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Effect of Microbes on the Growth and Physiology of the Dioecious Moss, *Ceratodon purpureus*

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Effect of Microbes on the Growth and Physiology of the Dioecious Moss,

Ceratodon purpureus

by

Caitlin Ann Maraist

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biology

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ABSTRACT

The microorganisms colonizing plants can have a significant effect on host phenotype, mediating such processes as pathogen resistance, stress tolerance, nutrient acquisition, growth, and reproduction. Research regarding plant-microbe interactions has focused almost exclusively on vascular plants, and we know comparatively little about how bryophytes – including mosses, liverworts, and hornworts – are influenced by their microbiomes. *Ceratodon purpureus* is a dioecious, cosmopolitan moss species that exhibits sex-specific fungal communities, yet we do not know whether these microbes have a differential effect on the growth and physiology of male and female genotypes. Using a common-garden design, we reared ten axenic genotypes of *C. purpureus* in a controlled environmental chamber. Clonal *C. purpureus* replicates, with and without the addition of a microbial inoculation, were used to test the effect of a mixed microbial community on vegetative growth, sex expression, photosynthetic efficiency (Fv/Fm and ETR), and chlorophyll content (CFR) for male and female mosses. We found that microbes had a negative impact on the growth and photosynthesis efficiency of *C. purpureus*, and this effect varied among genotypes of *C. purpureus* for ETR and growth. Microbes also had a positive, sex-specific effect on chlorophyll content in *C. purpureus*, with males exhibiting lower CFR values in the absence of microbes. *C. purpureus* sex expression was marginally negatively affected by microbe addition, but gametangia production was low overall in our experiment. We also conducted preliminary surveys using direct counts from moss ramets to assess the community composition of

epiphytic algae associated with our microbe addition and control *C. purpureus*.

These surveys identified three algal morphospecies in association with the microbe addition *C. purpureus* genotypes, as well as cyanobacteria, nematodes, rotifers, and testate amoeba. No algae, cyanobacteria, or micro-fauna were observed in the control plants. Transplantation of a mixed microbial community from field-to-laboratory conditions may be applied to other bryophyte species under varying environmental conditions to provide insight into how these diminutive yet important ecosystems will respond to environmental perturbation.

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TABLE OF CONTENTS

Abstract	i
Acknowledgements	iii
List of Figures	vi
Introduction.....	1
Materials & Methods.....	7
Plant material and growth conditions.....	7
Microbial treatments.....	10
Plant growth response	12
Photochemistry measurements.....	13
Sex expression ratio	15
Enumeration of epiphytic algae.....	16
Statistical analysis.....	18
Results	21
Plant growth response	21
Photochemistry measurements.....	21
Chlorophyll content	22
Sex expression	23
Algae species abundance and diversity	23
Discussion	25
Algal species diversity.....	25
Plant growth response	26
Photochemistry measurements.....	29
Chlorophyll content	33
Sex expression	36
Conclusion	41
References	48

LIST OF FIGURES

Figure 1: Percent growth (%) for microbe addition and control <i>Ceratodon purpureus</i> male and female genotypes.	42
Figure 2: Dark-adapted quantum efficiency (Fv/Fm) for microbe addition and control <i>Ceratodon purpureus</i> male and female genotypes.	43
Figure 3: Electron transport rate (ETR) for microbe addition and control <i>Ceratodon purpureus</i> male and female genotypes.	44
Figure 4: Chlorophyll fluorescence ratio (CFR) for microbe addition and control <i>Ceratodon purpureus</i> male and female genotypes.	45
Figure 5: Contingency plot of the total number of sexually expressing and non-expressing replicate individuals for ten <i>Ceratodon purpureus</i> genotypes.	46
Figure 6: The probability of interspecific encounter (a; PIE) and relative abundance (b; N_i/N) for algae morphospecies quantified from the upper stem region of one ramet collected from microbe addition and control <i>Ceratodon purpureus</i> genotypes.	47

INTRODUCTION

Plants and animals are colonized by a diversity of microorganisms interacting as mutualists, commensals and pathogens (reviewed by Zilber-Rosenberg & Rosenberg, 2008; Bulgarelli et al., 2013; Turner et al., 2013; Peñuelas & Terradas, 2014; Christian et al., 2015). These microbes can have profound effects on host phenotype by influencing pathogen resistance (Arnold et al., 2003; Blum et al., 2013; Innerebner et al., 2011; Li et al., 2015), nutrient acquisition (Dillon & Dillon, 2004; Zhang et al., 2009; Wang et al., 2011; Gonçalves et al., 2014), growth (Hornschuh et al., 2002, 2006; Storelli et al., 2011; Madhaiyan et al., 2005; Montañez et al., 2012), stress tolerance (Rodriguez et al., 2008), or by causing disease or mortality (Hentschel et al., 2000). Furthermore, the microbiome has the potential to affect mate choice in animals (Sharon et al. 2010) and pollinator visitation in plants (Vannette et al., 2013), which suggests microbes can mediate processes driving speciation within their host species.

The genetic background of a host also influences its assemblage of resident microbiota. This host-effect on the structure of the microbiome has been demonstrated for both animals and plants (e.g., Lundberg et al., 2012; Franzenburg et al., 2013; Chaston et al., 2016; Early et al., 2017). Depending on the physiology and genotype of the host, colonization by some species of microbes may be facilitated over others. Biochemical control over microbe community assembly has been suggested for species of *Hydra* that produce different expression profiles of antimicrobial peptides (Franzenburg et al., 2013)

and *Sphagnum* with unique profiles of secondary metabolites (Opelt et al., 2007b). In vascular plants, where the composition of the rhizosphere microbiota is markedly determined by soil-quality and climate, host genotype is implicated in causing some variation in the root-associated bacterial communities of *Arabidopsis thaliana* (Lundberg et al., 2012), potato (Weinert et al., 2011; Inceoğlu et al., 2011), maize (Bouffaud et al., 2012; Peiffer et al., 2013), barley (Bulgarelli et al., 2015), and lettuce (Cardinale et al., 2015; reviewed by Pérez-Jaramillo et al., 2016).

The bryophytes – which include the mosses, hornworts, and liverworts – diversified more than 400 million years ago (Renzaglia et al., 2007) and occupy a diversity of ecosystems worldwide, including some of the most physiologically demanding environments (see Shortlidge et al., 2017; Stark et al., 1998). According to recent molecular data, modern streptophyte algae (relatives to ancestors of early land-plants) possess microbial communities similar to species of liverworts growing in neighboring habitats, suggesting that plant-microbe interactions initially occurred early in plant evolution (Knack et al., 2015). Furthermore, when infected with individual strains of pathogenic fungi and bacteria, mosses utilize similar mechanisms of pathogen defense as found in higher plants (Oliver et al., 2009; Ponce de León et al., 2007; Ponce de León et al., 2012; Bressendorff et al., 2016; Overdijk et al., 2016). Yet despite numerous recent studies characterizing the microbial associations of vascular plants (reviewed extensively by Partida-Martinez & Heil, 2011; Bulgarelli et al., 2013; Turner et al., 2013; Peñuelas & Terradas, 2014; Berg et al., 2016), the

microbiomes of contemporary bryophytes are not as well explored (Knack et al., 2015). Most studies conducted to date that attempt to elucidate moss-microbe interactions have focused on characterizing the diversity and function of moss-associated fungi in natural ecosystems (Kausrud et al., 2008, Davey et al., 2012; Davey et al., 2014; Kachalkin & Yurkov, 2012; Osono et al., 2012; Yu et al., 2014), or the bacterial consortia affiliated with *Sphagnum* sp. (Opelt et al., 2007a; Opelt et al. 2007b; Bragina et al., 2012; Bragina et al., 2014; Bragina et al., 2015; reviewed in Kostka et al., 2016). Although *Methylobacteria* are known to enhance protonema development in *Funaria hygrometrica* (Hornschuh et al., 2002, 2006), and other growth-promoting bacteria occupy the *Sphagnum* microbiome (Opelt et al., 2007a; Opelt et al., 2007b; Shcherbakova et al., 2013), we know relatively little about how microbial communities as a whole influence moss growth and physiology metrics.

