0063s0067.1	Autosomal	2.2463	Iron-sulfur assembly protein IscA
Mapoly0064s0048.1	Autosomal	1.5588	Putative GDP-L-fucose synthase 2
Mapoly0066s0022.1	Autosomal	1.5588	Uncharacterized ABC transporter ATP-binding protein
Mapoly0067s0036.1	Autosomal	1.5588	Protein SERAC1
Mapoly0067s0061.1	Autosomal	1.5588	Codeine O-demethylase
Mapoly0068s0065.1	Autosomal	1.5588	Pentatricopeptide repeat-containing protein At3g18110
Mapoly0071s0008.1	Autosomal	1.5168	Serine/arginine-rich splicing factor RSZ23
Mapoly0088s0006.1	Autosomal	1.0503	Methyl-CpG-binding domain protein 4
Mapoly0088s0027.1	Autosomal	1.0503	Uncharacterized FAD-linked oxidoreductase YvdP
Mapoly0088s0041.1	Autosomal	1.0503	Zinc transporter 2
Mapoly0093s0044.1	Autosomal	1.0503	Zinc-regulated transporter 2
Mapoly0097s0084.1	Autosomal	1.0503	DNA-directed RNA polymerase subunit beta'

Table 5.1 (continued)

Mapoly0098s0044.1	Autosomal	1.0503	Exosome RNA helicase MTR4
Mapoly0098s0056.1	Autosomal	1.0503	Myb family transcription factor APL
Mapoly0106s0023.1	Autosomal	1.0503	Phosphate permease PHO89
Mapoly0131s0005.1	Autosomal	1.0503	Patatin-like protein 2
Mapoly0144s0027.1	Autosomal	1.0503	Protein tas
Mapoly0147s0041.1	Autosomal	1.0503	Alpha-N-acetylglucosaminidase
Mapoly0166s0010.1	Autosomal	1.0039	Endochitinase CH25
Mapoly0191s0007.1	Autosomal	1	Ethylene-responsive transcription factor ERF110
Mapoly0191s0015.1	Autosomal	1	Protein-L-isoaspartate O-methyltransferase
Mapoly0204s0015.1	Autosomal	1	Probable linoleate 9S-lipoxygenase 5
Mapoly0265s0001.1	Autosomal	1	Probable LRR receptor-like serine/threonine
Mapoly0643s0001.1	Autosomal	1	Probable LRR receptor-like serine/threonine
Mapoly1175s0002.1	Autosomal	1	Probable LRR receptor-like serine/threonine
Mapoly0030s0099.1	DhT related	3.1317	Aldehyde dehydrogenase family 3 member I1
Mp011-91	Mitochondria	1.309	orf 69
Mp067-91	Mitochondria	1.105	rpl 10
Mp048-91	Mitochondria	1.0398	orf 84
Mapoly_Y_A0049	Male	5.107	phosphatidylinositol-4,5-bisphosphate 3-kinase
Mapoly_Y_B0032	Male	2.3731	Unknown function
Mapoly_Y_B0003	Male	1.1008	Unknown function
Mapoly_Y_B0018	Male allele	1.3802	bHLH-MYC transcription factor
Mapoly_0018s0021	Female allele	4.2679	CCR4-NOT transcription related complex

CHAPTER SIX

VARIATION IN THE BACTERIOME OF THE TROPICAL LIVERWORT MARCHANTIA INFLEXA, BETWEEN THE SEXES AND ACROSS HABITATS

Reproduced with minor edits from: Marks, R. A., Smith, J. J., Cronk, Q. & McLetchie, D. N. Variation in the bacteriome of the tropical liverwort, *Marchantia inflexa*, between the sexes and across habitats. *Symbiosis* 1–9 (2017). doi:10.1007/s13199-017-0522-3

Introduction

Associations between prokaryotes and larger life forms are both ubiquitous and important (van der Heijden *et al.* 2016). Animal microbiomes are known to influence nutrient uptake, digestion (Hooper *et al.* 2002), metabolism (Claus *et al.* 2008), and even behavior (Ezenwa *et al.* 2012). Likewise, plant microbiomes can dramatically affect performance, physiology, and play a significant role in nutrient acquisition, plant-water relations, and stress responses (Turner *et al.* 2013; Panke-Buisse *et al.* 2015; Haney *et al.* 2015; Vandenkoornhuyse *et al.* 2015; Agler *et al.* 2016). Because the plant microbiome can dramatically impact plant health and performance, understanding the factors that modulate microbiome establishment and composition has implications for food security, agricultural productivity (Sessitsch and Mitter 2015), and ecological stability in natural systems (Vandenkoornhuyse *et al.* 2015).

That being said, we are only beginning to appreciate the complex dynamics of plant-microbe interactions. To date, most research efforts have focused on subsurface (rhizosphere) interactions with a particular concentration on agricultural species. Consequently, I have developed a sophisticated understanding of associations between plant roots and soil microbes (Berendsen *et al.* 2012; Vandenkoornhuyse *et al.* 2015). However, the microbiome in aboveground tissues (phyllosphere) is less well characterized, especially in non-agricultural systems (Turner *et al.* 2013). Nevertheless,

recent technological advances have made this area of investigation more accessible, and a number of contemporary studies have addressed related questions (Turner *et al.* 2013; Delaux *et al.* 2013; Bragina *et al.* 2014; Knack *et al.* 2015; Agler *et al.* 2016; van der Heijden *et al.* 2016). Some of these studies provide evidence suggesting that plant microbiomes are determined, at least in part, by associations that span the deep evolutionary history of plant lineages (Knack *et al.* 2015). In parallel, others have shown that random colonization (Lebeis 2014), nutrient availability (Turner *et al.* 2013), local ecology (Lundberg *et al.* 2012; Schlaeppi *et al.* 2014; Koua *et al.* 2014; Bragina *et al.* 2014), host genotype (Manter *et al.* 2010; Ofek *et al.* 2014; Agler *et al.* 2016), and host sex (Vega-Frutis and Guevara 2009; Ali Balkan 2016) can influence the community structure of plant microbiomes.

