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Isoprene Emission in Polytrichaceae Mosses

Timea Deakova
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Isoprene Emission in Polytrichaceae Mosses

by

Timea Deakova

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

Doctor of Philosophy
in
Biology

Dissertation Committee:
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Portland State University
2019

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Abstract

Our first aim was to identify and quantify Biological Volatile Organic Compound (BVOC) emissions, specifically emissions of isoprene, from the moss *Polytrichum juniperinum* during its earliest stage of life. Isoprene emission from mosses could be a significant component of the total global budget of BVOC emissions. Data concerning the spatial and temporal variability of these emissions are lacking due to poor characterization of the physical and biological factors controlling isoprene synthesis in both vascular and non-vascular plants. We found that *P. juniperinum* in its early life stage (protonema) can emit isoprene at detectable levels at day five after spore germination. These results suggest that protonema is capable of isoprene emission, shown here for the first time, and media affects emission rates. We saw a negative trend with leaf emergence and protonema growth. Chlorophyll fluorescence ratio had a positive correlation with isoprene emission, and isoprene emission was both temperature and light dependent in early protonema.

Our second aim was to determine the effects of sex and sexual expression on isoprene emission in mosses. Sex is a biologically costly, but it can result in genetic adaptability and variety for resulting offspring. Using three Polytrichaceae species, we measured isoprene emission between plants sexually expressing and non-expressing and between male and female moss plants. We found that non-sexually expressing gametophytes had significantly higher isoprene emission than gametophytes expressing sex, suggesting that sex expression may be costly and that additional resources are

allocated to isoprene emission when plants are not reproducing. Males emitted higher levels of isoprene than females, but surprisingly this difference occurred only when plants were not expressing sex. We found species and sex-specific differences in chlorophyll fluorescence ratio (CFR) and relative electron transport rate (RETR).

Our third aim was to investigate and categorize the effect of nitrogen addition on isoprene emission of *P. juniperinum* by creating an artificial nitrogen gradient with ammonium nitrate addition (NH_4NO_3). Current rates of anthropogenic N deposition are altering many biogeochemical processes. In these changing environments, increased nitrogen availability alters plant phenology, physiology, and the allocation of resources, but no information is available on whether additional N increases isoprene emissions in mosses. We used a manipulative experiment to measure the effects of nitrogen addition on moss isoprene emission, as well as on moss morphology and reproductive effort. We found site-specific differences in our morphological and physiological measurements. Isoprene emission was site, sex, and N addition specific, with the highest isoprene emission seen from our mid-level N addition, in both female- and male-majority pots. We found significant treatment and sex-specific interactions among our sites and within sites. We saw the highest reproductive effort counts from non-sexual expressive gametophytes, followed by high sporophyte count from female-majority pots. We also saw chlorophyll fluorescence ratio (CFR) differences between sites and treatments, but not between sexes.

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Table of Contents

Abstract.....	i
Acknowledgements.....	iii
List of Tables	vi
List of Figures	vii
Chapter 1	
Introduction.....	1
Chapter 2	
<i>Isoprene emission from Polytrichum juniperinum protonema.</i>	9
Chapter 3	
<i>The role of isoprene in Polytrichaceae sexual systems</i>	58
Chapter 4	
<i>The effect of nitrogen addition on moss morphology, sexual reproduction, and isoprene production.</i>	127
Chapter 5	
Conclusion	188
References.....	199

List of Tables

Table 2.1 Summary table of <i>Polytrichum juniperinum</i> populations used for protonema single spore and mixed spore experiments.	42
Table 3.1 Summary table of <i>P. juniperinum</i> populations used for non-sexually expressing isoprene emission (n=45).....	100
Table 3.2 Summary table of <i>P. juniperinum</i> populations used for sexually expressing isoprene emission (n=25).....	101
Table 3.3 Summary table of <i>Atrichum undulatum</i> , <i>Polytrichum juniperinum</i> and <i>Pogonatum urnigerum</i> populations used for sexually expressing isoprene emission (n=167).....	102
Table 3.4 Summary table for aboveground morphological trait measurements from <i>P. juniperinum</i> gametophytes to assess discernable characteristics and contributions to isoprene emission (n=45).....	104
Table 3.5 Summary table for belowground morphological trait measurements from <i>P. juniperinum</i> gametophytes to assess discernable characteristics and contributions to isoprene emission (n=45).....	105
Table 4.1 Summary table of <i>Polytrichum juniperinum</i> from six populations (n=60).....	165
Table 4.2 Site characteristics of <i>Polytrichum</i> moss collected in the winter of 2015.....	166
Table 4.3 Age classification rubric of <i>P. juniperinum</i> .populations.....	167
Table 4.4 Summary of all morphological measurements taken from <i>P. juniperinum</i> populations.....	168
Table 4.5 Experimental design set up showing three nitrogen additions.....	170

List of Figures

Figure 1.1 Conceptual model for exploring isoprene emission in moss.....	43
Figure 2.1 (a) Depiction of <i>P. juniperinum</i> spores before capsule sterilization from two sites.....	44
Figure 2.2 Isoprene emission (nmol m ⁻² s ⁻¹) versus <i>P. juniperinum</i> spore germinated protonema (n=21).	45
Figure 2.3 Isoprene emission (nmol m ⁻² s ⁻¹) versus media (agar versus BCD) in <i>P. juniperinum</i> protonema (n=52).	46
Figure 2.4 Image depicting <i>P. juniperinum</i> protonema growth versus day number (n=21).	47
Figure 2.5 Isoprene emission (nmol m ⁻² s ⁻¹) versus <i>P. juniperinum</i> protonema and leaf number (n=81).....	48
Figure 2.6 Isoprene emission (nmol m ⁻² s ⁻¹)/growth versus protonema growth (n=246).	49
Figure 2.7 <i>P. juniperinum</i> % change in protonema growth versus leaf emergence for three individual dates (n=246).	50
Figure 2.8 Isoprene emission (nmol m ⁻² s ⁻¹) versus Chlorophyll Fluorescence Ratio (CFR) in <i>P. juniperinum</i> protonema on agar and BCD media (n=52).....	51
Figure 2.9 Chlorophyll Fluorescence Ratio (CFR) versus <i>P. juniperinum</i> protonema on agar and BCD media (n=52).....	52
Figure 2.10 Relative Electron Transport Rate (RETR) (μmol m ⁻² s ⁻¹) from <i>P. juniperinum</i> protonema versus agar and BCD media under low-light setting (0-820 μmol m ⁻² s ⁻¹) PPFD (n=20).	53
Figure 2.11 Relative Electron Transport Rate (RETR) (μmol m ⁻² s ⁻¹) from <i>P. juniperinum</i> protonema versus agar and BCD media under high-light setting (0-1150 μmol m ⁻² s ⁻¹) PPFD (n=20).	54
Figure 2.12 Relative Electron Transport Rate (RETR) (μmol m ⁻² s ⁻¹) from <i>P. juniperinum</i> protonema versus combined agar and BCD media under low-light	

setting (0-820 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and high-light setting (0-1150 $\mu\text{mol m}^{-2} \text{s}^{-1}$) (n=40).	55
Figure 2.13 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) in <i>P. juniperinum</i> protonema versus temperature ($^{\circ}\text{C}$; n=38)	56
Figure 2.14 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) in <i>P. juniperinum</i> protonema versus Photosynthetic Photon Flux Density (PPFD) (n=53).....	57
Figure 3.1 Illustration of <i>P. juniperinum</i> costa cross section with number of lamellae counts (LN) measurement at the widest part of the costa cross section	106
Figure 3.2 Illustration on <i>P. juniperinum</i> costa cross section with five lamellae height (CLH) measurement at the widest part of the costa cross section.....	107
Figure 3.3 Illustration on <i>P. juniperinum</i> costa cross section of gametophyte stem after leaf removal.....	108
Figure 3.4 Setup for isoprene emission assessment for individual gametophyte emission using Dracast LED1000 Pro Daylight light panel, with light-emitting diode array (Photon Systems Instruments).....	109
Figure 3.5 Logged isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus above and belowground morphological variables in non-sexually expressing <i>P. juniperinum</i> gametophytes (n=45).....	110
Figure 3.6 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) best fit regression line versus stem area dry (SAD) mm^2 in <i>P. juniperinum</i> gametophytes (n=45).....	111
Figure 3.7 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus plant sex in <i>P. juniperinum</i> gametophytes that were not expressing sex during the experiment (n=300).....	112
Figure 3.8 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus species: <i>Atrichum undulatum</i> , <i>Pogonatum urnigerum</i> and <i>Polytrichum juniperinum</i> gametophytes (n=167).....	113
Figure 3.9 Logged isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus sex between species of <i>A. undulatum</i> , <i>P. urnigerum</i> and <i>P. juniperinum</i> gametophytes (n=167).....	114
Figure 3.10 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus sexes in Polytrichaceae gametophytes (n=167).....	115
Figure 3.11 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus site in <i>A. undulatum</i> gametophytes (n=70).....	116

Figure 3.12 Isoprene emission (nmol m ⁻² s ⁻¹) versus site in <i>P. juniperinum</i> gametophytes (n=56).....	117
Figure 3.13 Isoprene emission (nmol m ⁻² s ⁻¹) versus site in <i>P. urnigerum</i> gametophytes (n=41).....	118
Figure 3.14 Isoprene emission (nmol m ⁻² s ⁻¹) best fit regression line versus Chlorophyll Fluorescence Ratio (CFR) in <i>P. juniperinum</i> gametophytes (n=45).....	119
Figure 3.15 Logged isoprene emission (nmol m ⁻² s ⁻¹) best fit regression line versus Chlorophyll Fluorescence Ratio (CFR) in <i>P. juniperinum</i> gametophytes (n=28).....	120
Figure 3.16 Chlorophyll Fluorescence Ratio (CFR) versus sex in <i>P. juniperinum</i> (n=25)	121
Figure 3.17 Relative Electron Transport Rate (RETR) (μmols m ⁻² s ⁻¹) versus sex on BCD media in <i>P. juniperinum</i> and <i>A. undulatum</i> gametophytes (n=28).....	122
Figure 3.18 Isoprene emission (nmol m ⁻² s ⁻¹) best fit regression line versus Chlorophyll Fluorescence Ratio (CFR) in <i>P. juniperinum</i> gametophytes (n=28).....	123
Figure 3.19 Chlorophyll Fluorescence Ratio (CFR) versus species: <i>P. juniperinum</i> and <i>A. undulatum</i> gametophytes (n=28)	124
Figure 3.20 Relative Electron Transport Rate (RETR) (μmols m ⁻² s ⁻¹) versus Photosynthetic Photon Flux Density (PPFD) (μmols m ⁻² s ⁻¹) in <i>P. juniperinum</i> and <i>A. undulatum</i> gametophytes (n=28).....	125
Figure 3.21 Relative Electron Transport Rate (RETR) (μmols m ⁻² s ⁻¹) versus sex in <i>P. juniperinum</i> and <i>A. undulatum</i> gametophytes (n=28).....	126
Figure 4.1 Ammonium nitrate addition to <i>P. juniperinum</i> moss via 1 Liter (L) hand held pump mister sprayer (Solo INC., Model 418, Newport News, VA).....	171
Figure 4.2 Small 77mm × 77 mm × 97mm static chamber (magenta jar) created for headspace analyses using (Magenta vessel™).....	172
Figure 4.3 Magenta vessel™ used to collect isoprene emission data from <i>P. juniperinum</i> populations before and after nitrogen addition.....	173
Figure 4.4 Gametophyte length (GL) cm versus sites (1-6) in <i>P. juniperinum</i> (n=3596).....	174