Mosses provide a unique ecological niche to functionally diverse microbiota as a result of their physiology and structure. Mosses are haploid dominant with long-lived gametophytes in which dead, senescing, and photosynthetically active tissue persist, providing a continuum between the below- and above-ground components of the environment (Lindo & Gonzalez, 2010). Mosses are also poikilohydric, and their inability to internally regulate their water status relative to that of their external environment has restricted their stature to diminutive proportions (Roberts et al., 2012). Consequently, the microbes that colonize mosses must be adapted to the reduced size and conditional water status of their hosts (Döbbeler, 1997), as well as the extreme

fluctuations in temperature, high UV exposure, and nutrient deficit associated with the phyllosphere (Lindow & Brandl, 2003; Müller et al., 2016). However, the topographical features of the moss gametophore may provide relatively stable micro-environments for microbes to exploit, including uneven leaf surfaces, minute crevices situated at the leaf axes, and subterranean rhizoids (Felix, 1988; Döbbeler, 1997).

Lindo & Gonzalez (2010) argue that mosses represent a functional ecosystem (the “bryosphere”) that serves as a platform for extensive trophic networks, where the mosses intercept organic matter and airborne nutrients that support complex communities of microbes and micro-fauna. Mosses further mediate below-ground processes by influencing soil moisture and temperature conditions that control the rate of microbial decomposition of organic matter (Gornall et al., 2007). In high-latitude environments, the abundance and depth of ground-dwelling moss patches have been correlated with microbial activity and therefore carbon (Hobbie et al., 2000) and nitrogen (Gornall et al., 2007) accumulation in the soil. Recently, Bragina et al. (2015) found that the vegetation in Alpine bog ecosystems dominated by *Sphagnum* mosses is connected by a complex network of core bacteria, including nitrogen-fixing and methane-oxidizing species. The symbiotic methanotrophic bacteria in *Sphagnum* effectively trap carbon within the bryosphere by oxidizing methane – which is generated during decomposition processes occurring within *Sphagnum* bogs – and producing CO₂ that can again be assimilated by the host (Raghoebarsing et al., 2005). Insights from these experiments may be enhanced through an

understanding of the physiological effects of the moss microbiome for the purpose of improved prediction of how global nutrient cycling and ecosystem functioning will be influenced by environmental change (Lindo & Gonzalez, 2010; Kostka et al., 2016).

Ceratodon purpureus is a cosmopolitan moss with separate sexes (dioecy) that tolerates a range of habitats (Crum & Anderson, 1981; Jules & Shaw, 1994). Like many dioecious moss species, natural *C. purpureus* populations exhibit female bias (Shaw & Gaughan, 1993), the cause of which remains unknown. *Ceratodon purpureus* is gaining traction as a model organism in the evolutionary ecology of sexual systems, making it an ideal study species for experiments exploring sex- and genotype-specific microbe interactions, because it possesses sex chromosomes (McDaniel et al., 2007; McDaniel et al., 2013b) and exhibits sexual dimorphism in its physiology and morphology (Shaw & Gaughan, 1993; Shaw & Beer, 1999; Rosenstiel et al., 2012; Slate et al., 2017). Additionally, the *C. purpureus* genome is currently being sequenced (McDaniel & Perroud, 2012; <https://jgi.doe.gov/why-sequence-ceratodon-purpureus-moss/>) and transcriptomes have been assembled from male and female plants (Szövényi et al., 2015). Past studies have found that male and female *C. purpureus* plants differ in fungal abundance and composition (Balkan, 2016), but whether fungal community structure is influenced by the genotype of the moss or by complex interactions within the microbial community remains to be elucidated. However, the evidence that female *C. purpureus* individuals from different populations have more similar fungal community compositions to one

another than their male counterparts from the same population suggests female *C. purpureus* genotypes may influence their microbiomes (Balkan, 2016).

Here, we tested the hypothesis that the microbiome can have sex- and genotype-specific effects on the growth and physiology of the moss *C. purpureus*. To this end, we inoculated ten different axenic *C. purpureus* genotypes with a microbial community isolated from a population of *C. purpureus* occurring in Portland, Oregon USA, while establishing sterile controls for each genotype. We allowed the plants to grow for several months in a controlled growth chamber, and assessed whether microbial treatment and moss genotype affected vegetative growth, gametangia production (sex expression), photosynthetic efficiency (Fv/Fm and ETR), and chlorophyll content (CFR). We also conducted preliminary surveys using direct counts from moss ramets to assess the community composition of epiphytic algae associated with our microbe addition and control *C. purpureus*. Our results suggest that the moss microbiome can have a significant effect on moss growth, reproductive effort, and stress. The effect of our microbial inoculum on the growth and physiology of *C. purpureus* was more often negative than positive, and may depend upon the genotype and sex of the host.

MATERIALS AND METHODS

Plant material and growth conditions

Axenic *Ceratodon purpureus* tissue used in the experiments was provided by Dr. Stuart McDaniel at the University of Florida in September 2015. The *C. purpureus* tissue cultures included single male and female genotypes from ten different haploid-sibling families sourced from Alaska, Oregon, Connecticut, North Carolina, New York, Ecuador, and Chile. Stock axenic tissue cultures were raised in petri dishes covered with Parafilm on sterile BCD agar media (0.7 g agar, 250 mg MgSO₄, 250 mg KH₂PO₄, 1.01 g KNO₃, 1 mL Hoagland's A-Z trace element solution, 12.5 mg FeSO₄ • 7H₂O, and 1 L of DI H₂O; Ashton & Cove, 1977) supplemented with 0.925 g of ammonium tartrate and 1 M of calcium chloride. Stock tissue was subcultured onto fresh BCD agar media approximately every 3-4 months, or as needed, to ensure actively growing uncontaminated moss tissue. In December 2015 and February 2016, a subset of moss tissue from each genotype was sub-cultured onto 60 mL of sterile BCD agar in Magenta vessels (GA-7; Magenta Corp., Chicago, IL USA) and allowed to grow for 4-6 months until subcultured again for the experiment. All stock tissue cultures were maintained in a growth chamber (Conviron Adaptis: CMP 6010; Conviron, Winnipeg, CAN) with a 12 h light /dark cycle (17 h at 21 °C and 7 h at 15 °C) at 67 % relative humidity.

In June 2016, axenic *C. purpureus* tissue was gathered from the December 2015 and February 2016 Magenta vessel stock for the male and female haploid-sibs from Alaska, Portland, Connecticut, Ecuador and Chile (ten

genotypes). These populations were chosen for the experiment because they originate from a variety of climatic conditions with which to test the effects of climate origin on moss-microbe interactions. For each of the ten genotypes, eight clonal replicates were prepared by sterilely transferring 0.1 mL of tissue into independent Magenta vessels, each of which was filled about half-way with 160 mL of sterile Turface (Profile Products LLC, Illinois, USA) saturated with 85 mL of sterile tap water.

The clones were divided among two different treatments with four replicate Magenta vessels per treatment: (1) inoculation with a mixed microbial suspension in 0.01% Triton-X (microbial addition) and (2) inoculation with autoclave sterilized 0.01% Triton-X (control), although the treatments were not applied until later (see pgs. 10-12 for approach). Magenta vessels were sealed with Parafilm, placed in a growth chamber (Convion Adaptis: CMP 6010; Convion, Winnipeg, CAN) in a randomized pattern with respect to genotype and treatment, and allowed to grow in conditions of 12 h light/dark (17 h at 20 °C and 7 h at 10 °C) with 67% relative humidity for approximately two months before Parafilm was removed to improve gas exchange. Since the Convion Adaptis: CMP 6010 does not have a dehumidifier, the humidity of the growth chamber would vary from the established 67% depending upon the water content of the Magenta vessels in which the mosses were growing. Near the beginning (July 15-22, 2016) and the end (Oct. 19- Nov. 30, 2017) of the growing period for our mosses, we used a HOBO Pro v2 temp/RH data logger (Part No. U23-001; Onset Computer Corp., Massachusetts USA) to determine the average relative

humidity in our growth chamber during these time points. The average relative humidity of the growth chamber near the beginning and the end of the growing period was $75.62\% \pm 0.59$ SE and $57.21\% \pm 0.39$ SE, respectively.