While some specific plant-microbe relationships have been well studied, the bacteriome (the bacterial component of the microbiome) in bryophytes (mosses, hornworts and liverworts) is not well characterized. Only a few studies have described bacteriomes in liverworts (Koua *et al.* 2014; Knack *et al.* 2015) and mosses (Opelt and Berg 2004; Opelt, Berg, *et al.* 2007; Opelt, Chobot, *et al.* 2007; Bragina *et al.* 2012, 2014, 2015; Knack *et al.* 2015) despite the potential insight provided by such studies. It is worth noting that bryophyte microbiomes may differ substantially from the microbiomes of other plants in relevant ways. For example bryophytes are often early colonizers in ecological succession, and their propensity for drastic changes in water content may necessitate specific adaptations in the accompanying microorganisms (Opelt and Berg 2004; Proctor *et al.* 2007). Additionally, many bryophytes are found in harsh and nutrient limited environments (Goffinet 2008), which likely impacts any microbes associated with

these plants. A comprehensive understanding of the bryophyte microbiome may therefore offer insight into relationships that aid in stress tolerance and nutrient acquisition, both of which have practical applications in environmental and crop management.

In this study, I hypothesized that because the plant bacteriome can affect plant fitness, changes in the composition of a plant's bacteriome would be associated with the environment, possibly increasing local fitness. I further hypothesized that host plant sex would be associated with changes in bacteriome composition, due to differences in the specific functions of each sex. To test these hypotheses, I examined the bacteriome of the tropical liverwort, *Marchantia inflexa*, in male and female plants from multiple habitats (native stream sides, recently colonized roadsides, and a greenhouse common garden) by targeted sequencing of the bacterial 16S rRNA gene. I predicted that particular bacterial taxa would be enriched in the bacteriome of plants from different habitats and sexes. Other studies have demonstrated that the plant bacteriome is influenced by environmental variation, and I expected this study system to exhibit a similar pattern. Identification of habitat-specific associations could point toward functional relationships that aid in plant performance under specific environmental conditions, such as drought or nutrient limitation. Due to differences in the function, physiology, and morphology of the sexes I expected to detect sex-specific differences in the bacteriome of *M. inflexa.* Although sex-specific fungal interactions have been noted in angiosperms (Varga *et al.* 2013) and mosses (Ali Balkan 2016), a sex difference in the bacteriome of liverworts has not been documented.

Materials and Methods

Study organism

Marchantia inflexa Nees & Mont is a New World liverwort with unisexual individuals found from northern Venezuela to the southern United States (Bischler 1986). *Marchantia inflexa* grows as a dichotomously branching thallus with dorsiventral organization (Figure 6.1) and can reproduce asexually by fragmentation and the production of gemmae (asexual propagules), or sexually by spores. The dominant life phase of *M. inflexa* is the haploid gametophyte, which produces gametes, and sex is chromosomally determined (Bischler 1986). Male and female gametophytes have unique morphology during reproductive life stages (Figure 6.1) and can easily be sexed. *Marchantia inflexa* is typically found in low light, high humidity environments along streams, but it has recently been found colonizing more exposed and disturbed sites, such as roadsides (Groen *et al.* 2010; Brzyski *et al.* 2014).

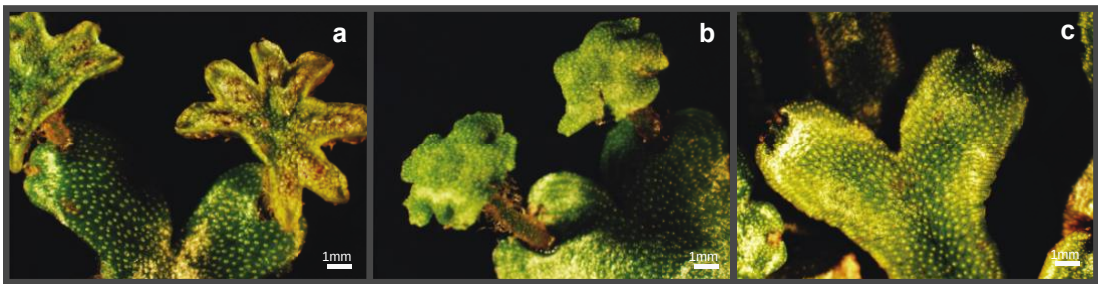

Figure 6.1. Anatomy and morphology of *Marchantia inflexa* plants. (a) Two fully-developed male sex structures (antheridiophores) emerging from the thallus. The antheridia are embedded in the tissue and sperm is released onto the top of the structure (seen on the surface of the right most antheridiophore above). (b) Two fully-developed, but unfertilized female sex structures (archegoniophores) and supporting thallus tissue. The female archegonia are located on the underside of the umbrella like structure. Fertilization occurs when water-dispersed sperm reaches the female archegonia. (c**)** vegetative thallus tissue. Greenhouse collections consisted of vegetative tissues only, whereas field samples included male or female sex structures (used to determine the sex of each sample).

Sampling and growth conditions

Specimens of *M. inflexa* were collected from the island of Trinidad, Republic of Trinidad and Tobago in 2009 and 2015 (Table 6.1). In 2009, vegetative thallus tissue from individual male and female plants was collected from populations along one stream (East Turure) and one road (Cumaca) (Table 6.1). Samples were physically separated from one another to ensure that individuals were genetically distinct, and the uniqueness of each isolate was confirmed by microsatellite analysis (Brzyski *et al.* 2014). Specimens were vouchered at the Missouri Botanical Garden (St Louis, MO, USA, specimen numbers M092113 and M092115) and at the National Herbarium of the Republic of Trinidad and Tobago (St Augustine, Trinidad, specimen number TRIN34616, D. N. McLetchie, collector). Specimens were transported to Kentucky, USA, planted on steam-sterilized soil in a climate-controlled greenhouse and maintained via vegetative propagation until sampling for the current study in 2015. All individual genotypes were maintained in lidded pots to prevent contamination or cross fertilization among specimens. Pots were placed on a capillary mat, kept wet by daily watering with dH_2O, and placed under shade cloth to mimic field light conditions. Plants were rotated periodically, and new clones of each genetic line were propagated by vegetative fragmentation on a semi-annual schedule. When sampled for the current study, greenhouse specimens had been in culture in Kentucky for over 5 years. For the current study, three vegetative thallus tips (~5x8 mm) from each genetic line were collected for DNA extractions. Only aerial tips growing with no soil contact and no evidence of reproductive structures were sampled, and tissues were collected directly into 100% EtOH. Total tissue sampled amounted to ~30 mg per genotype. Although female plants

of this species have higher growth rates than males (McLetchie and Puterbaugh 2000), the individual thalli sampled were similar in size across sexes and genotypes.