Figure 4.5 Gametophyte length (GL) cm versus sex and nitrogen treatment in <i>P. juniperinum</i> (n=3596).....	175
Figure 4.6 Gametophyte width (GW) cm versus sites (1-6) in <i>P. juniperinum</i> (n=3596).....	176
Figure 4.7 Gametophyte width (GW) cm versus sex and nitrogen treatment in <i>P. juniperinum</i> (n=3596).....	177
Figure 4.8 Reproductive effort versus sexual expression in <i>P. juniperinum</i> (n=1440).....	178
Figure 4.9 Isoprene emission (nmol m ⁻² s ⁻¹) in the winter of 2015 versus site-specific differences in <i>P. juniperinum</i> gametophytes (n=244).	179
Figure 4.10 Isoprene emission (nmol m ⁻² s ⁻¹) before N treatment versus within site-specific differences in <i>P. juniperinum</i> gametophytes (n=244)....	180
Figure 4.11 Isoprene emission (nmol m ⁻² s ⁻¹) before N treatment versus sex in <i>P. juniperinum</i> gametophytes (n=244)....	181
Figure 4.12 Isoprene emission (nmol m ⁻² s ⁻¹) after N treatment versus site-specific differences in <i>P. juniperinum</i> gametophytes (n=244).....	182
Figure 4.13 Isoprene emission (nmol m ⁻² s ⁻¹) after N treatment versus within site-specific differences in <i>P. juniperinum</i> gametophytes (n=244).....	183
Figure 4.14 Isoprene emission (nmol m ⁻² s ⁻¹) after N treatment versus sex in <i>P. juniperinum</i> gametophytes (n=244).....	184
Figure 4.15 Isoprene emission (nmol m ⁻² s ⁻¹) versus nitrogen treatment in <i>P. juniperinum</i> gametophytes (n=244)	185
Figure 4.16 Chlorophyll Fluorescence Ratio (CFR) measures versus site in <i>P. juniperinum</i> gametophytes (n=244).....	186
Figure 4.17 Chlorophyll Fluorescence Ratio (CFR) measures versus N treatment in <i>P. juniperinum</i> gametophytes (n=244).....	187

Chapter 1

Introduction

Global emissions of non-methane Biogenic Volatile Organic Compounds (BVOCs) produced by plants are estimated to be between 700 and 1150 Tg C yr⁻¹ (Guenther et al. 1995, 2006, 2012) and have significant effects on tropospheric chemistry at regional and global scales (Zimmerman et al. 1978; Fehsenfeld et al. 1992). Isoprene is highly reactive and in the presence of OH has a short atmospheric lifetime (Guenther et al. 1991, 1993; Funk et al. 2003; Fowler et al. 2009; Matsunaga et al. 2012; Seinfeld et al. 2012; Harrison et al. 2013). We must understand these emissions if we are to properly model regional atmospheric processes and chemistry transport models (CTMS).

Isoprene (2-methyl-1,3-butadiene; $\text{CH}_2=\text{C}(\text{CH}_3)\text{CH}=\text{CH}_2$), an unsaturated pentahydrocarbon, is the dominant non methane BVOC by mass, contributing about half of the global non methane BVOC flux (Stotzky et al. 1976; Guenther et al. 1995, 2006, 2012; Müller et al. 2008; Atkinson et al. 2003; Ashworth et al. 2013; Marmulla et al. 2014). In many ecosystems and even some urban areas, isoprene plays a significant, often dominant role, in photochemical reactions in the atmospheric boundary layer (Chameides et al. 1988; Goldstein et al. 1998). Modeled isoprene emission from urban areas with high nitrous oxides (NO_x) (Curci et al. 2009; Calfapietra et al. 2013) can be as significant as anthropogenic hydrocarbons input (Geron et al. 1994; Guenther et al. 1994; Biesenthal et al. 1997). High levels of NO_x , particularly in major cities where atmospheric photochemical reactivity and ozone production are high (Ling et al. 2014) are of global

concern (Fares et al. 2011; Dieleman et al. 2012; Lahr et al. 2015). In these environments a single isoprene molecule can lead to the production of multiple ozone (O_3) molecules (Zeng et al. 2008; Fini et al. 2017) through Secondary Organic Aerosol (SOA) production (Cai et al. 2006; Ng et al. 2008; Goldstein et al. 2009; Hallquist et al. 2009; Makkonen et al. 2012; Pratt et al. 2013; Rap et al. 2018; Tegen et al. 2018).

Daytime atmospheric chemistry of BVOCs, such as isoprene, is dominated by OH/isoprene reactions that result in SOA's and precursors to tropospheric ozone (Chameides et al. 1988; Goldan et al. 1995; Lerdau et al. 1997; Duane et al. 2002), and thus can contribute to a localized feedback on radiative forcing (Peñuelas et al. 2010; Bonan et al. 2011). Oxidation capacity of the boundary layer is therefore reduced with a positive feedback on climate change by increasing the lifetime of tropospheric methane (Lelieveld et al. 1998; Bell et al. 2003, 2007; Peñuelas et al. 2003, 2009, 2010). Under increased BVOC production due to warming, commiserate increases in SOA production is expected. Given that the atmospheric chemistry interactions of isoprene with OH and the reactive nitrogen species reactions are implicated in creating atmospheric ozone, with increased climate change we expect to see increased BVOC production, including increased isoprene, resulting in increased SOA (Ng et al. 2008; Bison et al. 2018; Jambert et al. 2017; Fiore et al. 2005) and boundary layer ozone production.

Persistent BVOCs can serve as a viable carbon source. For example, methane can account for 15% of the carbon source for Sphagnum mosses (Raghoebarsing et al. 2005; Larmola et al. 2010). Active consumption of isoprene in soil is performed by microbes that consume isoprene as a source of energy and carbon (Van Ginkel et al. 1987;

Pegoraro et al. 2006; Gray et al. 2015; McGenity et al. 2018). Rain or precipitation can transport BVOCs to soils, where microorganisms encounter BVOCs while living in or on plants (Atkinson et al. 2003; Fu et al. 2009; Ziemann et al. 2012). Specific bacteria that can degrade isoprene in soil, marine sediments, and on the leaves of plants have recently sparked interest in new areas of investigation (Crombie et al. 2019). These interactions regulate a considerable amount of soil communities carbon and energy budgets and affects chemical ecology both aboveground and belowground.

Organic molecules like isoprene are extremely costly for plants to produce (Sharkey et al. 2001), and the ability has been lost and gained multiple times during plant evolution (Sharkey et al. 2007, 2013; Fineschi et al. 2013; Monson et al. 2013; Dani et al. 2014; Loreto et al. 2015). Normal levels of isoprene production are greatly influenced by many abiotic and biotic factors, such as temperature, water stress, CO₂ and drought, and have been measured using a wide range of techniques in a number of higher plants (Zimmerman, 1979; Khalil et al. 1992; Fares et al. 2006; Tiiva et al. 2007a,b; Duncan et al. 2009; Pétron et al. 2001; Peñuelas et al. 2001, 2003, 2010; Rasulov et al. 2010, 2018; review Loreto et al. 2015; review Sharkey et al. 2014, 2017; Yu et al. 2017; Bamberger et al. 2017).

Speculation as to why plants spend so much of their resources on isoprene production and the functional role of isoprene emission continues (Saxena et al. 2019). Isoprene has mainly been investigated and explored in vascular plants, but its biological and environmental significance is still poorly understood (Sharkey et al. 2017). There is a complicated and poorly understood calculus between isoprene emission and biomass

accumulation in vascular plants (Hong et al. 2012). Understanding the reasons for the observed resource allocation will require a better understanding of the benefits to fitness of isoprene production and emission.

Isoprene emission has been observed in nonvascular plants (Hanson et al. 1999; Janson et al. 1999; Trapp et al. 2001; Haapanala et al. 2006; Tiiva et al. 2007a,b), in freshwater green algae (Disch et al. 1998), marine phytoplankton, cyanobacteria (Meskhidze et al. 2015; Dani et al. 2017), and macroalgae (seaweed) (Broadgate et al. 2004). Isoprene emission has roles in a wide array of interactions including evolutionary roles (Hanson et al. 1999; Harley et al. 1999; Sharkey et al. 2005b), biological roles (Fall et al. 1998; Logan et al. 2000; Loreto et al. 2001; Sharkey et al. 2001; Brillì et al. 2009; Tattini et al. 2014; Velikova et al. 2004, 2005, 2016; Sanadze et al. 2017; Wilson et al. 2018), and atmospheric roles (Lim et al. 2005; Zhang et al. 2007; Gerken et al. 2016). Isoprene is considered a true “building block in nature” (Barton et al. 1999; Morrison et al. 2002); however, our understanding of the role of isoprene emission in vascular plants is limited and in non-vascular plants it is extremely limited.

Purpose of Study

Bryophytes have been shown to be more responsive than vascular plants to nitrogen deposition and climate warming, and in a changing environment, they can serve as model systems in exploring biodiversity and ecosystem functioning (Becker Scarpitta et al. 2017). Bryophytes diverged from vascular plants with many unique morphological and biochemical adaptations (Roberts et al. 2012). Mosses contain a number of unique metabolites (Asakawa et al. 1981, 1995, 2007, 2011; Xie et al. 2009; Erxleben et al.

2012; Ruiz-Molina et al. 2015). Over 300 BVOC chemical compounds have been recorded in bryophytes (Rosenstiel et al. 2012), but a lack of mechanistic understanding of these compounds hinders our ability to interpret and predict the responses of mosses to anthropogenic drivers such as climate change and anthropogenic nitrogen addition (Vitousek et al. 1997; Royles et al. 2015; Patiño et al. 2016; Gavazov et al. 2018; Delgado-Baquerizo et al. 2018). This is particularly important today as researchers are trying to decode the moss genome to understand how climate change will affect plants (Ludwig et al. 2017) and what mechanisms enable moss to survive extremely unfavorable environments (Hartley et al. 2014; Machteld et al. 2017).

Data concerning the spatial and temporal variability of BVOC emissions are lacking due to poor characterization of the physical and biological factors controlling isoprene synthesis in non-vascular plants as most research has been focused on vascular plants (Sharkey et al. 2017). There is also scarce research into sex expression in bryophytes regarding BVOC emissions and cost benefit analyses for reproductive effort. Previous research in bryophytes suggests that some BVOC emissions are sex-specific and can influence fertilization in mosses by microarthropods (Rosenstiel et al. 2012). There exists modest evidence that physiological factors affect sex expression in bryophytes, and sex can be modified by these external physiological factors (Bowker et al. 2000; Rosenstiel et al. 2012; Shortlidge et al. 2014; Slate et al. 2017).

To test hypotheses concerning the biosynthetic, metabolic and functional processes relating to isoprene emission, we investigated isoprene emission in the Polytrichaceae. Polytrichaceae are morphologically diverse (Mishler et al. 2009),

perennial and evergreen, and are able to colonize a wide range of habitats on every continent (Bazzaz et al. 1970, Shortlidge et al. 2016) and are a significant pioneer species.