Plants were watered as needed to the top of the Turface level with sterile tap water about every 2-3 months. In July 2016, the perimeter of all moss colonies was fertilized with 1 mL of 15% modified BCD without agar at a distance of 1 mm from the moss colony to avoid potentially over-nutrifying the plants and causing stress. The modified BCD followed the same general formula as above, but included trace Hoaglands A-Z prepared from Hoagland Modified Basal Salt Mixture (Phytotechnology Laboratory, Kansas, USA) that already contained ammonium phosphate (0.115 mg/mL) and ferrous sulfate (0.0025 mg/mL); therefore, additional trace ferrous sulfate and ammonium tartrate were excluded from this recipe. In November 2016, all mosses were growing slowly and were fertilized a second time with 5 mL of 100% BCD without agar (applied along the perimeter of the moss colony at a distance of 1 mm) and 500 μ L of 15% BCD without agar (applied directly to the moss tissue) containing Hoaglands A-Z prepared from the Hoagland Modified Basal Salt Mixture (Phytotechnology Laboratory, Kansas, USA). During this round of fertilization, plants were provided the full amount of ferrous sulfate and ammonium tartrate used in the Ashton & Cove (1977) BCD recipe, in addition to the trace quantities present in the Hoagland Modified Basal Salt Mixture. Small quantities of nutrients were added in January and March 2017 to minimize chlorosis.

Microbial Treatments

To determine the effect of microbes on bryophyte growth and physiology, we applied the microbial treatments (microbial addition and control) to our experimental Magenta vessels in October 2016. For our microbial addition, we inoculated *Ceratodon purpureus* genotypes with a mixed microbial community isolated from a large population of *C. purpureus* located in Portland, Oregon (45°28'05.5"N, 122°38'53.1"W). During two different field sampling events occurring at most two days following rainfall, 19 patches of *C. purpureus* of 2.7 cm diameter were haphazardly collected in sterile 50 mL falcon tubes and immediately transported to the lab in double Ziploc freezer bags. The patches from which moss samples were collected on each day were situated approximately 18 meters apart. In the lab, rhizoids and soil were removed from all moss clumps by cutting away the base of each stem with sterile shears. All sporophyte capsules were similarly removed by cutting away along the seta to reduce the risk of contaminating the samples with moss spores of unknown genetic background. Angiosperm seeds and conifer needles were removed from the samples to ensure that microbes extracted from the tissue samples were predominantly associated with the phyllospheric region of the moss. On each day, moss tissue comprising stems ranging from 1-10 mm long were equally divided by fresh weight into four different sterile 50 mL falcon tubes. For the control treatment, four sterile falcon tubes were used in a simulation of the moss preparation steps. All moss and control samples were stored at 2 °C overnight before being processed further the following day.

The next day, all four falcon tubes containing moss and the controls were rinsed two times by vortexing in new falcon tubes of 30 mL sterile tap water for ten seconds. The wastewater was strained from the moss tissue after each rinsing step over a sterile 2 mm² stainless steel sieve to facilitate the exclusion of any soil particles or transient microbes. Next, all four tubes of moss and the controls were transferred to two separate sterile Erlenmeyer flasks that contained 100 mL of sterile 0.01% Triton-X. Dilute detergents like Triton-X have been used in past studies to remove epiphytic microbes from the leaf surfaces of angiosperms (Allen et al., 2006), and in preliminary experiments this concentration was not found to impact the growth of *C. purpureus* negatively. Moss tissue and controls were agitated in a rotary shaker at 250 rpm for one hour at room temperature to bring microbes into suspension (Allen et al., 2006). Following agitation, 200 µm pluriStrainers (PluriSelect PET mesh, #43-50020-03; Leipzig, Germany) were used to filter 25 mL of the microbe suspension and controls into four sterile falcon tubes each. All moss tissue was subsequently consolidated and dried at 60 °C to obtain a final dry mass (1.92 grams for day 1 and 1.81 grams for day 2). To inoculate the mosses, 2 mL of the microbe suspension or sterile 0.01% Triton-X was pipetted directly onto the moss tissue belonging to each treatment group. Once treated, all Magenta vessels were placed back in the growth chamber for the duration of the experiment where they were rotated biweekly to avoid placement effects. Half of the replicate Magenta vessels from each treatment group were treated on each day to minimize the effect of day on treatment.

One replicate Magenta vessel from the Oregon male genotype was removed early in the experiment to confirm that microbes were successfully added to the moss tissue. Additionally, one replicate pot each of the microbe addition and control Ecuador male and female genotypes were excluded from the photochemistry measurements because they were used in a separate preliminary experiment.

Plant Growth Response

To determine the rate of growth of each plant in each treatment, each Magenta vessel (replicate) was photographed monthly, beginning in mid-October 2016 and ending in mid-January 2017, to obtain regular photographs for growth measures across microbe addition and control mosses. All photographs were taken with a Canon Powershot G16 digital camera attached to a tripod orientated to permit canopy photos of the existing moss lawn in each Magenta vessel. In GIMP (2.8.22 MacOS, open-source software), each photo was horizontally aligned with respect to one panel of each Magenta vessel and then cropped to the outer-most edge of the Turface. For the October and January photos, percent cover of all green moss tissue in each Magenta vessel was estimated with the image analysis tool Canopeo (Patrignani & Ochsner, 2015; Oklahoma State University, Stillwater, OK, USA). The thresholding settings in Canopeo were optimized to maximize the selection of green tissue in all Magenta vessels while minimizing the selection of bleached or senescent moss tissue. The growth of the mosses was determined as the percent increase in green percent cover from

October to January. One replicate Magenta vessel from the control Chile male was unintentionally excluded from the dataset due to a failure to photograph in October 2016.

The depth of the Turface height in the Magenta vessels varied (min= 3.8 cm; max=5.1 cm), which could affect the area from which moss percent cover was estimated if the differences in the total Turface area for the minimum and maximum Turface heights were significant. Based on the dimensions of the Magenta vessels (L x W in cm, with container thickness deducted) at the minimum and maximum Turface heights, we estimated that the percent error for the Turface area was $\leq 2\%$.

Photochemistry Measurements

To determine whether *C. purpureus* physiology is affected by its microbial community, we measured maximum quantum efficiency (F_v/F_m) and the efficiency of photochemistry (Φ_{PSII}) of photosystem II for microbe addition and control genotypes derived from Alaska, Oregon, Connecticut, Ecuador, and Chile with a portable OS5p+ chlorophyll fluorimeter (Opti-Sciences Inc., Hudson, NH, USA). All moss colonies were watered no more than two months prior to the chlorophyll fluorescence measurements, and since some standing water was still present in the sealed Magenta vessels, no additional water was added to the moss colonies before the measurements were taken. All fluorescence measures were collected over the course of one month from April to May of 2017.

For the Fv/Fm measurements, mosses were dark adapted for at least 19 hours (5 hours at 20 °C and 14 hours at 10 °C) and measurements were collected from three haphazardly chosen locations in each Magenta vessel at approximately 0.5 cm distance from the highest point of the moss colony. All plants were dark adapted for an additional 20-30 minutes between each of the three Fv/Fm measurements collected from each Magenta vessel. Occasionally, if the moss colony was about the same diameter as the fluorimeter probe, only one region of the moss colony was sampled for fluorescence. The saturating light level was set at 40% of factory settings for the chlorophyll fluorimeter ($15,000 \mu\text{mol m}^{-2} \text{s}^{-1}$).

Once Fv/Fm measurements were completed, the plants were light adapted under an average photosynthetically active radiation (PAR) of 342-349 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under artificial light for 20 minutes. This light setting is close to the highest recorded light setting in the growth chamber in which the mosses were maintained ($310 \mu\text{mol m}^{-2} \text{s}^{-1}$). The ΦPSII measurements were collected for three haphazardly chosen locations for each moss colony at about 0.5 cm distance from the highest point of the moss colony. The same fluorimeter settings were used for ΦPSII as the Fv/Fm measurements, although the far-red light was applied to mimic its occurrence in sunlight. The probe was cleaned with 70% ethanol in between measurements from each Magenta vessel. If fluorescence was not detected by the probe for a particular location, a different region of the moss was haphazardly selected for analysis. One replicate Magenta vessel from the Chile male was excluded from the dataset due to significant tissue damage.

We calculated the electron transport rate (ETR) of photosystem II from ΦPSII using the equation:

$$\text{ETR} = \Phi\text{PSII} \times \text{PAR} \times 0.84 \times 0.5$$

Finally, to estimate chlorophyll content, chlorophyll fluorescence ratio was determined for each Magenta vessel at six random locations with a hand-held chlorophyll fluorimeter (Gitelson et al., 1999; CCM-300 Chlorophyll Content Meter; Opti-Sciences, Hudson, NH, USA). The probe was placed directly on the moss tissue to obtain each reading and the six readings from each Magenta vessel were averaged.