Additional specimens were collected directly from field sites in Trinidad, Republic of Trinidad and Tobago in March 2015 (Table 6.1). Plants were collected at three sites (Quare stream, Cumaca road and Guanapo road). Thus, there is potential genetic overlap among greenhouse and field samples from Cumaca road. Study sites in separate drainages were targeted to increase variation between populations. The closest and farthest sites were ~1 and ~11 km apart, respectively. Vegetative tissues (growing aerially with no soil contact) and attached reproductive structures (used to determine the sex of the specimens) were collected at each field site. In order to minimize the potential of collecting clones, all samples were at least 1 m apart. At each site a minimum of one and maximum of three plants of each sex were collected directly into 75% ethanol and transported to the University of Kentucky for subsequent DNA extraction. Field collections included reproductive structures, but greenhouse specimens were comprised of vegetative tissue only. Environmental soil samples were not collected due to the restrictions on the transport of soil across national borders.

Table 6.1. The name, location, and habitat type of collection locations in Trinidad, Republic of Trinidad and Tobago are listed along with the collection year. Cultivation indicates whether the plants were cultured in a common garden at the University of Kentucky or processed directly from field sites.

Habitat type	***Collection location***	***Coordinates***	***Collection Year***	***Cultivation***
Stream	East Turure	10°41'04"N 61°09'39"W	2009	Yes
Stream	Quare	10°40'37"N 61°11'40"W	2015	No
Road	Guanapo	10°41'08"N 61°15'49"W	2015	No
Road	Cumaca	10°41'11"N 61°09'45"W	2009 and 2015	Yes 2009 No 2015

To demonstrate that the field habitats were significantly distinct from one another in relative humidity and temperature, I collected environmental data in June 2016. I monitored the relative humidity and temperature at each field site at five-minute intervals for 4-6 days (overlapping dates when possible) using sensors integrated in the WatchDog™ model 450 data logger (Spectrum® Technologies, Inc. Plainfield, IL, USA). The resulting data were analyzed with a mixed effect linear model in JMP®, Version 10 (SAS Institute Inc., Cary, NC) to test the effect of habitat type and site (nested within habitat type) on relative humidity and temperature (date and time of day were included as random effects). Relative to the road sites, the stream sites were significantly more humid (mean: 99.8 ± 0.02% vs. 95.2 ± 0.17%, $F_{1,11} = 920.8$, $P = 0.0019$) and cooler (mean: 24.6 ± 0.04 °C. vs. 25.3 ± 0.08 °C., $F_{1,13} = 5.7$, $P = 0.032$). While these data clearly do not capture all differences between these two habitats, they demonstrate that the habitats differ significantly in temperature and humidity. Environmental data are available at Figshare (DOI:10.6084/m9.figshare.4823530). Historical weather data for

June 2009, June 2015, and June 2016 indicate that mean monthly temperatures varied by only 0.5 ^{o}C across sampling years (© Copyright 2017 The Weather Company, LLC).

Characterization of the M. inflexa bacteriome

Given evidence for a tightly associated and functionally diverse bacteriome in *M. inflexa* (unpublished data), I sought to characterize the taxonomic diversity of the *M. inflexa* bacteriome and assess variation across habitats and among the sexes. The composition and diversity of the *M. inflexa* bacteriome (including both surface dwelling and endophytic bacteria) was characterized in mature male and female plants from three habitat types: 1) streams in Trinidad (native habitat), 2) roadsides in Trinidad (recently colonized habitat), and 3) the greenhouse at the University of Kentucky (common garden environment). The reproductive structures of field samples were included, but greenhouse samples consisted of only vegetative tissue.

DNA was extracted from 20 plant samples via a modified CTAB extraction method (Doyle and Doyle 1987). Prior to DNA extraction samples were washed three times in distilled water to reduce surface contamination and remove any residual soil on samples. This washing was not intended to remove all surface bacteria, but rather to enrich the proportion of tightly associated and endophytic bacteria in my samples. In addition, I performed three DNA extractions from 100% EtOH without plant tissue to serve as negative controls. 16S rRNA gene sequencing libraries were generated for each sample by PCR amplification of the V4 region of the 16S bacterial rRNA gene. Barcoded primers 501-507F (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

)/701-706R
(5’GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCT
AATCC) were used to index samples. PCR reactions were conducted using Promega GoTaq 5X PCR buffer, 0.8μM of each primer (F/R), and 150-200 ng genomic DNA. The PCR cycling conditions consisted of initial denaturation for 5 minutes at 94 °C followed by 34 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 60 seconds and a final extension at 72 °C for 5 minutes. Samples that did not amplify were cleaned using AMPure magnetic beads, until adequate amplification was attained. All barcoded samples were pooled in approximately equi-molar concentration for sequencing (estimated by florescence on agarose gels). Sequencing of the 16S rRNA gene was conducted at the Advanced Genetic Technology Center, University of Kentucky on an Illumina MiSeq platform. Samples were demultiplexed by the Advanced Genetic Technology Center. Sequence data are available on the Sequence Read Archive of NCBI (BioProject accession number: PRJNA381821).

Read quality of the resulting sequence data was assessed with fastQC (Andrews 2010), and reads were merged using FLASH (Magoč and Salzberg 2011) with a minimum overlap of 20 base pairs (bp) and a maximum overlap of 250 bp. Merged reads were quality filtered with FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Bases with a quality score below 25, entire reads in which >20% of the bases had quality scores below 25 and samples with <1,000 reads were removed.