- In **Chapter 1**, I introduce isoprene emission as it relates to plant BVOC production in vascular and non-vascular plants. I explain the background for my dissertation, explaining the potential roles of nitrogen deposition and sexual allocation in influencing BVOC emissions.
- In **Chapter 2**, I describe isoprene emission in early *P. juniperinum* protonema by assessing and quantifying isoprene emission. Further, I explore effects on protonema growth and how physiological factors contribute to and are affected by these emissions.
- In **Chapter 3**, I assess and quantify how isoprene emission varies between species and sexes within the Polytrichaceae family. In addition, I measure morphological and physiological traits from the cell to population level to investigate how these traits can contribute to isoprene emission.
- In **Chapter 4**, I conduct a four-year nitrogen addition experiment with *P. juniperinum* and explore abiotic and biotic factors that influenced isoprene emission, sexual expression, reproductive effort, morphology and physiological diversity along an artificial nitrogen gradient.

A conceptual model of isoprene production by plants can be seen in Figure 1.1, where production is considered a result of morphological and physiological characteristics, though it can also be positively or negatively be influenced by genetics,

environmental factors, and atmospheric chemistry. This may shed light on community wide emission characteristics from different populations and site categories, both as snapshots and over time for spatial location of the colony within the population of both sexes, and time during ontogeny. This work will examine for the first time the hypothesis that isoprene-BVOC emission capabilities are present in early protonema in *P. juniperinum*. This work will also examine for the first time the hypothesis that sexual expression affects isoprene emission in Polytrichaceae species. Lastly, this work will examine for the first time the hypothesis that isoprene emission under nitrogen addition influences isoprene emission in *P. juniperinum*.

Isoprene emissions from mosses and their response to climate change could lead to an increase in landscape level BVOC emissions with profound implications for possible uncharacterized climate change feedback mechanisms. Currently, non-vascular plants are not included in atmospheric chemistry modeling, and after 60 years of research into isoprene emission with vascular plants, the reason why some plants emit and what provides the evolutionary pressure to do so remains poorly understood (Sharkey et al. 2017). With uncertainty in the capacity of largely overlooked non-vascular plants to emit isoprene it is hard to predict how these emissions will be affected by and in turn affect climate change in possible positive or negative feedback loops.

In **Chapters 1-4**, as outlined above, I discuss how I have conducted research to understand my research topic and explain what steps I have taken to complete this dissertation. In **Chapter 5**, I discuss the broader implications of this work for future studies of the role of moss isoprene emissions on Earth's early land plant evolution. The

results from this work will allow a preliminary parameterization of the emission of BVOC's, specifically isoprene, from this often overlooked but ubiquitous flora. I further give explanation for the work I did, which supports a role for bulk isoprene emission in the development of water stress management in mosses possibly going all the way back to the emergence of life onto land. The implications of mosses isoprene emissions driving local atmospheric hydrological processes are explored but their parameterizations are beyond the scope of this study and require further research.

Chapter 2

*Isoprene emission from *Polytrichum juniperinum* protonema*

Abstract

Isoprene (2-methyl-1, 3-butadiene) accounts for > 90% of non-methane Biogenic Volatile Organic Compounds (BVOCs) emissions from vegetation. In this study, working with the moss *Polytrichum juniperinum* (Polytrichaceae), we (1) determine when isoprene emission begins after spore germination, (2) quantify isoprene emission in single-spore isolated protonema (the earliest life stage after spore germination), (3) investigate physiological effects through laboratory gas exchange measurements, and (4) investigate light and temperature curves that influence and affect isoprene emission.

Here, we show for the first time that protonema from *P. juniperinum* emits isoprene, and a time lag after germination exists before isoprene production. We recorded an increase in isoprene emission with young protonema and measured emission at detectable levels between days 5-6, which declined over time with leaf expression. The emission is the first ever reported for *P. juniperinum*, or any moss protonema. We show that physiological measurements, such as relative electron transport rate, are comparable to what is known for macro algae. Isoprene emission is temperature and light dependent and these curves correspond to what is has been observed in vascular plant isoprene emissions. We think moss may provide a novel model system suited to assess further questions as to why early land plants emitted isoprene, why plants continue to emit isoprene and what factors contribute to these emissions.

Keywords: bryophyte, isoprene emission, life stage, *Polytrichum juniperinum*, protonema, relative electron transport rate

Introduction

Annually, plants assimilate 120 Picogram (Pg) of carbon dioxide through photosynthesis. Carbon dioxide has increased in the Earth's atmosphere due to anthropogenic emission factors (Bala et al. 2007; Pongratz et al. 2009; Reichstein et al. 2013; Ussiri et al. 2017; Doetterl et al. 2018; Mau et al. 2018). Atmospheric CO₂ is warming the planet, will alter carbon cycles, and is already intensifying shifts in ecosystem processes (Gorham et al. 1991; Heimann et al. 2008; Allison et al. 2010; Jiang et al. 2013; Pold et al. 2017; Unda et al. 2017; Yvon-Durocher et al. 2017; Quesada et al. 2018; Pellegrini et al. 2018). Plants use carbon dioxide for photosynthesis, but in the process can transform it into chemically complex molecules, some of which are re-emitted back into the atmosphere in the form of isoprene (Sharkey et al. 1993, 1995, 1996; Harley et al. 1994; Magel et al. 2006; Kuhn et al. 2007; Ghirardo et al. 2011, 2014). Isoprene, a terpenoid, is significantly more chemically reactive than CO₂ and may have a larger than suspected impact on atmospheric temperature through albedo effects via the formation of SOA (Mooney et al. 1987; Scholefield et al. 2004; Lohmann et al. 2005, Arneth et al. 2007; Heald et al. 2009; Bauwens et al. 2018).

We need to understand why and at what rate plants produce isoprene to fully understand the global carbon cycle and the effects of vegetative shifts on climate change. However, as of yet, our limited understanding of isoprene emission does not include a definitive reason as to why some plants emit isoprene and others do not. This is all the more remarkable given that isoprene production is so metabolically costly (Loreto et al. 2010; Behnke et al. 2007, 2009, 2012). Despite decades of work on this topic, the

physiological role and the evolutionary origins of isoprene emission is a matter of debate. Even within plant groups (e.g., flowering plants) isoprene emission is inconsistent (Hanson et al. 1999; Monson et al. 2013). Without an understanding of the fundamental motivations for isoprene production and emission, we have a limited ability to predict emissions with increasing climate change (Sharkey et al. 2017).

The process of isoprene synthesis and emission are biochemically expensive and energy dependent (Vickers et al. 2009; Loreto et al 2010; Sharkey et al. 2001; Behnke et al. 2012). Isoprene is produced from its five-carbon precursor, isopentenyl diphosphate (IPP) to its isomer, dimethylallyl diphosphate (DMAPP) (Lichtenthaler et al. 1997) by isoprene synthase enzyme (Kuzma et al. 1993; Schnitzler et al. 1996; Miller et al. 2001; Sharkey et al. 2005a,b). Two pathways are known for isoprene synthesis: the eukaryotic mevalonic acid pathway (MVA) in cytosol and the methylerythritol phosphate (MEP) pathway in plastids (Kuzuyama et al. 2002), with little crosstalk between the pathways (Eisenreich et al. 2001; Laule et al. 2003; Schuhr et al. 2003; Loreto et al. 2004; Peñuelas et al. 2005). Isoprene is not a general plant trait (Ekberg et al. 2011), but all plants possess the MEP pathway.

Isoprene emission is more common in mosses and ferns than in other major plant groups (Hanson et al. 1999), making bryophytes (liverworts, mosses, and hornworts) an important focus of research in isoprene emissions. Bryophytes were the earliest land plants observed in the fossil record and date back 475 million years (Wellman et al. 2003; Nickrent et al. 2000; Morris et al. 2018; Rensing et al. 2018a). Isoprene emission has been hypothesized as a thermo-tolerance mechanism beneficial for the radiation of early

plants on land (Hanson et al. 1999) through resistance to damage due to increased temperature variations that are part of an aerial existence. Later plants evolved other protective mechanism such as heat shock proteins (Waters et al. 1996; Heckathorn et al. 1998) to cope with these temperature swings. Isoprene emitted by these early plants should be accounted for in models of early climate and biological environmental engineering. Under current climate conditions, mosses occur on every continent, dominating observed biomass in the high arctic and can tolerate a wide array of temperature and water stresses (Ward 1960; Collins 1976; Corradini et al. 1999; Van der Velde et al. 2001, 2005; review Mishler et al. 2001; Wilson et al. 2003; Shafiqullina et al. 2018; Ojiodu et al. 2018). Global warming may lead to the expansion of isoprene emitting moss communities in some regions such as Antarctica and an increase in landscape level isoprene emissions with profound implications for climate change.

Isoprene emitting mosses have been found in the *Sphagnum* (Sphagnaceae) and *Warnstorfia* (Amblystegiaceae) genera, and several species from the Polytrichaceae family (Hanson et al. 1999). Research suggests that these mosses can emit substantial amounts of isoprene to the atmosphere with an annual emissions peak observed in the relative heat and high solar insolation days of both the northern and southern summers. Temperature, light level and time of day all contribute to diurnal and annual variation in isoprene emissions. Enhanced UV-B radiation leads to higher observed emissions which were explained by higher carbon assimilation (Sharkey et al. 1996, 1999; Singsaas et al. 1997, 1998; Rinne et al. 2002; Centritto et al. 2011; Fares et al. 2011; Ryan et al. 2014; Nogues et al. 2018; Niinemets et al. 2018). This research suggests that peatlands are

likely to be more important sources of isoprene than forests in the subarctic (Janson et al. 1998, 1999; Haapanala et al. 2006; Hellén et al. 2006; Tiiva et al. 2007a,b, 2009).

A time lag that exists between the onset of photosynthesis and isoprene emission has been observed in young vascular plants (Grinspoon et al. 1991; Rasulov et al. 2014, 2015). However, it is unknown when during development mosses start to emit isoprene, and whether the emission rates are similar to mature plants. Understanding how emission rates change during moss development will help us better estimate moss isoprene emissions on larger scales.

The objective of this study was to quantify moss isoprene emission across the earliest life history stages. After testing more than 90 species of moss for isoprene emissions including ten members of the Polytrichaceae family, all of which emitted isoprene, we chose the moss *Polytrichum juniperinum* as our test species because of its' high rate of emission, global distribution (Conard 1956; Cremer et al. 1965; Callaghan et al. 1978; Crum et al, 1981; Van der Velde 2000, 2003; Ryömä et al. 2005; Newton et al. 2000; Nickrent et al. 2000; Bell et al. 2010a,b; Yumol 2016) and status as a one of the planets most successful pioneer species (Scotter 1964; Sims et al. 1981; Foster 1985; Kim et al. 2014; Shortlidge et al. 2016). Comparable rates of emission are found coming from oak leaves $32.8 \pm 2.9 \text{ nmol m}^{-2} \text{ s}^{-1}$ at 30°C and $900 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (Sharkey et al. 1991) a widely distributed vascular counterpart. In this study, we investigated: 1) whether isoprene emission is present and quantifiable in *P. juniperinum* protonema and 2) how isoprene emission changes during gametophytic development. Additionally, we attained 1) repeated protonema growth measurements, 2) gas exchange measurements and

electron transport rates, and 3) light and temperature curves with respect to isoprene emissions. These data allow us to assess both the production of isoprene during the early life stages in this moss species, but also allows the first look at how this production may vary with environmental and growth parameters.