Sex Expression Ratio

To determine the difference in sex expression among the microbial treatments, stems were collected from ten random locations within each Magenta vessel (replicate) every two weeks for two months (May 2017-July 2017). Stems were removed from Magenta vessels with sterilized forceps, placed in coin envelopes, and dried for at least two days at 30 °C. Samples were not collected from Magenta vessels with fewer than 40 moss stems to avoid removing all mature ramets from a clone (N=64). From the stems collected in June 2017, rehydrated stems were dissected from each tissue collection to determine the fraction of expressing male or female stems for each of the ten microbe addition and control *C. purpureus* genotypes. We initially quantified the extent of sex expression in half of our samples by dissecting all stems randomly collected from each Magenta vessel. Since sex expression was rare among these samples, we

chose to dissect only ten random stems from the remaining half of the total samples collected. Often stems would possess more than one developing apex with the potential of producing gametangia; each potentially expressing apex (referred to as “buds”) was dissected on each of the stems to evaluate the extent of sex expression in the *C. purpureus* genotypes. Since preliminary surveys of the stems collected in May 2017 suggested sex expression was nearly absent in all samples, and this trend had not changed significantly in the June 2017 samples, we did not dissect stems from the remaining four tissue collections.

Enumeration of Epiphytic Algae

Algae appeared to be the most abundant and microscopically discernible organisms occurring epiphytically on our microbe treated plants, and so we decided to focus on this group of microbes for our initial assessments of epiphyte diversity among our treatment groups. We used direct counts to determine the relative abundance of the common algae occurring epiphytically on microbe addition and control moss stems for the female *C. purpureus* genotypes (N=2-4). For direct counts, a random moss stem was collected from each Magenta vessel with sterile forceps and mounted on a slide with Protoslo. To quantify non-cyanobacteria algae, one side of the top segment of each moss stem was visualized in phase contrast at 200x through a compound microscope with an Axiocam 105 Color camera attachment (Carl Zeiss; Oberkochen, Germany). Micrographs were taken at this location using Zen Lite 2012 (Carl Zeiss; Oberkochen, Germany; image dimensions were 632.12 μm x 474.09 μm). We

photographed at the second-to-top whirl of leaves for each moss stem, at a region of the whirl where a gap was visible between stacked leaves to ensure visibility of algae present in the water column surrounding the moss stem. If an accessory bud was present on the moss stem, we photographed the upper leaf whirl associated with the main stem. Photographs were taken at various depths-of-field to ensure as many epiphytes were in focus as possible.

To enumerate algae associated with the top portion of each moss stem from each replicate Magenta vessel, a grid overlay with 6 rows and 6 grid columns (36 cells total) was stacked on top of each image in Zen Lite 2012. Within each grid cell, we quantified the density of three different alga morphospecies: (1) *Keratococcus* (oval specimen with slightly curved end-points. Approximately 20 μm in length. Stained positively with Lugol's iodine for true starch), (2) *Tetraselmis* (circular to oval shaped cell with a large pyrenoid; light-green to golden in color. Approximately 15-25 μm in length. Stained positively with Lugol's iodine for true starch), and (3) unidentified filamentous alga (elongated, narrow specimen potentially with slightly curved endpoints. One end point was generally more curved than the other. Roughly 60-80 μm in length. Did not stain positively for true starch with Lugol's iodine). To estimate total algal cell number for each replicate Magenta vessel, we combined the cell counts for each morphospecies estimated from each grid cell. We used the Probability of Interspecific Encounter diversity index ($\text{PIE} = 1.0 - \sum(n/N)^2$; Hurlbert, 1971) to estimate algal morphospecies diversity among our microbe treated and control female *C. purpureus* genotypes. For replicate pots where the three target algae

species were absent, we recorded a PIE value of “0”.

The three algae morphospecies observed in association with the moss stems were not easily identifiable to genus from the stem mounts alone, in particular because it is difficult to observe motile algae when Protoslo is used as a mounting agent. To further characterize the genera commonly observed on our moss stems, we collected water samples from one of the replicate pots for the microbe addition Alaska male. Observations were made of algae motility before the sample was stained with Lugol’s iodine to determine whether algae contained true starch (a characteristic of green algae). Algae were identified to genus following Wehr et al. (2015).

Statistical Analysis

We used a generalized linear model (GLM) with a Poisson error structure (assuming equal mean and variance) and an identity link function to determine whether the percent growth of *C. purpureus* from October 2016 to January 2017 was affected by the experimental treatment (microbe addition and control), moss genotype (ten genotypes), or the interaction between treatment and genotype. Although our percent growth data were not count data, we used a Poisson error structure because the structure of our data most closely approximated a Poisson distribution. Furthermore, this model provided the best fit to our data based on the significance of the Pearson goodness of fit statistic and the likelihood ratio (McCullagh & Nelder, 1989). We also tested the effect of sex (independent of genotype) on the percent growth of *C. purpureus*, but

excluded it from the final model because it was not a significant factor in determining growth. Due to negative percent increase values, four replicate Magenta vessels in the microbe addition group (one Alaska female, two Chile females and one Connecticut female) were dropped from the data analysis.

To evaluate the response of *C. purpureus* genotypes to microbe addition in their photosynthesis efficiency, we constructed a standard GLM (Gaussian error structure with an identity link function) testing the effect of genotype, treatment, and the interaction between these two factors on Fv/Fm and ETR. We also tested the effect of sex on Fv/Fm and ETR but excluded this factor from our final models after finding that it had no effect on either measurement. We used a standard nested GLM (Gaussian error structure with an identity link function) to evaluate whether genotype (nested in sex), treatment, sex, and the interaction between sex and treatment determined chlorophyll content (CFR) in *C. purpureus*.

To determine the effect of treatment and genotype on sex expression in *C. purpureus*, replicate samples were first categorized as expressing (number of expressing buds >0) or non-expressing (number of expressing buds=0). Chi-square analysis was used to evaluate whether treatment and genotype determined the probability that each replicate individual of the ten *C. purpureus* genotypes were expressing or non-expressing. We tested the effect of sex on the probability that each *C. purpureus* replicate was expressing but excluded this factor from the final model because it showed no effect.

To evaluate the effect of treatment, genotype, and the interaction

between the two factors on algal species diversity (estimated using the PIE diversity index) and total algal cell count in microbe treated and control female *C. purpureus* genotypes, we used a standard GLM (Gaussian error structure with an identity link function). We also determined the effect of treatment, genotype, and the interaction between the two factors on the relative abundance of the three algal morphospecies using three separate standard GLM (Gaussian error structure with an identity link function). For each analysis, significant differences among factors were determined through a post-hoc contrast analysis. All analyses were conducted in JMP 13.0.0 (SAS Institute 2015) and R version 3.3.2.

RESULTS

Plant Growth Response

Our experimental treatments (microbe addition and control) significantly decreased the percent growth of *C. purpureus* from October 2016 to January 2017 (df=1; $X^2=6498.83$; $p<0.0001$; see Figure 1 inset); plants with microbial addition had lower percent growth ($309\% \pm 50$ SE) than the control plants ($820\% \pm 74$ SE). The genotype of the moss (df=9; $X^2=6597.25$; $p<0.0001$) and the interaction between moss genotype and treatment (df=9; $X^2=2925.06$; $p<0.0001$) also significantly affected the percent growth of *C. purpureus*. The post-hoc contrast analysis showed that the Chile male, Ecuador female, Ecuador male, Connecticut male, and Connecticut female genotypes exhibited significant percent growth differences between microbe addition and control replicates. The remaining genotypes showed no significant difference in growth between replicates in the different microbial treatments (see Figure 1 main panel). The sex of the plants did not significantly affect plant growth (df=1; $X^2=2.24$; $p=0.13$).

Photochemistry measurements

The maximum quantum efficiency (Fv/Fm) of *C. purpureus* was significantly decreased by microbial treatment (df=1; $X^2=44.80$; $p<0.0001$; see Figure 2 inset). In plants with the microbial addition, Fv/Fm was lower (0.57 ± 0.02 SE) than that of the control plants (0.67 ± 0.00 SE). The genotype of the moss also significantly influenced Fv/Fm (df=9; $X^2=24.45$; $p=0.004$; see Figure 2

main panel), while the interaction between moss genotype and microbial treatment was not significant ($df=9$; $X^2=9.38$; $p=0.40$).