To characterize the bacteriome composition and address questions of sex and habitat specificity, bacteriome composition and diversity were analyzed using QIIME (Caporaso *et al.* 2010). FASTA files were labeled, combined into a single file, and *de*

novo operational taxonomic units (OTUs) were picked using a 97% similarity threshold. All OTUs found in negative controls and all chloroplast sequences were removed from downstream analyses.

To test for differences in the magnitude of bacterial diversity among habitat types, alpha diversity was assessed. In order to account for differences in the number of reads recovered for each sample, rarefaction analysis was conducted. The data set was normalized to 7,174 counts/sample (the median number of counts/sample) before calculating the Chao1 metric, and the difference in the magnitude of diversity between habitats was tested for significance using a dissimilarity matrix.

To test for differences in bacteriome composition between habitats, beta diversity was analyzed using the Unweighted UniFrac metric (Caporaso *et al.* 2010; Lozupone *et al.* 2011), and the number of counts/sample was normalized to the 1,139 (the minimum number of counts/sample in my dataset). Principal coordinates analysis (PCoA) plots were generated to characterize the differences between all samples. The diversity between habitats was compared to the diversity within habitats using a 999 Monte Carlo permutations based on the Unweighted UniFrac metric, and P-values were adjusted using the Bonferroni correction for multiple comparisons.

Differences in the relative abundance of each OTU among habitats were tested for significance using the Kruskal Wallis test. To reduce statistical problems associated with multiple comparisons the OTU table was filtered to retain only OTUs observed in 25% or more of the samples prior to running this test (Caporaso *et al.* 2010). P-values were corrected for multiple comparisons with the False Discovery Rate (FDR) adjustment.

I tested for a sex difference in the bacteriome of the entire data set, but also in a

reduced OTU table containing only greenhouse samples. Analyses of sex differences in alpha diversity, beta diversity, and differential abundance of taxa (analogous to those described above) were conducted using the complete OTU table and an OTU table containing only the greenhouse samples. Again, all p-values were adjusted using the Bonferroni correction in order to account for multiple comparisons.

To characterize similarities in the bacteriome, I described the shared bacteriome of *M. inflexa.* I defined a shared bacteriome that includes all OTUs found in at least 75% of samples. This definition is analogous to the definition of the core bacteriome (Bragina *et al.* 2015), but because my study included only a limited number of *M. inflexa* populations I prefer to be conservative and refrain from defining a core bacteriome. Differences in taxon abundance among habitats within the shared bacteriome were identified using a Kruskal Wallis test, and the FDR correction was employed to correct for false positives.

Results

Targeted sequencing of the 16S rRNA gene in *M. inflexa* males and females from three habitats revealed high diversity in the bacteriome of *M. inflexa*. Overall, I identified 10,337 unique OTUs, representing 618 bacterial genera. The most abundant phylum was Proteobacteria, and within that phylum, the order Rhizobiales was prevalent. Other abundant phyla included Bacteroidetes, Verrucomicrobia, Cyanobacteria, Acidobacteria and Actinobacteria (Figure 6.2). Although hundreds of different bacterial genera were present at all collection sites, there was no difference in the magnitude of alpha diversity among habitats (Chao1 values for each habitat: stream=2,050; road=1,827; and greenhouse=1.383).

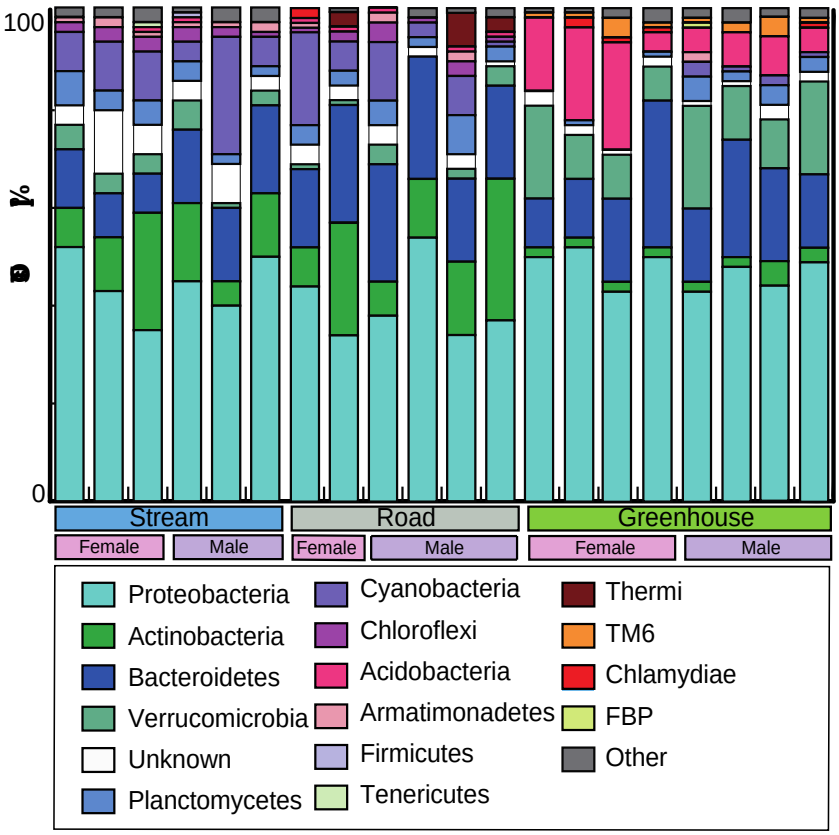

Figure 6.2. The 16 dominant bacterial phyla (major groups) in the bacteriome of *Marchantia inflexa* are shown as percentage of total OTUs. Each stacked column depicts the bacteriome of a single sample and colors represent different phyla. All phyla that comprise <1% of the total OTUs for a given sample are combined and labeled as "other". Samples are grouped by habitat and then by sex.