Materials and Methods

Study species

We used *Polytrichum juniperinum* (Hedw.) (Polytrichaceae), a cosmopolitan species, for the experiments described in this study (Smith 1972; Forrest et al. 1995; Zouhair et al. 2000; Magombo et al. 2003; Hyvönen et al. 2004; Bell et al. 2010 a,b). *P. juniperinum* grows on every continent (Conard 1956; Cremer et al. 1965; Callaghan et al. 1978; Crum et al, 1981; Van der Velde 2000, 2003; Ryömä et al. 2005; Newton et al. 2000; Nickrent et al. 2000; Bell et al. 2010a,b; Yumol 2016) and is a pioneer moss species (Scotter 1964; Sims et al. 1981; Foster 1985; Kim et al. 2014). It is often found in disturbed habitats and on recently burned soil (Rundel et al. 1977; Rencz, et al. 1978; Fryer 2008; Depante et al. 2018; Maslov et al. 2018; Shafigullina et al. 2018) where it can rapidly outcompete non-colonizing species (Duncan et al. 1982; Delach et al. 2002; Gonzáles et al. 2013; Juutinen et al. 2015). This moss has been observed to vary in physiology and morphology to adapt to fluctuating environments (Bazzaz et al. 1970; Sarafis 1971; Collins 1976; Potter et al. 1995; Callaghan et al. 1997; Shafigullina et al. 2018), can sustain high light level fluctuations (Lloyd et al. 2003; Marschall et al. 2004; Proctor et al. 2018), and withstand increased summer temperatures (Potter et al. 1995;

Richter et al. 2000; O'Neill et al. 2006; Oishi 2018). This observed resilience and strategy for broad temperature tolerance and make it of particular interest as a model species for observing potential plant physiological changes in the face of a warming environment.

Protonema from mixed spore samples

To assess detectable levels of isoprene emission from *P. juniperinum*, ten intact adult mature sporophytes were collected randomly from three locations in and around Portland, OR during the summer of 2015 (Table 2.1). Mature spores were inspected under a Leica DME compound microscope (Figure 2.1a) to ensure sporophytes had intact protective cover (calyptra) over sporophytes and were not damaged. Calyptra and seta were detached using forceps before sterilization. Using sterile techniques, the calyptra was removed from each sporophyte (n=7), and sterilization of mature sporophytes was achieved by using sodium hypochlorite following (McDaniel et al. 2007). We sterilized the capsules in 1.0 % solution of sodium hypochlorite for 60 seconds and subsequently washed them in tap water for 60 seconds repeating this three times in three Eppendorf tubes inside a laminar flow hood. The fourth Eppendorf tube contained 1 ml water in which the sporophyte was ruptured using sterilized forceps and spores released.

Agar and nutritive BCD media were prepared following protocol outlined by Wang et al. (2015). Sixty milliliters of prepared media was poured into base of 9.5 cm x 9.5 cm x 3.5 cm PlantCon™ containers (MP Biomedicals, 2017) and sterilized before the addition of spore mixtures under UV light. The container lid was placed atop the base

which measured 9.5 cm x 9.5 cm x 7.0 cm. PlantCon™ containers are made of clear polyvinyl chloride (PVC) and are designed for plant culture applications because they allow light transmission and produce optimal growth. The PlantCon™ container eliminated bacterial growth and allowed for easy sampling (Figure 2.1b).

Three sporophytes from the same location were chosen at random and the capsule of the three sporophytes were excavated and vortexed together to ensure enough spores would be viable to achieve detectable levels of isoprene emission. Once thoroughly blended, the mix was plated in PlantCon container on agar or BCD media. Spores emissions were assessed daily until detectable isoprene emission was observed. The *P. juniperinum* protonema samples were grouped into a binary system of emitting and non-emitting samples for analyses.

Protonema from single spores

To assess isoprene emission from single-spores of *P. juniperinum*, a single capsule was obtained from a rural site outside of Portland, Oregon (Table 2.1). Using the protocol described above and using standard microbiological procedures, the single healthy sporophyte was opened using sterile forceps and ~100 spores per 90-mm-diameter Petri dish were spread on agar following Cove et al. (2009). This allowed dilution of the spores from the plate and made it possible to transfer individual spores under a Leica microscope (Leitz DME, Leica, Wetzlar, Germany) with a AxioCam 105 Color camera and Zen Blue software (version 1.1.2.0; Carl Zeiss Microscopy). Using sterile forceps single spore isolates were transferred onto individual 90-mm Petri dishes

with agar media which then were sealed with Parafilm wax film (Pechiney Plastic Packaging, Menasha, WI). Eighty one single-spore isolates were allowed to grow for 2 months under standardized growth conditions of ($55\text{--}70 \mu\text{mol m}^{-2} \text{s}^{-1}$) light intensity and a photoperiod of 16 h light to 8 h dark at $22^\circ\text{C} \pm 2$ SEM using Adaptis1000 Conviron growth chambers (Pembina, ND).

P. juniperinum moss spores germinate on agar in ~ 2 days, and protonema develops in 7 days (Nehlsen 1979; Duncan et al. 1982). Every two week, we tested the single-spore germinated protonema for isoprene emission under controlled light level and basal rate (maintained at $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$, ± 100 SEM) and $30 \pm 2^\circ\text{C}$ using a Reduced Gas Detector (RGD2, Trace Analytical, Menlo Park, CA). Light intensity was assessed every other day to maintain consistency and samples were rotated clockwise each day to avoid edge effects and allow light optimum to penetrate each Petri dish.

Two isoprene emission sampling events occurred for single-spore isolated protonema. First, isoprene emission was assessed at from day 1 after spore plating and germination until day 45, and second after 75 days. *P. juniperinum* spores need 20-40 days for complete protonema formation (de Carvalho et al. 2011). In the first set of isoprene emission at day 45, we sampled isoprene from three sites in two growth media and performed physiological measurements. In the second set, our assessment consisted of single-spore isolated protonema, where we investigated whether protonemal growth had an effect on isoprene emission. We used 81 half-siblings from one capsule to eliminate environmental variability and variance in maternal effects (Shaw 1985; Vanderpoorten et al. 2002; Budke et al. 2012, 2013). These spores were assessed in two-

week intervals, starting from assessment one on day 75 on September 2, 2016. The second isoprene assessment on day 89 occurred on September 15. The last isoprene assessment on day 103 occurred on September 28, 2016. During the last two isoprene assessments (assessments 2 and 3), protonema began to sprout phyllids (leaves), and plants were now considered to be in the gametophytic stage. Therefore, the last two isoprene emission quantifications included leaves. Quantitative analyses using Reduced Gas Detector (RGD2, Trace Analytical, Menlo Park, CA) picked up detectable levels of isoprene emission.

Using a Leica microscope (Leitz DME, Leica, Wetzlar, Germany), single-spore grown protonema were quantified with a bright-field microscope and inspected for health. Differential interference contrast images were captured using automatic exposure when taking images of the protonema; the drawing tool was used to manually circle the area of moss protonema growth using Leica Application Suite software. A 2 mm stage micrometer (Ward's Science, cat. #949910) was used to calibrate the camera drawing tool, and we used calibrated images from Zen software version 1.1.2.0 (Carl Zeiss Microscopy).

Isoprene measurement method of *P. juniperinum* protonema

To measure isoprene emission in *P. juniperinum* protonema, we used a nondestructive method, headspace sampling, which gives a spatially realistic profile of emission for sampling volatiles in an ecological context. Headspace gas chromatography using gas extraction was used after a specified time interval, and a small headspace

sample was injected with a syringe directly into packed columns in the Gas Chromatograph with a reduction gas detector (GC/RGD2). Each sample was allowed an acclimation period before the headspace was sampled under the standard conditions of controlled light level (maintained at $1000 \mu\text{mol m}^{-2} \text{s}^{-1} \pm 100 \text{ SEM}$), also known as the basal rate of isoprene emission. We tested isoprene emission during timed intervals with 3 samples of protonema and 3 replicates and found that most of the isoprene was emitted within the first five minutes of incubation. The moss protonema were photographed under a Leica microscope (Leitz DME, Leica, Wetzlar, Germany), to establish the area of each sample allowing us to use area in the equation for isoprene emission (Equation 1.).

All protonema level isoprene emission samples were collected in 2 ml Pressure-Lok® Precision Analytical Syringe (VICI Precision Sampling Inc., Baton Rouge, LA, USA) for laboratory analyses. These samples were injected into 1 ml sample loop within 10 minutes. Prior to use, 20 ml round bottom vials were cleaned by rinsing with 3 ml methanol and then baked at 60 °C for a minimum of 2 hours on a MaxQ 4000 ventilated shaker (Barnstead Lab-Line, Thermo Scientific; Logan, UT). Vials were removed from heat and immediately capped with clean septa and caps that had been washed using DI water and baked at 60 °C alongside the vials. Each moss protonema clump was placed in a clean 20 ml vial with Teflon backed 6mm silicone septa (Supelco, Sigma-Aldrich; Bellefonte, PA). After 10 minutes, 1 ml of air was removed via Pressure-Lok® precision analytical syringe (VICI Precision Sampling Inc., Baton Rouge, LA) and injected into a reduction gas detector (RGD2, Trace Analytical, Menlo Park, CA). Isoprene was separated isothermally (100 °C) in a stainless-steel column (1.3 m long x 2 mm inner

diameter i.d.) packed with UNI 8 Beads 3S 60/80 6' x 1/8", 0.085 SS (Alicat Scientific, Inc; Tucson, AZ).

Peak times and areas were recorded using a commercial integrator (Model 3396, Hewlett-Packard, Avondale, Pennsylvania) and transformed to parts per million of isoprene. The GC-RGD2 was calibrated each day using a standard cylinder containing 71 ppb (v/v) isoprene, referenced to a National Institute of Standard and Technology (NIST) and helium (He) standard (SRM 1660a; 1 ppm He in N₂, Rochester, NY; Praxair Inc., Specialty Gases, Portland, OR) with a ten point standard dilution of isoprene and Helium as a carrier gas. Blanks were run every five samples to ensure quality assessment and to detect contamination within the column. Isoprene gas was mixed with high purity helium using a mass flow controller calibrated to a flow rate of 21 ml/min (Alicat Scientific, Inc; Tucson, AZ). One ml of the calibration gas mixture was removed with a syringe from a mixing chamber and injected into the GC-RGD2 to create the calibration curve and obtain slope of the equation see (Equation 1.).

Isoprene GC-analyses of *P. juniperinum* protonema

Two types of isoprene measurements were performed to determine isoprene emission from *P. juniperinum* protonema. First, we tested whether isoprene emission occurs in early protonema. We found no previous reports on isoprene emission from moss protonema. To test for isoprene emission in protonema, we used protonema derived from spores grown from several sporophytes (see above "Protonema from mixed spore samples"). Second, we wanted to assess quantity of isoprene emission from single-spore

isolates grown into protonema after a period of adjustment (see above “Protonema from single spores”). Variation in isoprene emission during three separate time intervals was assessed and labeled; assessment 1 (September 2, 2016), assessment 2 (September 15, 2016), and assessment 3 (September 28, 2016).