The electron-transport rate (ETR) of *C. purpureus* was significantly decreased by microbial treatment ($df=1$; $X^2=15.68$; $p<0.0001$, see Figure 3 inset). The ETR of the plants with microbial addition was lower (56.79 ± 1.97 SE) than that of the control plants (64.40 ± 1.89 SE). The genotype of the moss ($df=9$; $X^2=42.42$; $p<0.0001$) and the interaction between treatment and moss genotype ($df=9$; $X^2=19.69$; $p=0.02$) were significant factors in determining ETR in *C. purpureus*. The post-hoc contrast analysis showed that the Alaska female and Ecuador male genotypes had significantly different ETR values between microbe addition and control replicates. The remaining genotypes showed no significant difference in their ETR values for replicates in the different microbial treatments (see Figure 3 main panel). The sex of the *C. purpureus* genotypes did not significantly affect the Fv/Fm ($df=1$; $X^2=0.77$; $p=0.38$) or the ETR values of the plants ($df=1$; $X^2=1.21$; $p=0.27$).

Chlorophyll content

The microbial treatment significantly increased the chlorophyll content (CFR) of *C. purpureus* ($df=1$; $X^2=11.58$; $p=0.0007$); CFR was greater in plants with microbial addition (0.68 ± 0.01 SE) than in the control plants (0.65 ± 0.01 SE). Moss genotype nested within sex had a significant effect on the CFR ($df=8$; $X^2=32.63$; $p<0.0001$), although sex was not a significant factor by itself ($df=1$; $X^2=0.66$; $p=0.42$). The interaction between the sex of the plant and the microbial

treatment was a significant factor in determining CFR ($df=1$; $X^2=7.44$; $p=0.006$). Male plants in the microbe addition and control groups had significantly different CFR levels while female plants did not significantly differ between treatments ($df=1$; $X^2=17.55$; $p<0.0001$; $df=1$; $X^2=0.28$; $p=0.60$, respectively; see Figure 4).

Sex Expression

Our experimental treatments marginally significantly affected sex expression in *C. purpureus* ($d=1$; $X^2=3.08$; $p=0.08$). For plants with microbe addition, the total number of sexually expressing replicate individuals was lower (total=1, N=30) than in the control plants (total=6, N=34; see Figure 5 inset). Only 3.33% of replicate samples in the microbe addition group had sexually expressing buds, while 17.65% of replicate samples in the control group had sexually expressing buds. The effect of moss genotype on sex expression was also marginally significant ($d=9$; $X^2=14.88$; $p=0.09$; see Figure 5 main panel).

Algal species abundance and diversity

The relative abundances of *Keratococcus* and *Tetraselmis* associated with the female *C. purpureus* ramets were affected by our experimental treatment (*Keratococcus*: $df=1$, $X^2=2.08$, $p<0.0001$; *Tetraselmis*: $df=1$, $X^2=0.52$, $p=0.0045$), but were not affected by moss genotype (*Keratococcus*: $df=4$, $X^2=0.34$, $p=0.4888$; *Tetraselmis*: $df=4$, $X^2=0.16$, $p=0.6454$) nor the interaction between treatment and moss genotype (*Keratococcus*: $df=4$, $X^2=0.33$, $p=0.5091$; *Tetraselmis*: $df=4$, $X^2=0.16$, $p=0.6558$). The relative abundance of the

unidentified filamentous algae was not affected by treatment ($df=1$, $X^2=0.05$, $p=0.1489$), moss genotype ($df=4$, $X^2=0.10$, $p=0.3821$), or the interaction between treatment and moss genotype ($df=4$, $X^2=0.09$, $p=0.4373$). *Keratococcus* (mean= 0.53 ± 0.11 SE) was on average more abundant than *Tetraselmis* (mean= 0.27 ± 0.08 SE) and the unidentified filamentous algae (mean= 0.08 ± 0.05 SE) for the upper-region of the microbe addition female *C. purpureus* ramets (see Figure 6a). We did not observe *Keratococcus*, *Tetraselmis*, or the unidentified filamentous algae among ramets collected from our control group (*Keratococcus*: mean= 0 ± 0 SE; *Tetraselmis*: mean= 0 ± 0 SE; unidentified filamentous: mean= 0 ± 0 SE).

Our experimental treatment also had a significant effect on the PIE diversity index for algae enumerated from the female *C. purpureus* stems ($df=1$; $X^2=0.33$; $p<0.0001$). Species diversity was higher among microbe addition stems (mean= 0.21 ± 0.05 SE; see Figure 6b) than control stems (mean= 0 ± 0 SE). Neither moss genotype ($df=4$; $X^2=0.07$; $p=0.4763$) nor the interaction between treatment and moss genotype ($df=4$; $X^2=0.05$; $p=0.6177$) significantly determined the PIE species diversity index for the female *C. purpureus* stems.

DISCUSSION

The results of this study suggest that the microbial community with which male and female *Ceratodon purpureus* genotypes are inoculated can have a significant effect on the growth and physiology of the moss, and that this impact can vary with host sex and genetic identity. Since the phyllosphere is colonized by a diversity of microbes that can function as commensals, pathogens, and beneficials (Turner et al., 2013), and the effect that different strains of microbes have on a host plant can vary depending upon the identities of co-occurring micro-organisms (Berendsen et al., 2012), more studies are needed that quantify the influence of microbes on plant growth using a mixed microbial community to obtain ecologically relevant data.

Algal species diversity

We observed three algal morphospecies colonizing female *C. purpureus* moss stems in the microbe addition treatment, and no algal individuals were observed among ramets from our control treatment. Among the algal morphospecies quantified in our microbe addition samples during the direct counts were *Keratococcus*, *Tetraselmis*, and an unidentified, non-Chlorophyta filamentous alga. While the diversity and relative abundance of the algal morphospecies varied between the microbial treatments, we failed to see an effect of *C. purpureus* genotype on the relative abundance of algae associated with the upper portions of the moss ramets. These data suggest that host plant genotype does not affect the ability of epiphytic algae to colonize moss tissues,

although we did find genotype-specific responses to microbes in the physiology of *C. purpureus*.

During separate, non-quantitative observations occurring sporadically from May to December 2017, we also noted the presence of *Arcella* (a genus of testate amoeba in the order Arcellinida), cyanobacteria, nematodes, and rotifers associated with the moss stems collected from our microbe addition Magenta vessels, but observed none of these organisms among ramets from control vessels. These observations suggest a diverse detrital trophic network of early-colonizing microbiota (Lindo & Gonzalez, 2010) was successfully introduced to and maintained in our microcosms throughout the duration of our experiment.

Plant Growth Response

We found that transplanting microbes onto axenic *C. purpureus* resulted in significant reductions in lateral growth (determined by the change in percent cover) over a three-month period compared to plants without microbe additions, suggesting a potentially antagonistic effect of the microbial community with which our plants were inoculated. Some of the moss genotypes showed more significant reductions in growth due to treatment (eg. Chile male, Ecuador female, Ecuador male, Connecticut male, and Connecticut female) than other genotypes (eg. Alaska female, Alaska male, Chile female, Oregon female , and Oregon male), and the variation in the severity of the lateral growth reduction among the genotypes may be due to differences in their immune responses or interactions with the members of their microbial community.

The microbiome is known to affect growth and development in several metazoan model systems (Shin et al., 2011; Storelli et al., 2011; Rawls et al., 2004; Houthoofd et al., 2002). Shin *et al.* (2011) found that *D. melanogaster* larvae raised in axenic conditions developed slower than larvae raised in the presence of commensal bacteria. The bacterium *Acetobacter pomorum* (Acetobacteriaceae) was specifically found to assist in the growth and development of *D. melanogaster* provided with a standard or low nutrient diet by modulating insulin/insulin-like growth factor signaling (IIS) in the host (Shin et al., 2011). In a separate study where Acetobacteriaceae were absent in the guts of *D. melanogaster*, *Lactobacillus plantarum* was responsible for the growth promoting effect of the microbiome observed in flies provided a low nutrient diet (Storelli et al., 2011). Together, the results of these studies suggest functional redundancy in the gut microbiota that can associate with metazoan hosts, resulting in a growth-promoting influence of the microbiome despite variation in its species composition.