Comparisons among samples derived from stream, roadside, and greenhouse habitats revealed substantial differences in community composition. There was significantly higher diversity among habitats compared to within habitat diversity ($P < 0.0001$), and samples from the same habitat clustered with one another and were distinct from other habitats in PCoA plots of bacteriome composition (Figure 6.3). The variation detected may be driven by standing differences in the bacterial communities at these collection sites, or differences in plant-microbe interactions that are impacted by habitat differences, but I am unable to distinguish among these alternate explanations. Greenhouse plants were originally collected from different habitats (roads and streams)

~five years prior to the study, but habitat of origin did not significantly impact bacteriome composition of common garden plants.

Figure 6.3. Unweighted Unifrac principal coordinates analysis (PCoA) showing the diversity among samples along PC1 and PC2. Each point represents a sample, and samples are color coded by habitat. Within greenhouse plants, dark green plants were originally collected from Cumaca road, whereas light green plants were collected from East Turure stream. The sexes appear as separate shapes (squares designate males, and circles represent females). Habitat differences in the bacteriome are significant, but sex differences are evident only within greenhouse samples. No additional separation was evident when comparing PC2 and PC3, and consequently that relationship is not shown here.

Two hundred and ninety-one bacterial genera exhibited significant differences in abundance among habitats. Of these, 140 belonged to the phylum Proteobacteria, including the subgroups Rhizobiales (known to be involved in nitrogen fixation, stress protection, auxin and vitamin production (Erlacher *et al.* 2015)) and Gammaproteobacteria, which function in carbon processing and sulfur oxidation (Gifford *et al.* 2014). Forty-three genera belonged to the phylum Actinobacteria. Many subgroups of this phylum are involved in nitrogen fixation, antimicrobial compound production, and antioxidant production (Newton *et al.* 2008). These genera were most abundant in the field sites. Cyanobacteria (10 genera) some of which are known to fix nitrogen in bryophyte tissues (DeLuca *et al.* 2002), Amaimonadetes (5 genera) (Lee *et al.* 2014) and the filamentous Chloroflexi (5 genera) (Björnsson *et al.* 2002) were most abundant in the stream site. In the greenhouse, the carbon processing Bacteroidetes (27 genera) and

Verrucomicrobia (14 genera) (Thomas *et al.* 2011) were abundant. Planctomycetes (14 genera), some of which can metabolize ammonia (Fuerst and Sagulenko 2011), Acidobacteria (12 genera) which are important in nutrient cycling (Naether *et al.* 2012), and Tenericutes (2 genera) were also more abundant in the greenhouse. The remainder of differentially abundant genera are unclassified taxa.

No significant sex differences were detected in the *M. inflexa* bacteriome when the entire data set was analyzed. Both alpha and beta diversity were assessed between the sexes and found to be non-significant. Additionally, no specific taxa differed significantly in abundance between the sexes. However, analysis of greenhouse samples only revealed that diversity between the sexes was significantly higher than diversity within the sexes (T_5=4.22, P=0.03), indicating sex specificity in the bacteriome under common conditions.

The shared bacteriome of *M. inflexa* contained 34 OTUs from 26 genera that are commonly associated with this plant. The most abundant orders of bacteria found in the shared bacteriome were Caulobacterales, Rhizobiales, Acidobacteriales, Saprospirales, Actinomycetales and Rhodospirillales (Table 6.2). Like the entire bacteriome, some taxa in the shared bacteriome varied in abundance among habitats (Figure 6.4).

Table 6.2. The orders of the shared bacteriome and percent that each comprises are listed in decreasing abundance. The shared bacteriome was defined to include only OTUs found in 75% or more of the samples. Orders comprising less than 1% of the shared bacteriome are not shown.

Bacterial Order	***Percent of shared bacteriome (%)***
Caulobacterales	18.59
Rhizobiales	15.73
Acidobacteriales	13.44
Saprospirales	11.50
Actinomycetales	9.03
Rhodospirillales	8.65
Xanthomonadales	7.99
Sphingobacteriales	6.10
Chthoniobacterales	2.43
BD7-3	2.02
Cytophagales	1.58

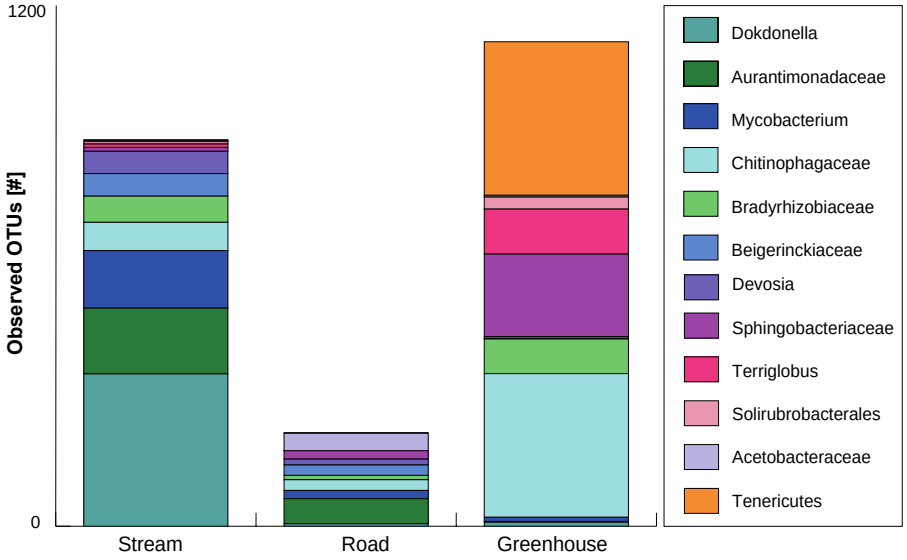

Figure 6.4. Genera of the shared bacteriome showing differential abundance among habitats. Abundance is the mean number of observed OTUs in each habitat.