Isoprene emission measurement was calculated as follows, using Peak Area Units (PAU) from the standard calibration curve $y = mx + b$, at GC temperature of 298 Kelvin (K), and Atmospheric Pressure (P) of 760 Torr.

$$\begin{aligned}
 &= \left(\frac{\text{PAU} + 20.69}{2.4703} \right) \times \left(\frac{1 \text{ nmol/L}}{\text{ppb}} \right) \times (.0005) \times \left(\frac{\text{std nl}}{\text{nl}} \right) \times \left(\frac{760 \text{ Torr}}{298 \text{ K}} \right) \times \left(\frac{378 \text{ K}}{771.8 \text{ Torr}} \right) \\
 &\times \left(\frac{1 \text{ nmol}}{22.4 \text{ std nl}} \right) \times \left(\frac{\text{volume vial (ml)}}{.5 \text{ ml}} \right) \times 1 \text{ (factor for loop recovery)} \\
 &\times \left(\frac{1}{\text{area/m}^2} \right) = (\text{nmol m}^2 \text{ s}^{-1})
 \end{aligned}$$

Equation 1. Formula used to convert isoprene from calibration curve, $y = mx + b$, and PAU to isoprene ($\text{nmol m}^{-2} \text{ s}^{-1}$).

We obtained the volume-mixing ratio of isoprene (ppb) using Equation 1 to determine protonema isoprene emission flux. We determined the area of the protonema using AxioCam 105 Color camera, and Zen Blue software, version 1.1.2.0 (Carl Zeiss Microscopy).

Peak identification was performed with the PEAK software (Stein 1999) that integrates raw peak area units and retention times. To identify isoprene, a custom calibration curve was created from isoprene standard (Sigma, Deisenhofen, Germany). For quantification, peak areas of isoprene were determined after baseline correction. Levels of background contamination were determined from blanks. Analytical quality was analyzed by using Standard Reference Material of isoprene (NIST SRM 1515,

National Institute of Standards and Technology, Gaithersburg, MD). The standard material was used in the analyses to create calibration curves for quality assurance and quality control purposes.

Laboratory gas exchange measurements of *P. juniperinum* protonema

To investigate physiological factors affecting isoprene emission from *P. juniperinum*, we conducted measurements of photosynthetic parameters of mature photosynthetic green protonema tissue using Junior Pulse-amplitude modulated (JUNIOR-PAM) chlorophyll fluorometer and WinControl Software (Walz, Effeltrich, Germany). All gas exchange measurements in the lab were conducted using the protocol outlined by JR-PAM, with a single 100 cm plastic fiber, 1.5 mm in diameter. The Junior-PAM (Walz, Germany, <http://www.walz.com/>) was used according to the manufacturer's instructions with the following parameters: light – saturation pulse intensity 6, pulse frequency 20 sec, actinic light intensity 8. The effective photochemical quantum yield of photosystem II [Y (II)] was calculated as described by Genty *et al.* (1989).

Our second physiology measure was chlorophyll fluorescence ratio (CFR), as a non-intrusive tool in using it as a proxy to establish plant stress and gain information on photosynthetic reactions in the chloroplast (Schreiber *et al.* 1987). The fluorescence-emission spectra read out two maxima in the 690 nm and the 735 nm region. The fluorescence ratio F690/F735 for green leaves is higher (values of 0.8 to 1.1), than in the yellow to red wavelength region (525 to 633 nm), which only yields values for F690/F735 of 0.5 to 0.7 (Rinderle *et al.* 1988). Chlorophyll fluorescence ratio

(CFR) was measured with a chlorophyll content meter (OPTI-Sciences model CCM-300, Hudson, USA).

Relative Electron Transport Rate (RETR) was calculated with JUNIOR-PAM chlorophyll fluorometer and WinControl Software (Walz, Effeltrich, Germany). Modulated excitation energy and the fluorescence signal were transmitted through a bifurcated fiber-optic cable. *P. juniperinum* protonema samples were placed in a cuvette containing moist cotton ball to provide adequate moisture through the assessment. Measurements with the Junior-PAM were conducted under constant fluorescent lighting in the laboratory at mean photosynthetically active radiation (PAR) value of $250 \mu\text{mol m}^{-2} \text{s}^{-1} \pm 50 \text{ SEM}$. Relative electron transport rate (RETR) can be obtained as $\text{PFD} \times (\Phi)_{\text{PSII}}$ (Bilger et al. 1995). Junior-PAM recorded electron transport rate, photosynthetic yield and PAR light levels ranging from 0-1500 PAR $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Light and temperature response curves of *P. juniperinum* protonema

Isoprene emission rates were quantified at different levels of incident light of photosynthetic photon flux density (PPFD). Temperature curve measurements were controlled by a water bath. Sample container leaf temperatures were monitored with thermocouples and adjusted as needed. Experiments were carried out under standard conditions (30°C and $1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density at leaf level (PPFD)), unless otherwise noted. Light was provided with a Dracast LED1000 Pro Daylight light panel, with a light-emitting diode array (Photon Systems Instruments, Albuquerque, NM).

Light and temperature curve measurements were made using JUNIOR-PAM chlorophyll fluorometer and WinControl Software (Walz, Effeltrich, Germany). For temperature response curves, we subjected *P. juniperinum* protonema (n=17), with 3 replicates for each sample with temperature increasing from 0-35.5°C under constant irradiance of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to establish basal emission rates. The temperature at the time of the measurement correlated to inside temperature of the PlantCon™ containers (MP Biomedicals, 2017), which was 0.5 cm away from the sample. For light measurements we used (n=28) with three replicates per protonema under constant temperature of 30°C and increasing light from 0-2250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD).

Statistical analyses

Statistical analyses of the data were performed using R version 3.4.2 (R Studio 2017) and JMP Pro 14.0 (SAS Institute, Cary, North Carolina, USA). To determine the effects of day number, site, and media on isoprene emission in protonema, we used Chi-square Goodness of Fit with a statistical significance level of $p=0.05$. Day number, site and media using binary code of isoprene emitters (1), and non-isoprene emitters (0) was used to analyze the dataset. The chi-square statistic quantified how much of the observed distribution of isoprene emitter counts varied from a hypothesized uniform distribution.

To determine the effects of media and site on protonema isoprene emission up until day forty-five, we used an ANOVA to compare means of protonema emission from each site and each media to assess whether the means of emission by media and site

stayed consistent. We ran a second ANOVA to test the effects of leaf number on isoprene emission from our half sibling isoprene emission experiment. For our continuous half-sibling experiment early *P. juniperinum* protonema, we ran repeated measure ANOVA (RM ANOVA) to test the effects of media and time on isoprene emission during our three assessments. Data met the assumptions of the model.

We used regression analysis to examine the relationship between isoprene emission and protonemal growth along with percentage change in protonema growth. Logged isoprene emission was classified as the response variable, and protonemal growth was classified as the continuous predictor.

To determine the effect of media on CFR, we used an ANOVA. We used regression analysis to examine the relationship between CFR and isoprene emission. To determine the effect of media on Relative Electron Transport Rate (RETR), we used an ANOVA. We also used regression to examine the relationship between RETR and isoprene emission. Similarly, we used regression analysis to examine the relationship between isoprene emission and light, as well as isoprene and temperature.

Results

Early isoprene emission in *Polytrichum juniperinum* protonema

Day number and initial collection site contributed significantly to our model fit in predicting isoprene emission in early *P. juniperinum* protonema. There was a significant difference between effect of day (DF=7; $X^2=149.6$; $P < 0.0001$) for total isoprene emitting samples of *P. juniperinum* and site-specific effects (DF=2; $X^2=6.015$; $P \leq 0.0009$). Not

all samples emitted isoprene at the same time, and we saw site-specific differences in emission ability and timing. Overall, day contributed to 78% of variation in emission, while site contributed only 2% of variation in isoprene emission, with the rest of the contribution coming from residual effects (Figure 2.2). On day five, 14 % of the samples (3 of 21) emitted isoprene, and plants from two of the three sites were emitting at detectable levels. The last site started emitting the following day. By day six 62 % of the samples (13 of 21) were emitting, and plants from all three sites were emitting at different rates. This increased to 95% (20 of 21 samples) emitting isoprene by day eight, and we saw emissions from all samples and sites on day ten. From three sites, site 6 vs. site 8, and site 8 vs. site 9 were statistically significant from each other, while site 6 vs. site 9 had no statistical significance. We found no effect of media on isoprene emission at this early stage ($P = 0.44$).

The effects of media and time on isoprene emission in early *Polytrichum juniperinum* protonema

Isoprene emission was greatly influenced by the media on which it was grown by day forty-five (ANOVA; $DF=1$; $F= 7.4082$; $P < 0.0001$; Figure 2.3). Isoprene emission from single spore isolated protonema showed higher levels of emissions on BCD media than agar. Mean isoprene emission on agar media was lower than BCD media. On agar isoprene emission mean value was $26.2 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 8.093 \text{ SEM}$ ($n=9$), on BCD media mean isoprene emission value was $62.56 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 9.887 \text{ SEM}$ ($n=9$). All surviving protonema in its resting phase has increased in growth, but none of the samples

differentiated into gametophytic tissue in the first assessment (Figure 2.4). Protonema by day 45 was embedded into the agar and separation was not possible without breaking the integrated protonemal network. Overall, media contributed 58% of the total variation in isoprene emission, protonema on agar had 42% less isoprene emission than protonema on BCD, while we have found no statistical significance between sites ($P=0.1325$).

In our second assessment, we found that isoprene emission was significantly affected by leaf number for single spore isolates of eighty-one siblings (ANOVA; $DF=4$; $F=4.01$; $P=0.0036$; Figure 2.5), with isoprene emission decreasing as leaf number increased. Total leaf count was 35 by our second assessment ($n=81$), and 51 on our third assessment. Mean isoprene emission was significantly different between days tested and leaf number (RM ANOVA; $DF=1$; $X^2=4.83$; $P \leq 0.0001$). Mean isoprene emission from the three individual time assessments was $9.76 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.55 \text{ SEM}$ ($n=243$), and ranged from 7.23 to $11.41 \text{ nmol m}^{-2} \text{ s}^{-1}$ ($n=243$), with the lowest isoprene emission seen on our last assessment.

Isoprene emission and growth in *Polytrichum juniperinum* protonema

We found a significant correlation between isoprene emission and protonema growth. Protonema growth showed a negative trend against isoprene emission with larger diameter protonema emitting less isoprene than protonema with smaller diameter. Logarithmic line equation for first assessment was $Y=-1.15\ln(x) + 3.02$; and for the second assessment was $Y= -1.02\ln(x) + 2.72$ (Regression; $r^2= 66.18$; $P<0.0001$; Figure 2.6). Isoprene emission increased in the beginning, plateaued midway and as soon as leaf

emergence took place isoprene emission dropped significantly. Collectively, mean isoprene emission from single spore isolates from all dates were $27.4 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 4.378 \text{ SEM}$ (n=243), which were higher than from protonema grown from ~ 100 spores. Isoprene emission ranges during September 2, 2016, September 15, 2016 and September 28, 2016 were from $13.92 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 1.217 \text{ SEM}$ to $40.89 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 8.434 \text{ SEM}$ (n=162).

Mean protonema growth for the three time intervals (September 2, 2016; September 15, 2016; September 28, 2016) was $1.456 \text{ mm}^2 \pm 0.0657 \text{ SEM}$ (n=243). Protonema growth ranges were from $1.04 \text{ mm}^2 \pm 0.089 \text{ SEM}$ to $1.872 \text{ mm}^2 \pm 0.072 \text{ SEM}$ (n=162). Overall protonema growth contributed to 52% of total variation in isoprene emission in the first assessment from September 2, 2016 to September 15, 2016 and contributed to 23% of total variation in isoprene in the second assessment from September 15, 2016 to September 28, 2016.