The microbiome of plants harbors growth promoting micro-organisms as well (aka PGPM; reviewed by Rodriguez et al., 2009; Weyens et al., 2009; Partida-Martinez & Heil, 2011; Schlaeppi & Bulgarelli, 2015). Some of the mechanisms by which microbes have been shown to augment plant growth include enhancing host access to nutrients (Iniguez et al., 2004; Wang et al., 2011; Gonçalves et al., 2014), releasing phytohormones (Madhaiyan et al., 2005; Hornschuh et al., 2006), decreasing ethylene levels (Mayak et al., 2004), and minimizing pathogen growth (Shcherbakova et al., 2013; reviewed by

Weyens et al., 2009 and Schlaeppi & Bulgarelli, 2015). PGPMs have been identified from the gametophyte surfaces of the common moss species *Funaria hygrometrica* (Hornschuh et al., 2002; Schauer & Kutschera, 2011) and *Sphagnum* (Opelt et al., 2007a; Opelt et al., 2007b; Shcherbakova et al., 2013).

Montañez et al (2012) found that there was a plant-genotype specific effect of 15 different strains of PGPM on the seedling growth of two different maize cultivars. For instance, three strains of *Pseudomonas fluorescens* and *Enterobacter* enhanced shoot growth in one cultivar of maize, but did not improve shoot growth in the second cultivar (Montañez et al., 2012). The results of the maize experiment suggest that host genotype can play a role in determining the outcomes of plant-microbe interactions, and our data further support this conclusion because the growth of some *C. purpureus* genotypes was more negatively affected by the addition of microbes than other genotypes. Notably, in our experiment both of the Alaska and Portland genotypes did not exhibit a significant difference in growth between microbe addition and control plants, while both of the Connecticut and Ecuador genotypes were significantly stunted by the presence of microbes.

We hypothesize that our results may indicate that the different genotypes may be tolerant of – or even reliant upon – different microbial strains, however this hypothesis has yet to be tested. In an experiment evaluating the effects of “home” versus “foreign” soil inoculum on the growth of different plant species, Pizano et al. (2014) found evidence that some fast-growing grass and tree species perform better in soil containing “foreign” microbe inoculum than in

soil inoculum collected from where the plant species typically occur. The improved growth of these plants in foreign soil may be due to the pioneer plant species being released from neighborhood antagonistic microbes (Pizano et al., 2014). Conversely, shade-tolerant trees performed better in soil inoculum collected from the sites in which they originally occurred, perhaps due to their increased reliance on arbuscular mycorrhizal fungi present in those soils (Pizano et al., 2014).

In our experiment, we measured the lateral growth of *C. purpureus* in response to microbe treatment by estimating the change in the green percent cover of the moss over time. However, this approach does not account for variation in stem density or height that may occur among different genotypes, or between male and female mosses (Moore et al., 2016). Future studies estimating stem density, height, and cross-sectional area for male and female *C. purpureus* inoculated with a mixed microbial community would shed light on whether the microbiome can significantly affect moss clump morphology.

Photochemistry measurements

Chlorophyll fluorescence measurements are widely used as a non-invasive method of determining how efficiently photosystem II (PSII) is functioning in plant tissues (Maxwell & Johnson, 2000; Murchie & Lawson, 2013). For instance, the dark-adapted maximum quantum efficiency of PSII (Fv/Fm) can be used to detect compromised PSII machinery in dark-adapted plants, with values lower than the optimum of about 0.83 indicating photoinhibition or

increased non-photochemical quenching (Maxwell & Johnson, 2000; Murchie & Lawson, 2013). Chlorophyll fluorescence measurements have been extensively utilized to evaluate bryophyte ecophysiology in response to desiccation (Csintalan et al., 1999; Proctor & Smirnoff, 2000; Proctor 2003; Proctor et al., 2007; Cruz de Carvalho et al., 2011), nutrients (Arróniz-Crespo et al., 2008), salinity (Bates et al., 2009), heavy metals (Tuba et al., 2010), and temperature (Jägerbrand & Kudo, 2016; Shortlidge et al., 2017). Yet to our knowledge, no experiments have been conducted that attempt to evaluate the effects of the microbiome on the quantum efficiency of photosynthesis among moss genotypes.

In our experiment, we observed overall low average Fv/Fm values in ten *C. purpureus* genotypes, ranging from 0.47 to 0.68 for microbe addition plants and 0.63 to 0.70 for control plants (Figure 2). The maximum values are within the range observed for *C. purpureus* from previous studies (Slate et al., 2017). Therefore, these values hint at lower optimal Fv/Fm in this moss than what is generally expected of vascular plants. However, the results of our chlorophyll fluorescence measurements also demonstrate that the microbe treatment negatively impacted photosynthesis in *C. purpureus* because both dark-adapted (Fv/Fm) and light-adapted (ETR) quantum efficiencies were lower in the microbe addition plants than the control plants.

We found significant differences among *C. purpureus* genotypes in their Fv/Fm and ETR values, regardless of microbial treatment, revealing inherent variation in the capacity of the genotypes to tolerate stress. Notably, the Chile

male genotype exhibited lower average Fv/Fm (0.55) and ETR (49.7) values than the remaining nine genotypes, consistent with compromised photosynthetic machinery. The results of our ETR measurements further implicate the microbial addition in negatively impacting the electron transport efficiency of PSII in our Alaska female and Ecuador male genotypes. For these genotypes, ETR was significantly lower in the microbe addition replicates (Alaska female mean=49.6; Ecuador male mean=60.6; Figure 3) than the control replicates (Alaska female mean=66.4; Ecuador male mean=88.5), which suggests the electron transport systems of these two genotypes were more vulnerable to biotic stress than systems in the other eight *C. purpureus* genotypes. To our knowledge, these data are the first to demonstrate that a microbial consortium can alter the photosynthetic efficiency of plants.

For vascular plants, both pathogenic and beneficial microbes have been evaluated for their effects on the photosynthesis of host plants in experiments that generally involve one focal microbe. In *Arabidopsis thaliana* seedlings, volatile organic compounds emitted from the plant growth promoting rhizobacterium (PGPR) *Bacillus subtilis* optimized the dark-adapted quantum efficiency (Fv/Fm) of the host by enhancing iron-uptake by the roots of plants grown in low-to-high iron conditions (Zhang et al., 2009). The volatiles emitted by *B. subtilis* can further amplify the photosynthesis efficiency of *A. thaliana* seedlings by silencing the hexokinase-dependent glucose sensing pathway responsible for inhibiting photosynthesis and triggering sugar storage when glucose is plentiful in the plant (Zhang et al., 2008). Ramping up the

photosynthetic machinery of the host by improving nutrient acquisition or diverting glucose signaling pathways increases primary production in host tissues and therefore the amount of nutrients available to sustain microbial growth (Zhang et al., 2008; Zhang et al., 2009).

Pathogens have likewise been hypothesized to increase photosynthesis in their hosts because the physical and biochemical defense strategies plants utilize upon infection are energetically demanding and photosynthesis generates the carbohydrates necessary to fuel those processes (reviewed by Bolton, 2009). However, several studies focusing on vascular plants have found reductions in chlorophyll fluorescence due to pathogen colonization, particularly at sites where infection is initiated (eg. Berger et al., 2004; Scharte et al., 2005; Bonfig et al., 2006; Swarbrick et al., 2008). This local reduction in photosynthesis may be due to the coordinated suppression of the expression of source-specific genes associated with photosynthesis, such as Rubisco coding genes (Somssich & Hahlbrook, 1998), with the concomitant increased expression of sink-related genes to provide energy for expensive defense mechanisms (Berger et al., 2004; Scharte et al., 2005; Bolton, 2009). The hypersensitive response (HR) to a pathogen may also reduce photosynthesis in higher plants by causing oxidative damage in chloroplasts due to the increased production of reactive oxygen species (ROS) in infected cells (Bechtold et al., 2005; Bolton, 2009). Although HR defense pathways are still being unmasked in lower plant lineages (Overdijk et al., 2016), *Physcomitrella patens* infected with the necrotroph *Botrytis cinerea* exhibit increased cellular ROS concentrations when the pathogen is present,

suggesting an HR-like response is possible in bryophytes (Ponce de León et al., 2012; Ponce de León & Montesano, 2017).