Discussion

Here, I demonstrate that there are significant patterns of association between bacteriome composition and habitat in *M. inflexa*. There was no comprehensive sex difference in bacteriome composition or diversity. However, I did detect a sex difference in the bacteriome of greenhouse-grown specimens, which I speculate is linked to sex function, sex related physiology (e.g. nutrient requirements for the production of gametes and maturation of sexual offspring) and sex-specific morphology (e.g. aiding in the dispersal and capture of gametes). It is likely that this sex effect is only identifiable under controlled conditions, due to an overwhelming effect of habitat on the bacteriome. Notably, I identified multiple groups of nitrogen fixing bacteria associated with *M. inflexa*. These taxa likely serve nutrient acquisition roles, as has been shown for other non-vascular plants (Bragina *et al.* 2012; Knack *et al.* 2015), suggesting that bacterial nitrogen fixation may be prevalent among bryophyte lineages.

Recent studies have shown that the bacteriomes of mosses (Opelt and Berg 2004; Opelt, Berg, *et al.* 2007; Opelt, Chobot, *et al.* 2007; Bragina *et al.* 2012, 2014, 2015) and liverworts (Knack *et al.* 2015) exhibit high diversity, and I found that the bacteriome of *M. inflexa* is similarly diverse. Variation in the taxonomic composition of the *M. inflexa* bacteriome is largely explained by habitat type. This variation may derive from standing differences in local bacterial pools across environments. Alternatively, it may arise from plant directed recruitment of symbiotic microbes, or selection within *M. inflexa*. The plant microbiome, as a reservoir of additional genes, may serve as the plant's first line of defense against changing environmental conditions (Vandenkoornhuyse *et al.* 2015), and therefore differences in the bacteriome correlated to habitat are expected. Interestingly,

although greenhouse specimens were derived from both road and stream habitats originally, there was no persistent effect of native habitat on bacteriome composition. This suggests that current environmental conditions have a dominant effect on the bacteriome of these plants, and that a substantial fraction of the bacteriome is relatively transient.

Notably, I detected an abundance of nitrogen fixing species, which were substantially enriched in putatively low nutrient environments (field sites). It is well known that some cyanobacteria associated with mosses, hornworts and liverworts aid in nitrogen fixation (During and Tooren 1990; DeLuca *et al.* 2002), but it has only recently become clear that rhizobium-like bacteria may play an equally important role in nutrient acquisition for the living relatives of early diverging land plants (Knack *et al.* 2015). In the current study, Rhizobiales were among the most abundant and consistently detected microbial taxa. Additionally, the richness of Actinobacteria in the field, but not the greenhouse, might suggest a role for the bacteriome in stress tolerance. In addition to antimicrobial compounds, some Actinobacteria species are known to produce mycothinol, a glutathione like antioxidant, that could be important in reactive oxygen species scavenging during stress response (Newton *et al.* 2008). These taxa would aid plants growing in field sites where stressful drying events are more common than in the climate-controlled greenhouse.

I did not detect comprehensive evidence of an association between bacteriome composition and plant sex. However, under common conditions I identified a sex difference in the bacteriome. This general pattern is analogous to studies of plant rhizosphere interactions, showing that the strongest driver of microbial community

composition is soil type (or land use in the case of air born microbial communities (Bowers *et al.* 2011)), yet within a single soil type plant genotype, cultivar, and species can influence microbiome assemblage (Manter *et al.* 2010; Ofek *et al.* 2014). Because plants were grown under common conditions, the identified sex difference cannot be explained by exposure to different microbial communities. Thus, I speculate that the detected difference derives from sex specific plant-microbe interactions, or sex specific morphology and physiology. It seems possible that these differences impact the retention and recruitment of associated microbes. However, these effects are subtle enough to be overwhelmed by the dominant effect of habitat on the bacteriome composition. In the common garden, I detected a higher abundance of Rhizobiales in females, including Brydyrhizobium, Agrobacterium and Rhizobium. In addition, females harbored a substantially more Terriglobus and Pseudonocardia. Males, on the other hand, had a greater abundance of the nitrogen fixing cyanobacteria, Nostoc, multiple groups of the aquatic Planctomyces and Chitinophagaceae. Taken together these differences in taxon abundance point towards alternative strategies for nitrogen acquisition among the sexes. While females host more Rhizobiales, males appear to associate preferentially with Nostoc. Additionally, the detection of Pseudonocardia in females suggests that the bacteriome may aid in stress tolerance, as these taxa have been implicated in detoxification and protection roles (Jafari *et al.* 2014). Consequently, I speculate that sex differences in microbial communities may be correlated to a previously identified sex difference in dehydration tolerance under identical growth conditions (Marks *et al.* 2016), but additional studies will be needed to confirm this.

I identified 26 bacterial genera shared in the majority of my samples, indicating that specific taxa are closely associated with *M. inflexa*, despite changing habitats and sexes, although it is possible that these taxa are simply ubiquitous. The composition of the shared *M. inflexa* bacteriome shows similarities with other bryophytes, including an abundance of Proteobacteria and a richness of Rhizobiales, Actinobacteria, and methanogenic bacteria (Juottonen *et al.* 2005; Knack *et al.* 2015). Actinobacteria are also abundant in seed plant bacteriomes indicating that this group may be a common feature of all plant bacteriomes (Schlaeppi *et al.* 2014). These genera are interesting candidates for future studies investigating relationships that are critical for *M. inflexa* across habitats.

In summary, I present evidence supporting the hypothesis that the *M. inflexa* bacteriome varies across habitats, and I show that under common conditions, host sex can modulate bacteriome composition. Furthermore, these data suggest that habitat dependent differences in the *M. inflexa* bacteriome may be functionally relevant because particular taxa that may aid in plant performance in specific conditions were enriched in these environments, but this speculation must be confirmed by additional studies. Other work in the field has indicated that plant bacteriome composition can be determined by lineage (Knack *et al.* 2015), and also that community composition depends on environmental factors (Bulgarelli *et al.* 2012; Schlaeppi *et al.* 2014) . My study suggests that both of these factors influence bacteriome diversity and assemblage in *M. inflexa*.