We found significant differences in percentage change in protonema growth versus leaf emergence (ANOVA; DF= 2; F=67.23; P= <0.0001; Figure 2.7). In the first assessment we saw an increase in the percentage change in protonema growth up until leaf emergence, but with leaves % change in protonema growth declined with more horizontal, rather than vertical growth development.

Laboratory gas exchange measurements

We have found that media significantly affects isoprene emission (DF=1; $X^2=21.25$; P <0.0001), as well as isoprene emission and CFR (DF=1; $X^2=5.85$; P < 0.0156).

The line slopes between media for CFR vs. isoprene emission are statistically significantly different with a regression line equation for agar $Y=3.2774*x + 0.5941$, and for BCD $Y=0.7023*x + 2.641$ ($F= 37.96$; $P<0.0001$; Figure 2.8). We found media significantly affects CFR measures (ANOVA; $DF=1$; $F= 11.6593$; $P <0.0013$; Figure 2.9).

Mean isoprene emission values overall were $16.18 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 1.614 \text{ SEM}$ ($n=52$). While on agar media mean isoprene emission were $9.267 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 1.137 \text{ SEM}$ ($n=26$), and ranged from 3.021 to 22.901 $\text{nmol m}^{-2} \text{ s}^{-1}$ ($n=26$). Mean isoprene emission values on BCD were $23.140 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 2.348 \text{ SEM}$ ($n=26$), and ranged from 4.274 to 57.680 $\text{nmol m}^{-2} \text{ s}^{-1}$ ($n=26$). On agar media, we saw a reduction of isoprene emission and on BCD isoprene emission was 40% higher than on agar media. CFR measures together explained 60% variability in isoprene emission seen from *P. juniperinum* protonema.

Mean CFR values collectively were $0.489 \pm 0.015 \text{ SEM}$ ($n=52$). On agar and BCD media CFR values ranged from 0.33 to 0.74 ($n=26$), but mean CFR values on BCD media was higher ($0.5377 \pm 0.012 \text{ SEM}$, $n=26$), than on agar media ($0.442 \pm 0.010 \text{ SEM}$, $n=26$). On agar media, CFR contributed 39% of variation in isoprene emission, and on BCD media, CFR contributed 21% of variation in isoprene emission.

We also took two light setting measurements for Relative Electron Transport Rate (RETR) curves for plants on two media (agar and BCD). Under low light setting ($0\text{-}820 \mu\text{mol m}^{-2} \text{ s}^{-1}$), we found no statistical differences in RETR curves between media ($P = 0.81$; Figure 2.10). Mean RETR values were $33.06 \mu\text{mol m}^{-2} \text{ s}^{-1} \pm 3.367 \text{ SEM}$ ($n=40$).

While on agar media under low light setting (0, 66, 90, 125, 190, 285, 420, 625, 820 $\mu\text{mol m}^{-2} \text{s}^{-1}$), RETR mean values were $32.18 \mu\text{mol m}^{-2} \text{s}^{-1} \pm 3.328 \text{ SEM}$ (n=20) on agar; and on BCD media mean RETR were $33.94 \pm 3.406 \text{ SEM}$ (n=20). Our regression line equation under agar was $Y = 0.1067x + 1.812$, and under BCD was $Y = 0.1102x + 0.8975$.

Under high light setting, we found statistical difference in media (RM ANOVA; DF=1; F= 3.9027; P < 0.05; Figure 2.11). Regression line equation for RETR measures on agar being $Y = 0.1116x + 0.9509$, and under BCD were $Y = 0.1486x + 2.885$ (RM ANOVA; DF= 1; $x^2 = 3.65$; P= 0.056). Under higher light settings (236, 302, 325, 361, 425, 520, 655, 860, 1055 $\mu\text{mol m}^{-2} \text{s}^{-1}$), mean RETR values were $74.06 \mu\text{mol m}^{-2} \text{s}^{-1} \pm 4.746 \text{ SEM}$ (n=40). Mean RETR values on agar were $68.06 \mu\text{mol m}^{-2} \text{s}^{-1} \pm 4.28 \text{ SEM}$ (n=20) and on BCD media were $81.14 \mu\text{mol m}^{-2} \text{s}^{-1} \pm 5.212 \text{ SEM}$ (n=20). RETR values contributed to 78.5% variation under low light setting (0-820 $\mu\text{mol m}^{-2} \text{s}^{-1}$) on agar media, and RETR values contributed 51% variation under high light setting (0-1150 $\mu\text{mol m}^{-2} \text{s}^{-1}$) on agar media with PAR levels selected as random effects. Our combined low agar and low BCD media RETR values were $26.2 \mu\text{mol m}^{-2} \text{s}^{-1} \pm 8.093 \text{ SEM}$ (n=20); whereas the combined high agar and BCD values were $62.56 \mu\text{mol m}^{-2} \text{s}^{-1} \pm 9.887 \text{ SEM}$ (n=20), with a regression line equation for low agar and BCD; Low $Y = .01087x + 1.355$, and for high agar and BCD; High $Y = 0.13012x + 6.197$ (Figure 2.12).

Light and temperature response curves of *P. juniperinum* protonema

We found that isoprene emission and light in *P. juniperinum* were statistically correlated across temperature. Our regression line equation for isoprene emission and

temperature was $Y = 0.4798 * x - 4.791$ (Regression; $r^2 = 0.56$; $P < 0.0027$; Figure 2.13).

Light correlated logarithmically to PPFD with a nonlinear lognormal line equation of $Y = -49.69 \ln(x) + 34.2$ (Regression; $r^2 = 0.62$; $P < 0.0004$; Figure 2.14). Under varying temperature and constant light (PPFD) *P. juniperinum* mean isoprene emission were $11.7 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.8862 \text{ SEM}$ (n=38). Under varying light (PPFD) and constant temperature mean isoprene emission were $7.267 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.421 \text{ SEM}$ (n=53).

Discussion

Isoprene emission in *Polytrichum juniperinum* protonema

Sampling detectable levels of isoprene emission from *P. juniperinum* protonema was accomplished here for the first time. We demonstrate that protonema from mature sporophytes (Figure 2.1) can be successfully assessed for isoprene emission in both as single spore isolates from half siblings and from mixed spores. In the earliest life stage in the moss life cycle, *P. juniperinum* can emit detectable levels of isoprene (Figure 2.2). Our aim was to assess and quantify isoprene emission in moss protonema. We have found that mosses can emit isoprene as early as five days after spore germination, and we demonstrate that a time lag exists from the beginning of protonemal development to isoprene emission. In vascular plants, it is already established that a time lag exists between onset of photosynthesis and isoprene emission (Rasulov et al. 2014). Vascular leaves will take days to weeks to be able to produce isoprene depending on photosynthetic adequacy (Sharkey et al. 1993; Monson et al. 1994; Goldstein et al. 1998; Kuhn et al. 2004; Mayrhofer et al. 2005; Wiberley et al. 2005; Sharkey et al. 2007). We

can confirm that we observed moss protonema isoprene emission time lag that is comparable to the time lag observed in vascular plants where isoprene emission develops by day six after new leaf emergence (Velikova et al. 2008; Rasulov et al. 2014). Our results indicate that isoprene emission initiates during the first moss protonemal stage, a stage which can last indefinitely in many mosses (Rensing et al. 2007, 2018a,b; Reski et al. 2018a,b; Ryo et al. 2018).

Additionally, our results suggest that isoprene emission capacity is higher for young moss tissue compared with older tissue. We found average isoprene emission for protonema to be $45.85 \text{ nmol m}^{-2} \text{ s}^{-1}$ (at $1000 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ and 30°C). This range is higher than what is reported for *P. juniperinum* gametophytes of $37 \text{ nmol m}^{-2} \text{ s}^{-1}$ (at $1600 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ and 30°C) (Hanson et al. 1999). Isoprene emission from protonema was highly variable, in both mixed sporophytes and half sibling surveys, suggesting genetic differences within moss capsules. Our data suggest that isoprene production in *P. juniperinum* is influenced by site-specific effects suggesting both genetic control and environmental effects in isoprene emission. These results are similar to those found in vascular plants, where large genetic differences between plants have been recorded (Loreto et al. 1998, 2009; Velikova et al. 2014). In addition, in vascular plants, isoprene emission has been seen to be highly developmentally variable within the same plant when sampled between the lower branches and top branches of a single tree, with emissions higher in the top of the tree (Harley et al. 1997).

For our spore mixtures from multiple sporophyte assessment, isoprene emission increased on nutritive BCD media as opposed to non-nutritive agar. This suggests that the

young protonema on nutrient rich media was able to utilize the resources available for secondary metabolite production rather than expend the resource on growth (Figure 2.4). We observed more isoprene emission from spores plated on BCD suggesting that the protonema can utilize environmental sources of nutrients to fix carbon for isoprene emission. On agar media, observed isoprene emission was lower as demand for resources to expand and grow initiated a higher demand than available supply allowed (Lambers 1993; Kuhn et al. 2004; Owen et al. 2005; Rosenstiel et al. 2004; Brilli et al. 2007; Loreto et al. 2007a,b; Sharkey et al. 2007; Morfopoulos et al. 2014). Moss protonema development provides useful insight into what factors control life cycle phases of mosses (Niklas et al. 2010; Hackenberg et al. 2016; Silva-e-Costa et al. 2017).

Light and temperature are closely linked to protonema development (Cove et al. 1978), as is bioavailable nitrogen (Alcade et al. 1996). Other factors that can contribute to protonema development include macronutrient availability (Basile et al. 1975), cell growth by osmotic cell properties (George et al. 2008), and the effects of moisture conditions (Pakarinen et al. 1977; Chopra et al. 1981; Chaban et al. 1999; Silva et al. 2010; Sabovljević et al. 2014; Nishihama et al. 2015; Liu et al. 2016). However, no other study has looked at isoprene emission and protonema development and considered this as a factor when assessing growth and resources allocation, as well as carbon allocation between growth and isoprene emission.

For our half sibling experiment, using single-spore isolates, we saw high isoprene values initially. We observed a decrease in isoprene emissions by our second assessment when leaves had first emerged from the young protonema. For the third and final

isoprene assessment, we saw more leaves and the lowest amount of isoprene collectively (Figure 2.5), suggesting a metabolic cost to isoprene emission. The 81 half siblings reached peaked protonema growth just as leaves emerged and dropped significantly as more leaves grew perhaps as a result of exhausted resources and accumulation of stress factors within the PlantCon™ container.

Isoprene synthase enzyme is developmentally regulated and for mature vascular leaves, emission rates depend on DMADP (dimethylallyl diphosphate) availability, whereas in young leaves emission is also influenced by isoprene synthase protein availability (Vickers et al. 2010). Differences have been observed in vascular plant isoprene emission, where its capacity was 124% higher in young leaves than in older mature leaves (Alves et al. 2014). We suspect that we could also find young to mature gametophyte *P. juniperinum* isoprene emission differences as we saw protonema age and leaf development with decreased isoprene emission.