In our experiment, *C. purpureus* plants with microbial addition exhibited chlorotic chloronema and browning moss tissue, which may have been indicative of pathogen infection in these plants. In the subsequent months during which chlorophyll fluorescence measurements were taken, the chlorotic appearance of portions of the microbe addition mosses had largely subsided. Future studies should aim to determine whether elevated defense mechanisms or oxidative damage are associated with reduced chlorophyll fluorescence in *C. purpureus* inoculated with a mixed microbial consortium.

Our results suggest that in *C. purpureus* Fv/FM and ETR did not differ significantly between male and female plants. Previously, Slate *et al.* (2017) found that male and female *C. purpureus* had significantly different mean Fv/Fm values ranging from about 0.68-0.75 for females and 0.63-0.72 for males over the course of about three months, using three Portland, OR populations (Slate et al., 2017). We used genotypes from a broad geographic distribution (Alaska, Oregon, Connecticut, Chile and Ecuador), and thus genotype differences may have been larger than sex-specific differences for these measurements in plants grown under similar light conditions in an environmental growth chamber.

Chlorophyll content

The results of our experiment suggest that the sex of *C. purpureus* predicts how chlorophyll content in moss tissues is affected by microbial

inoculation; male *C. purpureus* genotypes exhibited significantly greater chlorophyll content for microbe addition genotypes than control genotypes, while female *C. purpureus* exhibited no effect of treatment on chlorophyll content. The difference in chlorophyll content among the treatment groups for the males was largely driven by the Chilean male genotype, which had significantly lower chlorophyll content in the control group than with microbe addition.

One possible explanation for the unique phenotype exhibited by the Chilean male *C. purpureus* genotype is nutrient deficiency. Plants deprived of nutrients essential to normal chloroplast development can exhibit reduced chlorophyll content. For instance, nitrogen plays a critical role in photosynthesis because it is a significant component of chlorophyll pigments and proteins involved in the Calvin Cycle (Evans, 1983; Evans, 1989). The nitrogen concentration in plant tissues is strongly correlated with the chlorophyll content of plants, with plants containing low total nitrogen (mmol m^{-2}) exhibiting proportionally lower chlorophyll concentrations (mmol m^{-2} ; Evans, 1983; Evans, 1989). Other nutrients can cause reductions in chlorophyll content in plants as well. For example, sugar concentrations increase in magnesium (Mg) deficient *A. thaliana* leaves, resulting in down-regulation of photosynthesis-related genes (ie. the chlorophyll a/b binding protein) and a concomitant decrease in chlorophyll production and photosynthesis (reviewed by Verbruggen & Hermans, 2013). The magnesium deficient plants may eventually exhibit interveinal leaf chlorosis as a consequence of the reduced chlorophyll synthesis (Verbruggen & Hermans, 2013). Iron (Fe^{2+}) deficiency likewise causes chlorosis in vascular plants due to

the role this cation plays as an enzyme cofactor in the synthesis of the chlorophyll intermediate molecule protochlorophyllide (Zhang et al., 2009).

Nutrient deficiencies in mosses are relatively understudied because mosses are not generally regarded as “nutrient deficient” due to their lower requirements compared to vascular plants (Glime, 2017b). However, mosses grown in controlled conditions in the absence of nitrogen, iron, or magnesium eventually develop chlorosis and exhibit inhibited protonemal differentiation (Hoffman, 1966; Dietert, 1979; Glime, 2017b). The plants in our experiment were provided with equal amounts of nutrient amendments to avoid symptoms of deficiency and the Chilean male was the only genotype where gametophore development appeared severely inhibited in the control, but otherwise normal in the microbe addition treatment. These control plants also developed signs of chlorosis, as indicated by their low chlorophyll contents. If not nutrient deficient, the difference in phenotype in the control Chilean male may in part suggest an absence of sufficient phytohormones – such as cytokinins – which have previously been shown to promote the formation of buds on protonema filaments in mosses (reviewed by Reski, 1997). Cytokinins and other growth hormones may be exogenously acquired by mosses from epiphytic microbes such as the *Methylobacteria* (Hornschuh et al., 2002; Hornschuh et al., 2006). Hornschuh et al. (2002) found that axenic *Funaria hygrometrica* protonema grown in the presence of *Methylobacteria* experienced greater cell growth and bud induction than protonema grown without the bacterium.

Chlorophyll content did not significantly differ between the microbe addition and control female *C. purpureus* genotypes, suggesting chlorophyll content was otherwise stable across treatments for the females. Previously conducted studies on tobacco leaves inoculated with the necrotizing pathogen *Phytophthora nicotianae* found a reduction in photosynthesis, but consistent leaf chlorophyll concentrations until much later during a 14 hr course of infection (Scharte et al., 2005). The researchers suggested that the integrity of the light-harvesting complex in tobacco was not compromised by *P. nicotianae*, although transcription of the *Cab* gene was down-regulated by the sugar-sensing pathway invoked upon pathogen infection (Scharte et al., 2005; Bolton, 2009). Instead, Scharte et al. (2005) concluded that the reductions in photosynthesis observed in infected tobacco leaves were due to compromised electron transport between PSII and PSI, potentially due to a decrease in plastocyanin, which is a critical electron carrier in the electron transport chain for photosynthesis.

Sex Expression

To our knowledge, this is the first experiment to attempt to determine the impact of microbes on sex expression in a dioecious moss species. We found that microbe addition was associated with a marginally significant decline in gametangia production in *C. purpureus*, but that sex expression was low overall (1.07 %). Although female stems generally outnumber male stems in naturally occurring populations of dioecious mosses (Shaw & Gaughan, 1993; Stark et al., 1998; Bisang et al., 2004), we found no significant difference in gametangia

density between the sexes, and a marginally significant difference among genotypes.

Contrasting with angiosperms, where roughly 6% of species are dioecious (Barrett et al., 2010), bryophytes are overwhelmingly unisexual (~60% of taxa; Shaw & Gaughan, 1993; McDaniel et al., 2013a; Haig, 2016). Sexual expression in mosses is indicated by the production of sperm-containing capsules (antheridia) in males and egg-bearing structures (archegonia) in females (Glime, 2017a). However, moss ramets may fail to develop gametangia, and some moss populations are dominated by non-expressing (or “sterile”) individuals (Shaw & Gaughan, 1993; Stark et al., 1998; Bowker et al., 2000; Benassi et al., 2011). In sexually reproducing moss populations, the biflagellate sperm of a male is exuded from mature antheridia as a mass and must travel to a receptive female in a film of water, potentially traversing as far as 34 cm from their point of origin (Bisang et al., 2004; Shortlidge et al., 2012). The minimal distance that moss sperm can travel, combined with the low expression rate of some mosses can result in populations with low rates of successful fertilization, as indicated by paltry sporophyte densities in dioecious moss patches (Bowker et al., 2000; Bisang et al., 2004). If barriers to reproduction (such as skewed sex-ratios and distance limitations) result in fewer successful fertilization events, populations that can persist either clonally or sexually should invest less energy in producing costly gametangia (Haig et al., 2016). Consequently, many dioecious mosses rely on asexual (vegetative) propagation to persist, which can result in the establishment of unisexual moss patches due to clonal growth (Haig,

2016). Furthermore, most dioecious moss populations surveyed to date which sexually express are female-biased, also suggesting sex-specific differences in mortality at the spore or gametophyte stage of development, or in gametangia induction (Shaw & Gaughan. 1993; Stark et al., 1998; Stark et al., 2000; Bisang et al., 2004).

Surveys of 11 *C. purpureus* populations in the Eastern United States have indicated either low or no sexual expression for some populations (Shaw & Gaughan, 1993). We found that sexual expression was virtually absent in microbe treated *C. purpureus* genotypes in our experiment, but that a limited number of gametangia were produced in the control (no microbe) group. The overall low sex expression in our experiment occurred irrespective of the sex of the moss, which conflicts with previous research suggesting females express more frequently than males in most *C. purpureus* populations (Shaw & Gaughan, 1993). However, if males are limited in their sex expression by extreme environmental conditions (e.g. Stark et al., 1998; Bowker et al., 2000; Shortlidge et al., 2017), the relatively balanced incidence of expression among males and females in the control group may be due to the moderate growth chamber conditions we used in our experiment.