CONCLUSIONS AND FUTURE DIRECTIONS

Collectively, this work sheds light on intraspecific variability in water stress tolerance in *Marchantia inflexa* on ecological, physiological, and genomic levels. In a world where plants are threatened by changing land use practices and abiotic stresses associated with climate change, there is an urgent need to develop scientific solutions to mitigate crop losses and meet increased demands for human consumption. Dehydration tolerance (DhT, also dehydration tolerant), among other mechanisms of water stress tolerance, offers a promising path towards developing stress tolerant crops and better managing natural resources. Building a comprehensive understanding of the evolution, ecology, physiology, and genetics of stress tolerance will allow for innovations that enhance management in both natural and agricultural settings. Here, I sought to contribute to the growing understanding of plant stress tolerance by investigating the ecological patterns and genomic mechanisms of DhT in the tropical liverwort *M. inflexa*. This work was carried out on multiple scales ranging from field ecology, to physiology, to genomics and provides novel insight into the complexities of water stress tolerance in plants (Figure 7.1).

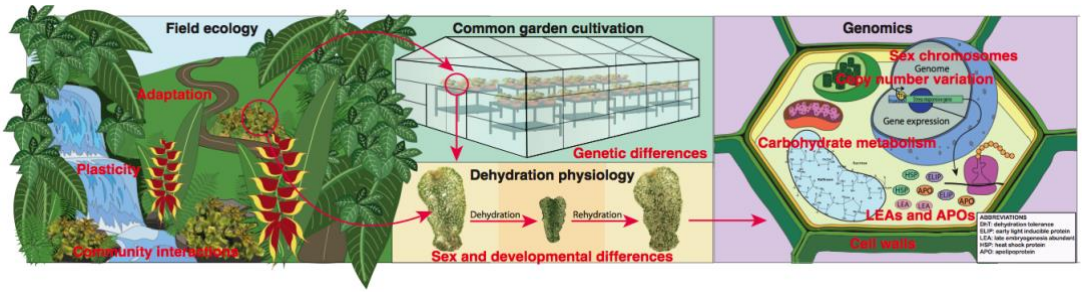

Figure 7.1. This schematic depicts my approach to studying DhT and highlights my findings. Field studies quantified environmental variation, identified population differences in DhT, the impact of differences in DhT on population sex ratios, and plant-microbe interactions. Common garden experiments quantified the intensity of dehydration that *M. inflexa* can tolerate, identified genetic and plastic mechanisms of DhT, and characterized sex and developmental differences in DhT. Genomic and transcriptomic approaches were utilized to characterize gene expression during

dehydration and rehydration and identify genes that may impact DhT. I found evidence to suggest that cell wall composition and modifications, expression of apolipoproteins and late embryogenesis abundant proteins, carbohydrate metabolism, sex linked genes, and copy number variation of stress related genes are involved in DhT.

Natural variation in DhT

Extensive natural variation in DhT exists within *M. inflexa*, is quantifiable, and can be leveraged to gain insight into complex phenotypes. The existence of natural variation in DhT is not, in and of itself a revolutionary observation. However, it is worth highlighting because the extent, distribution, and consequences of natural variation in stress tolerance can impact population dynamics, species distributions, and ultimately environmental stability and productivity. Genetic variation in functional traits is critical for adaptation to novel habitats and can allow populations to survive in a changing word (Jump and Penuelas 2005). Consequently, understanding the patterns of natural variation can be used to predict shifting species distributions, community dynamics, and ecosystem functioning under climate change. Further, quantifying natural variation (and differentiating among plastic and genetic mechanisms) offers a promising path towards understanding the molecular intricacies of complex phenotypes. Here, I detect intricate patterns of natural variation in DhT in *M. inflexa* (both genetic and plastic), which appear to be impacted by both plant sex and environmental selection. Because my approach is integrated, drawing on principles and techniques from ecology, physiology, and genomics I was able to link phenotype (in the field and in a common garden) to genotype (on transcriptional and genomic levels) (Figure 7.1). Ultimately, this multi-level approach allowed for a broad understanding of DhT in *M. inflexa*. Future studies that take advantage of natural variation in DhT (and other traits) are needed to better predict

ecological outcomes and develop management practices to mitigate drought induced losses under climate change.

This work indicates that quantifiable variation in DhT exists among populations of *M. inflexa.* As predicted, DhT is measurably higher in populations with increased exposure to dry conditions. Population differences in DhT appear to derive from a combination of plasticity (chapter 2) and genetic differences (chapter 3). I speculate that plasticity serves as an initial and temporary adjustment for rapid fluctuations in water availability, whereas genetic differences accrue over extended periods of consistent selection for increased DhT. Understanding the relative contribution of plasticity and genetic differences to variation in DhT can be used to predict adaptive potential of populations, define targets for breeding programs aimed at increasing DhT, and estimate range shifts (Jump and Penuelas 2005). Most studies that have documented population differences in DT have done so using field collected material, and thus cannot distinguish among genetic and plastic mechanisms of tolerance (Oliver *et al.* 1993; Farrant *et al.* 2009). I speculate that much of the documented variation in DhT and DT derives primarily from plasticity, but additional studies where plants are grown in a common garden to remove field effects are needed to confirm this speculation. If, on the other hand, heritable genetic differences driving variation in DhT can be identified (as my work suggests), they could provide an important resource for identifying tolerance enhancing genes. Distinguishing among plastic and genetic differences in tolerance is therefore critical for optimizing management practices and defining reasonable targets for breeding and bioengineering. Future studies that rigorously test the limits of plasticity in DT and DhT are needed and should use plants cultivated under common conditions.

Sexual dimorphism in DhT

Much of this work focuses on understanding the complexity of sex-specific biology and secondary sexual dimorphisms in stress tolerance. A growing body of work shows that *M. inflexa* exhibits numerous sexual dimorphisms, some of which may be directly related to reproductive function and others that have likely arisen due to tradeoffs and trait correlations (McLetchie and Puterbaugh 2000; Groen *et al.* 2010ab; Brzyski *et al.* 2014; Marks *et al.* 2016). The consequences of sexual dimorphisms (especially in stress tolerance traits) could have major implications for population sex ratios and ultimately the reproductive success of a population (Bierzychudek and Eckhart 1988). When the sexes exhibit unequal stress tolerance the more tolerant sex may out compete the less tolerant sex under stressful conditions or expand into marginal habitats where the other sex is rare, resulting in spatial segregation of the sexes and biased population sex ratios (Juvany and Munné-Bosch 2015). This difference in stress tolerance of the sexes could have major ramifications for sexual reproduction, adaptation, and species persistence. Thus, a detailed understanding of the sex-specific biology of stress tolerance should be used to inform ecological predictions.