Isoprene emission and growth in *Polytrichum juniperinum* protonema

In vascular plants, each isoprene molecule costs carbon and energy. The energy and carbon cost have been summarized by Sharkey et al (2001), with each isoprene molecule costing nine carbon atoms, 24 molecules of ATP, and 14 molecules of NADPH when using the MVA isoprene production pathway (Sharkey et al. 2001; Niinemets et al. 1999; Sanadze 2010; Sanadze et al. 2016; Fini et al. 2017). Whether the cost in non-vascular plants is the same as it is in vascular plants is yet to be determined. However, to be able to emit isoprene in mosses, photosynthetic capability must be adequate as it is in

vascular plants (Schnitzler et al. 2005), and carbon that is used for isoprene emission may not be available for growth. Isoprene emission rate and photosynthetic rate generally correlates, but both processes can have independent responses to plant growth (Monson et al. 1994).

In vascular plants under increased CO₂ growth of biomass can be enhanced, but isoprene emission is reduced, suggesting leaf level suppression of isoprene emission given increased CO₂ (Rosenstiel et al. 2003). We explored protonema growth as one of the costs to isoprene emission and found that plants with small protonema growth had higher isoprene emission than plants with higher rates of growth (Figure 2.6) suggesting a trade-off between growth and isoprene emission for carbon resource allocation in early protonema development. It was very interesting to find that some moss protonema allocated enough resources to isoprene production that growth was significantly delayed indicating that isoprene production and emission can play a role in early development whose importance is on par with the imperative of early growth.

Similar trade-offs have been reported in vascular plants. In overexpressed *Populus canescens* isoprene synthase gene in non-isoprene emitter *Camelina sativa* lead to alterations in its growth and metabolism. This resulted in shorter and smaller leaves than wild types, suggesting a trade-off between biomass production and isoprene emission (Rossi et al. 2017).

We observed a significant trade off associated with growth and isoprene emission when we assessed the percentage change in protonema growth compared to isoprene emission as new leaves began to emerge. Then with increased growth as evidenced by

more leaves emerging, and *P. juniperinum* spent resources on horizontal, rather than vertical growth (Figure 2.7) isoprene emissions leveled off and then fell. The advantages that plants receive must outweigh the cost to keep isoprene emission as an evolutionary trait present in many species. This observation makes studies showing that North American oaks all emit isoprene, but many of the European oaks do not (Loreto et al. 1998; Kesselmeier et al. 1999) that much more confounding in our search to understand a mechanistic model that provides a cost benefit analysis between isoprene emission and growth.

Mosses can serve as an ideal system to study isoprene emission, as many of the necessary traits such as isoprene emission were established in early land plants. Isoprene emission was conserved in some mosses and vascular plants, but not all plants emit isoprene. Since plants, including mosses have lost and gained this trait multiple times, it is possible to trace evolutionary linkages with gene targeting to understand what the evolutionary advantage is that repeatedly selected for this mechanism (Reski 1998; Wood et al. 2000; Stenøien et al. 2001, 2005; Fattash et al. 2007; Cove et al. 2009; Reski 2018b; Lang et al. 2018; Zhao et al. 2018).

Laboratory gas exchange measurements

We wanted to find out whether isoprene-emitting protonema are able to maintain typical levels of photosynthesis as stress levels change and if we could observe a relationship between isoprene emission and either chlorophyll fluorescence ratio (CFR) or Relative Electron Transport Rate measures (RETR). Polytrichaceae gametophytes

have been found to have a good negative exponential saturation curve in high irradiances, and this moss light saturation curve approaches and can exceed full midday sun (Proctor et al. 2005, 2006). However, no protonema electron transport curves have been published to our knowledge. We know that there is a strong positive correlation between photosynthetic electron transport rate and isoprene emission (Warren et al. 2000; Dani et al 2015). Even under moderate stress, electron transport rate values can be maintained (Genard et al. 2014). In vascular plants, mild stress can decrease stomata opening and affect carbon assimilation, while isoprene emission and electron transport rate maintenance is not affected (Niinemets et al. 2010).

Desiccation tolerant bryophytes and desiccation tolerant dicots conserve chlorophyll during desiccation (Tuba et al. 1996; Porembski et al. 2000), suggesting chlorophyll loss is an acceptable cost associated with desiccation tolerance (Hinshiri et al. 1971; Alpert 2000; Oliver et al. 2005). We have seen in our protonema that the low values we obtained for chlorophyll fluorescence ratio (CFR) was due to phenotypic plasticity to desiccation. Chlorophyll fluorescence is sensitive to moss water content and during desiccation, mosses suffer low capacity to maintain photosynthetic efficiency (Robinson et al. 2000). Mosses are C3 plants and the relationship between CO₂ gas exchange and ETR are complex and different from what has been found in higher plants (Green et al. 1998). We found a statistically significant relationship between chlorophyll fluorescence ratio on agar versus BCD media, and we saw a statistically significant relationship between media and CFR values affecting isoprene emission (Figure 2.8; Figure 2.9).

Relative electron transport rate measures stress and enables dissipation through photorespiration or photochemical quenching. Changes in isoprene emission have been strongly correlated with changes in leaf ATP content suggesting that the rate of photosynthetic electron transport (ETR) may exert control over isoprene production and emission (Loreto et al. 1990; Niinemets et al. 1999). RETR was measured independently using fluorescence and was not coupled with LiCor measurements and should only be taken as indicative and not as absolute measures (Dani et al. 2014). The values presented here for low light RETR, high RETR and combined low and high RETR curves were assessed for stress. Our results correspond under both high and low light conditions to what has been documented for macroalgae RETR ranges (Franklin et al. 2001; Figueroa et al. 2003), where a good correlation can be found between RETR and O₂ evolution (Beer et al. 2000; Longstaff et al. 2002). Our ranges are considered to be under low stress, neither of our low or high RETR curves reached saturation, meaning that protonema is capable of exceeding our values once saturation is reached (Figure 2.10, 2.11, 2.12).

Light and temperature response curves of *P. juniperinum* protonema

Isoprene emission saturation values for light (PPFD) and temperature in *P. juniperinum* are much lower than reported for mature vascular plants, where isoprene emission can increase with photosynthetic photon flux density of up to 3000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (at 30°C) and can continue to increase after photosynthesis is saturated at 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Sharkey et al. 1993). We found a strong effect on isoprene emission of both

temperature and light, indicating that isoprene emission can increase with photon flux up to $2250 \mu\text{mol m}^{-2} \text{s}^{-1}$ and temperature for up to 35°C . Our ranges for emission maxima were between $40\text{-}45^\circ\text{C}$ and $800\text{-}1200 \mu\text{mol m}^{-2} \text{s}^{-1}$, which is close to saturation level of young leaves in vascular plants (Alves et al. 2014). Isoprene saturation in moss protonema occurred at $1500 \text{PPFD } \mu\text{mol m}^{-2} \text{s}^{-1}$, and isoprene emission did not saturate for our temperature curves. This suggests that *P. juniperinum* protonema is sensitive to changes in light, but whether it is able to tolerate higher temperature values to reach saturation remains to be seen. Others have shown that at 40°C and $1000 \text{PPFD } \mu\text{mol m}^{-2} \text{s}^{-1}$ isoprene emission reaches maximum level and can produce over $200 \text{nmol m}^{-2} \text{s}^{-1}$ in kudzu leaves (Sharkey et al. 1993). Water stress plays a large role in emission as was shown by Sharkey where at 35°C and $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ isoprene emission in proportion to photosynthesis increased from 24% to 67% (Sharkey et al. 1993).

The positive correlation in this study between isoprene emission and temperature (Figure 2.13) as well as between isoprene emission and light (Figure 2.14) confirm temperature dependency and light dependency in moss protonema, as has been reported from vascular plants (Sanadze et al. 1966, 1969; Tingey et al. 1979; Monson et al. 1989, 1992; Sanadze 2004; Sasaki et al. 2005). Many protonemal samples did not respond to either light or temperature and remained flat lined through the trials, while higher temperatures caused the chloroplast to be released from the protonema membrane at 40°C . This resulted in complete chloroplast loss as evident the next day with protonema that turned translucent. High variability in isoprene emission seen in our light and temperature trials indicates that there is a close relationship between photosynthesis and

isoprene emission (Kuhn et al. 2002). We need to understand how much carbon taken up by photosynthesis is released as isoprene in mosses. In vascular plants, the loss of carbon that is fixed by photosynthesis can vary, and it can be as high as 20% (Sharkey et al. 1991, 1993). It has been shown in vascular plants that carbon fixed by photosynthesis and re-emitted as isoprene is 53% higher in sun leaves than in shade leaves (Harley et al. 1996). Further investigation is required to understand isoprene emission in moss species such as the Polytrichaceae, which ecologically occupy a wide range of habitats from xerophytes capable of withstanding high light to species which thrive in deep shaded wet habitat.

Tables and Figures

Table 2.1 Summary table of *Polytrichum juniperinum* populations used for protonema single spore and mixed spore experiments.

Experiment	Latitude	Longitude	Elevation (meters)
Protonema from single spore samples	45°41'38.34"N	122°52'14.23"W	15
Protonema from mixed spore samples Location 1	45°47'17"N	122°56'16.00"W	241
Protonema from mixed spore samples Location 2	45°41'50.54"N	122°52'40.83"W	145
Protonema from mixed spore samples Location 3	45°46' 58.78" N	123°22'14.14" W	315

Figure 1.1 Conceptual model for exploring isoprene emission in moss. Factors in this study excluded from analyses were Environmental factors and Atmospheric Chemistry differences associated with isoprene emission.

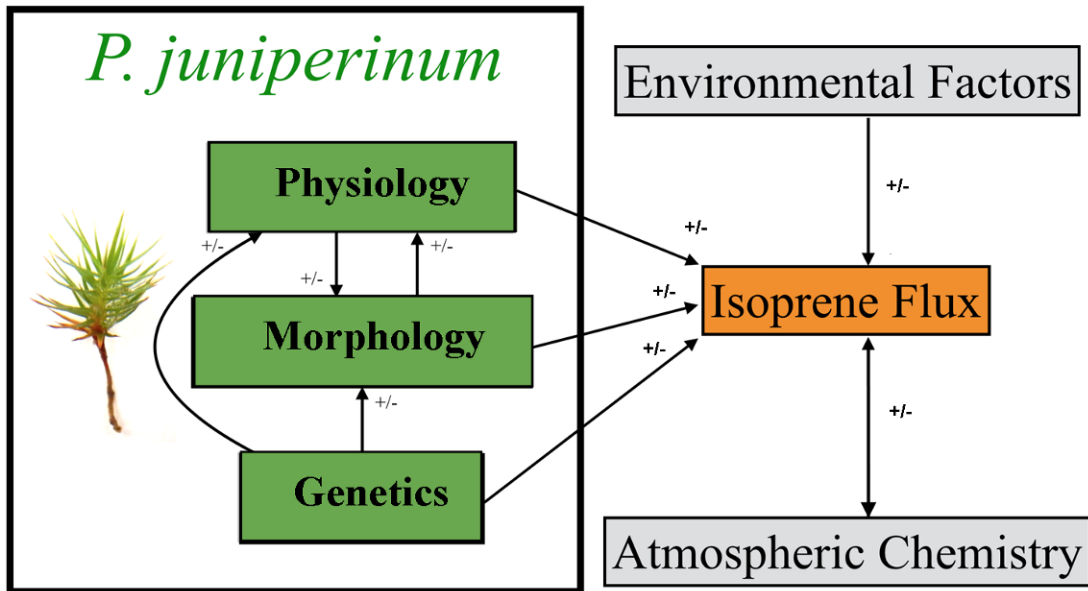


Figure 2.1 (a) Depiction of *P. juniperinum* spores before capsule sterilization from two sites (upper right rural forest site), and (lower left high elevation mountain site); (b) forty-five day old *P. juniperinum* protonema growing in BCD media in PlantCon™ containers.