Skewed sex-ratios in naturally occurring dioecious moss populations may also be the result of the different realized cost of sexual reproduction experienced by male and female mosses (Stark et al., 2000; but see Bisang et al., 2006). For the dioecious desert moss *Syntrichia caninervis*, Stark et al. (2000) found males are more likely to bear the full cost of reproduction upon

gametangia induction because they produce more gametangia per inflorescence, with a greater biomass than females of the same species (Stark et al., 2000). Conversely, female *S. caninervis* only realize their full reproductive cost upon fertilization, which may occur only rarely in mixed populations with spatially segregated sexes (Stark et al., 2000). Due to the differences in the cost of reproductive effort (sexual expression) among the sexes, males may require more nutrients than females to produce gametangia (Stark et al., 2000). The equal provisioning of nutrients to male and female genotypes in our experiment – in addition to a lack of competition among the sexes for those resources (since males and females were raised in separate containers) – may explain the relatively balanced density of sex expressing stems observed in male and female *C. purpureus* in our control group.

The near absence of sexual expression in our microbe treated plants is unsurprising in light of the reduced lateral growth exhibited by the mosses as a result of inoculation (Figure 1). Plant defense trade-offs occur when nutrients that could be devoted to biomass accumulation and reproduction are instead directed towards constructing physical barriers or a chemical arsenal against antagonists, consequently reducing growth (reviewed by Heil, 2002). However, it is impossible to say whether the observed failure of sexual expression in our microbe treated plants indicates a defense trade-off or a limitation in our study design. Collecting stems over a longer period of time will help determine whether sex-expression in *C. purpureus* is severely reduced, or simply delayed by the addition of microbes in controlled environmental conditions. Future studies could also determine

whether *C. purpureus* defense related genes are up-regulated in response to inoculation with a mixed microbial community, and whether this is associated with a concomitant reduction in sex-expression among infected stems.

We are aware of no other studies that have attempted to determine whether a microbiome can impact sex expression in dioecious mosses. However, previous studies have shown that soil microbiomes may impact the flowering phenology of *Boechera stricta* (a relative to *Arabidopsis*; Wagner et al., 2014), *A. thaliana*, and *Brassica rapa* (Panke-Buisse et al., 2014). For *A. thaliana* and *B. rapa*, microbiome selection resulted in communities that caused reproducible delays or advancements in flowering time, the mechanism for which remains unknown (Panke-Buisse et al., 2014). However, the soil microbial community associated with late-flowering plants caused a significant increase in extracellular enzyme activity, which the researchers hypothesized might improve nitrogen and phosphorus mineralization in the rhizosphere (Panke-Buisse et al., 2014). Consequently, late-flowering *A. thaliana* and *B. rapa* were less likely to undergo stress-induced bolting and devoted more resources overall to developing flowers (Panke-Buisse et al., 2014). Additionally, some species of soil bacteria and fungi possess metabolic pathways for producing phytohormones – such as auxin, cytokinin, and gibberellin – that are known to modify the growth and reproductive development of plants (reviewed by Baca & Elmerich, 2003). Previous work suggests that bryophyte gametangia induction is differentially suppressed or promoted by various nutrients and phytohormones (reviewed by Chopra & Bhatla, 1983), yet we were unable to determine whether these factors played a

role in the gametangia suppression observed in our microbe-treated mosses. More experimentation is needed to understand whether different functional classes of microbes can influence the timing of gametangia induction in bryophytes, and whether such an effect is due to nutrient mineralization or phytohormone production by microorganisms.

Conclusion

Mounting evidence suggest microbes play a critical role in phenotypic expression among tracheophytes, but information largely falls short of making clear the influence of microbes on bryophyte traits. To our knowledge, this is the first study to test whether different genotypes of a moss species exhibit significant impacts to their photosynthesis, chlorophyll content, and growth due to inoculation with a phyllosphere microbial community in controlled environmental conditions. Transplantation of a mixed microbial community from field-to-laboratory conditions may be applied to other bryophyte species under varying environmental conditions to provide insight into how these diminutive yet important ecosystems will respond to environmental perturbation.

FIGURES

Figure 1: Percent growth (%) for microbe addition and control *Ceratodon purpureus* male and female genotypes. Main panel: bars represent the mean percent growth for ten microbe addition and control *C. purpureus* genotypes $\pm$ S.E. $N=4$ for all groups except for the microbe addition Oregon ♂ ($N=3$) and the control Chile ♂ ($N=3$). Different letters represent significant differences among *C. purpureus* genotypes for percent growth ($p<0.05$; Tukey HSD). Genotypes with an asterisk (*) were significantly different in percent growth between treatments ($p<0.05$). **Inset:** bars represent the mean percent growth for *C. purpureus* genotypes across treatment $\pm$ S.E. ($N=39$). The asterisk indicates that *C.purpureus* percent growth was significantly different between treatments ($p<0.05$).

Figure 2: Dark-adapted quantum efficiency (Fv/Fm) for microbe addition and control *Ceratodon purpureus* male and female genotypes. Main panel: bars represent the mean Fv/Fm for ten microbe addition and control *C. purpureus* genotypes $\pm$ S.E. N=4 for all groups except microbe addition Oregon ♂, Ecuador ♀, & Ecuador ♂ and control Ecuador ♀ & Ecuador ♂ (N=3). Different letters represent significant differences among *C. purpureus* genotypes for Fv/Fm ($p < 0.05$; Tukey HSD). Genotypes with an asterisk (*) were significantly different in Fv/Fm between treatments ($p < 0.05$). **Inset:** bars represent the mean Fv/Fm for *C. purpureus* genotypes across treatment $\pm$ S.E. For the microbe addition group N=37 and for the control N=38. The asterisk indicates that *C. purpureus* Fv/Fm was significantly different between treatments ($p < 0.05$).

Figure 3: Electron transport rate (ETR) for microbe addition and control *Ceratodon purpureus* male and female genotypes. Main panel: Bars represent the mean ETR for ten microbe addition and control *C. purpureus* genotypes $\pm$ S.E. N=4 for all groups except microbe addition Oregon ♂, Chile ♂, Ecuador ♀ & ♂ and control Ecuador ♀ & ♂ (N=3). Different letters represent significant differences among *C. purpureus* genotypes for ETR ($p < 0.05$; Tukey HSD). Genotypes with an asterisk (*) were significantly different in ETR between treatments ($p < 0.05$). **Inset:** bars represent the mean ETR for *C. purpureus* genotypes across treatment $\pm$ S.E. For the microbe addition group N=36 and for the control N=38. The asterisk indicates that *C. purpureus* ETR was significantly different between treatments ($p < 0.05$).

Figure 4: Chlorophyll fluorescence ratio (CFR) for microbe addition and control *Ceratodon purpureus* male and female genotypes. Bars represent the mean CFR for male and female microbe addition and control *C. purpureus* genotypes $\pm$ S.E. N=19 for all groups except the microbe addition male, where N=18. Mean values with the same letter are not significantly different. The statistical significance of the GLM is denoted as ^{NS}p>0.05, *p<0.05, **p<0.01, and ***p<0.001, with T=treatment (microbial addition and control), S=sex (female or male), and SxT (interaction term). Mean values with the same letter are not significantly different (p<0.05; Tukey HSD).

Figure 5: Contingency plot of sexually expressing and non-expressing replicate individuals for ten *Ceratodon purpureus* genotypes. Main panel: bars represent the total number of expressing (“Yes”) and non-expressing (“No”) replicate individuals for ten *C. purpureus* genotypes. N=7 for all genotypes except the Alaska ♀ (N=8), Chile ♂ (N=3), Ecuador ♀ (N=6), Ecuador ♂ (N=4) and the Connecticut ♂ (N=8). Inset: bars represent the total number of expressing and non-expressing replicate individuals for microbe addition and control *C. purpureus* genotypes. N=30 for the microbe addition group and N=34 for the control group. The results of the chi-square analyses indicate treatment and genotype had a marginal significant effect on whether individuals were sexually expressing ($p<0.1$).

Figure 6: The relative abundance (a; N_i/N) and probability of interspecific encounter (b; PIE) for algae morphospecies quantified from the upper stem region of one ramet collected from microbe addition and control *Ceratodon purpureus* genotypes. (a) Bars represent the mean abundance for the algal morphospecies associated with the female microbe addition *C. purpureus* genotypes $\pm$ S.E. $N=16$ for each algae morphospecies. (b) Bars represent the mean PIE for algae associated with female microbe addition and control *C. purpureus* genotypes $\pm$ S.E. $N=16$ for the microbe addition group and $N=14$ for the control group. The asterisk indicates that the PIE for algae associated with *C. purpureus* was significantly different between microbe treatments ($p < 0.05$).

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