Here, I show that *M. inflexa* exhibits complex patterns of sexual dimorphism in DhT. Initially, I found that females were more DhT than males (chapter 2), which I speculated allowed females to persist for extended periods of time to mature offspring. However, subsequent studies identified populations in which males were more DhT than females (chapter 3). While this pattern of sex differences is surprising and somewhat counterintuitive, I speculate that environmental differences among sites alter the strength

of selective pressures in a sex-specific manner. I find that males are more tolerant than females in the driest sites, whereas females are more DhT in moister sites. It is possible that males only experience selection for DhT in sites where drying is frequent and intense, while in moister sites they complete their reproductive cycle without encountering a drying event and thus are not under selection for DhT. Females, on the other hand may experience selective pressure to maintain DhT at all sites because the time and resources required for reproduction are increased in females relative to males. Interestingly, I found that the fluctuating sexual dimorphism in DhT in *M. inflexa* is driven by male variability suggesting that the male reproductive biology may be more flexible and less constrained than female reproductive biology. Interestingly, my genomic work in *M. inflexa* shows that males harbor considerably higher substitution rates in sex-linked genes than females (chapter 5), indicating that males may be able to rapidly adapt to changing environments. Additional studies that quantify sex differences in stress tolerance are needed to fully understand the mechanism and consequences of sexual dimorphisms in stress tolerance and should include individuals from multiple populations, as my work indicates that sexual dimorphisms can fluctuate across the landscape (chapter 3).

Genomics of DhT

Our work demonstrates that intraspecific variation can be leveraged to gain insight into the underlying molecular mechanisms of stress tolerance. Background genetic differences between samples are minimized when making intraspecific comparisons, which allows for targeted identification of meaningful variation. I utilized

this approach to identify genes underlying elevated DhT in females and meristems of *M. inflexa* (chapter 4). My analyses detected a characteristic accumulation of late embryogenesis abundant (LEA) proteins, substantial modifications to carbohydrate metabolic processes, and changes in lipid transport during the dehydration – rehydration process. These findings are consistent with current models of DhT and DT (Dinakar *et al.* 2012; Costa *et al.* 2017; Zhang and Bartels 2018). Additionally, targeted analyses of sex and tissues specific gene expression indicates that baseline variation in physiology and cell wall characteristics impact the relative tolerance of the sexes and tissues. A similar approach could be applied to physiological, morphological, and metabolic traits and future work should leverage natural variation to better dissect the nuances of DT.

Previous studies comparing DT and DS species have provided insight into the central components of DT. Here, I build on this work by providing insight into the intermediate DhT phenotype. In doing so, I refine our understanding of the essential suite of DT enhancing mechanisms and begin to distinguish among common and unique components of water stress tolerance mechanisms. I detect notable differences between DT and DhT. In my characterization of DhT, I noted key characteristics that were absent in *M. inflexa* (and possibly other DhT plants) but are common in DT plants. These included a putative reduction in temporal regulation during dehydration and rehydration and a conspicuous absence of early light inducible protein (ELIP) and heat shock protein (HSP) expression during dehydration. Future studies on additional DhT species are needed to validate these findings.

Finally, I present an initial characterization of *M. inflexa's* microbiome and detect extensive natural variation in microbiome composition consistent with patterns of

variation in DhT (chapter 6). This work clearly demonstrates that differences in microbiome composition among populations of *M. inflexa* exist. Additional variation in microbiome composition was detected among the sexes, but only when plants were grown under common conditions. I speculate that the plant's microbiome can impact DhT, but additional studies are needed to test this hypothesis.

Future directions

Future work on *M. inflexa* should aim to test the limits of DhT and fully characterize the extent of plasticity in this trait. These studies should include tests of additional developmental stages and tissues, modified treatments including pre-hydration of samples to minimize damage caused by imbibition, and hardening treatments to increase tolerance via growth-based methods. Future studies should seek to characterize DhT in additional populations, and along additional environmental gradients (i.e. temperature, humidity, disturbance) to better define the extent and patterns of standing variation in DhT in *M. inflexa*, which will inform predictions on population persistence and range expansion under climate change. Additionally, characterization of within population sex differences may help to untangle the puzzle of fluctuating sexual dimorphisms in DhT.

The genomic resources for *M. inflexa* presented here can (and should) be improved with additional studies. Candidate genes identified via RNAseq (chapter 4) could be validated (via qPCR) in additional specimens from other populations to test for differences in patterns of gene expression during dehydration and rehydration. Additionally, many unannotated transcripts showed hydration responsive transcription

and characterizing these transcripts may provide insight into novel components of the DhT mechanism. The *M. inflexa* genome (chapter 5) should be improved with additional sequencing efforts to generate a more contiguous assembly, and genome annotation could be enhanced by integrating RNAseq information. Additionally, population genetics work on *M. inflexa* would greatly enhance this system by allowing for estimation of gene flow, migration rates, and tests for loci under selection for DhT. Finally, rigorous experiments to test for a link between microbiome composition and DhT in *M. inflexa* could be conducted to quantify the impact of microbial interactions on DhT. If such a relationship can be established, it would provide an alternative path for developing treatments and practices to increase DhT in both natural and agricultural settings.

To gain deeper insight into DT more broadly, future studies that characterize additional DhT species to validate these findings and better differentiate among general stress responses and critical components underling DT are needed. Further, studies of DT (and other traits) should seek to quantify natural variation. Not only is natural variation seemingly common, but quantifying natural variation can enhance ecological predictions and be used to tease apart the relative contribution of plasticity and genetic differences to changing stress tolerance. Finally, studies on additional dioecious species are needed to gain a better understanding of the causes and consequences of sexual dimorphism in stress tolerance traits, which can have major implications for population dynamics.

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