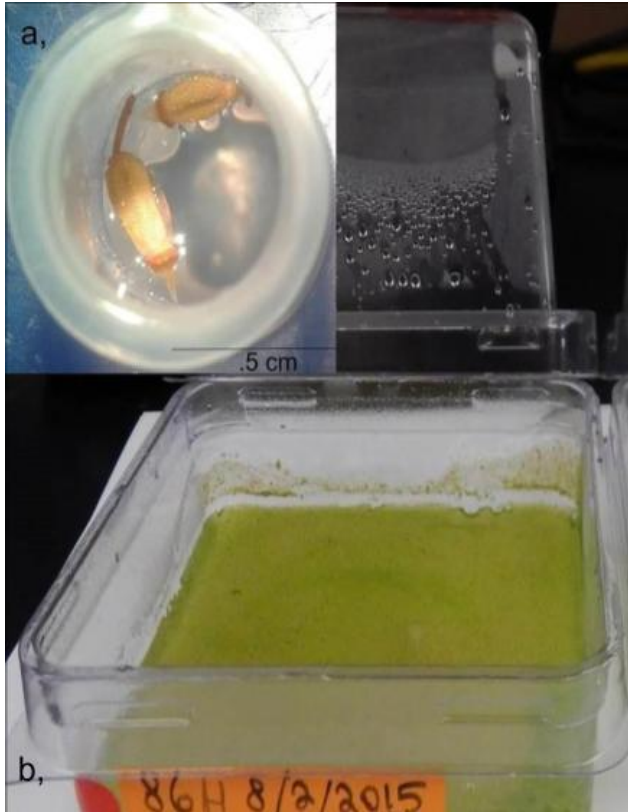


Figure 2.2 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus *P. juniperinum* spore germinated protonema ($n=21$). Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) by day is plotted on the x-axis from day 1 to day 10. Nominal Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between isoprene emitting samples among days. Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

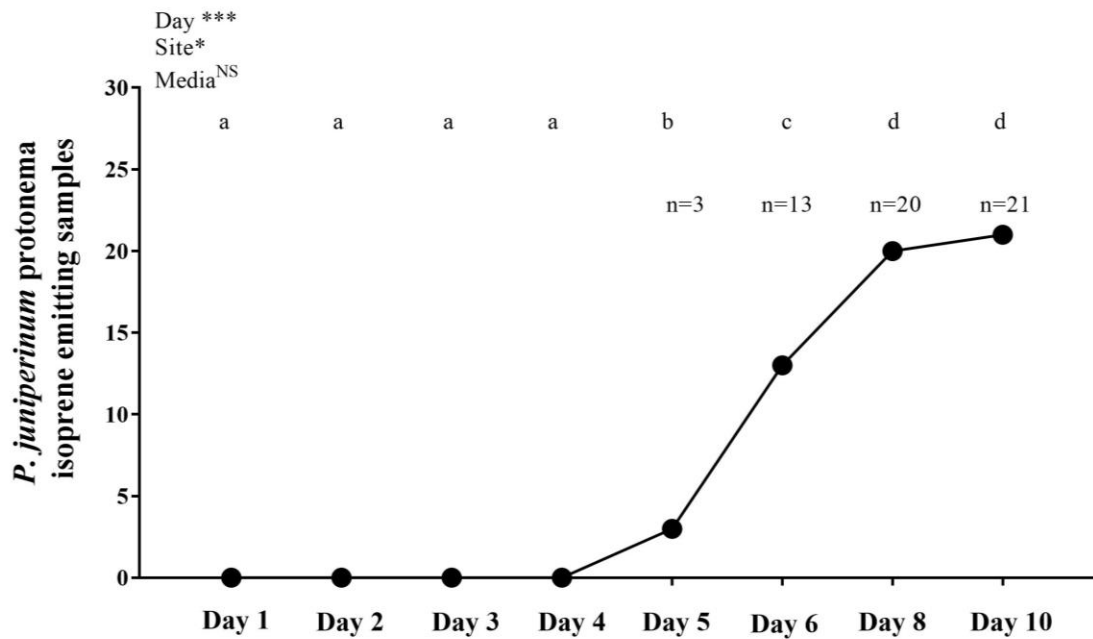


Figure 2.3 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus media (agar versus BCD) in *P. juniperinum* protonema (n=52). Media source is plotted on the x-axis. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

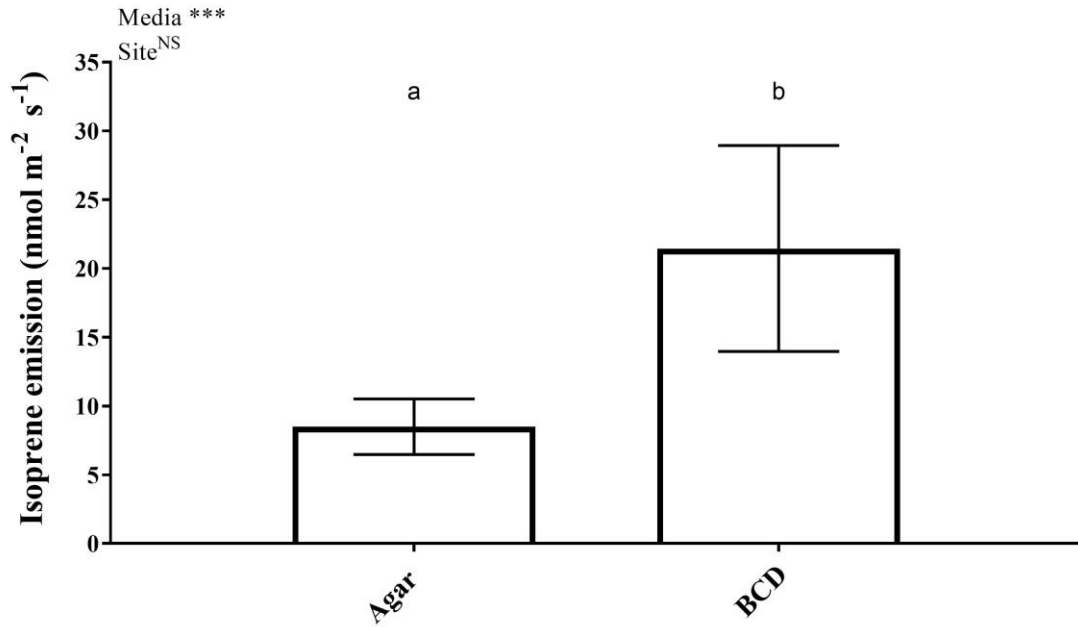


Figure 2.4 Image depicting *P. juniperinum* protonema growth versus day number (n=21). Detail is shown for average protonema growth for each day. Growth of protonema was measured using upright bright-field microscopy (Leitz DME, Leica, Wetzlar, Germany) equipped with AxioCam 105 Color camera and Zen Blue software (version 1.1.2.0; Carl Zeiss Microscopy).

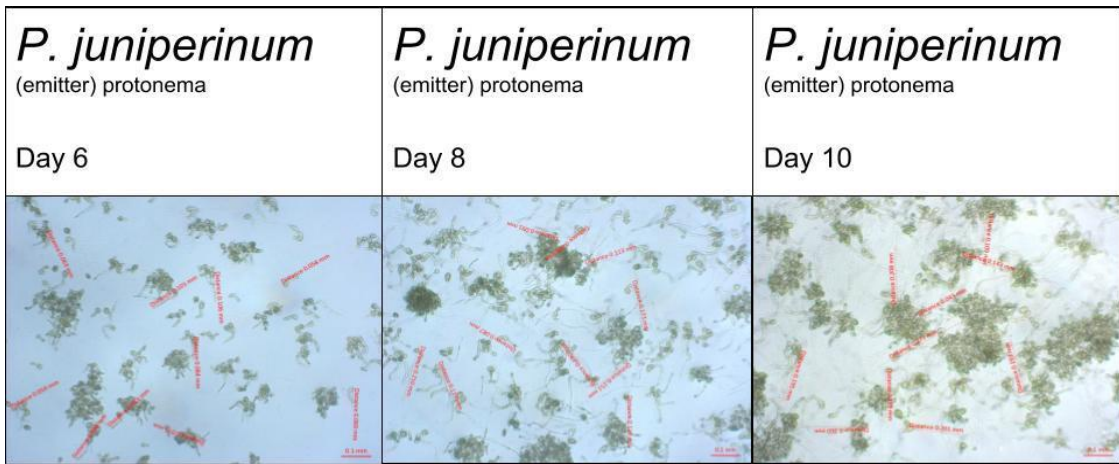


Figure 2.5 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus *P. juniperinum* protonema and leaf number ($n=81$). Assessment date is plotted on the x-axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) vs. leaf number. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

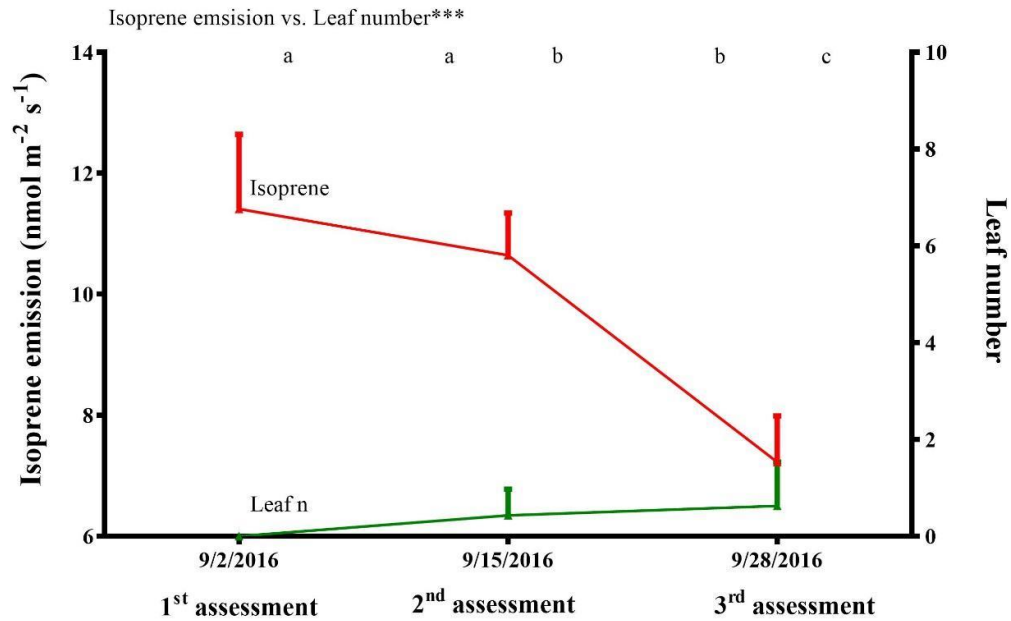


Figure 2.6 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$)/growth versus protonema growth ($n=246$). Protonema growth is plotted on the x-axis. The line slopes between two assessments are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance. Logarithmic line equations are shown for both time intervals.

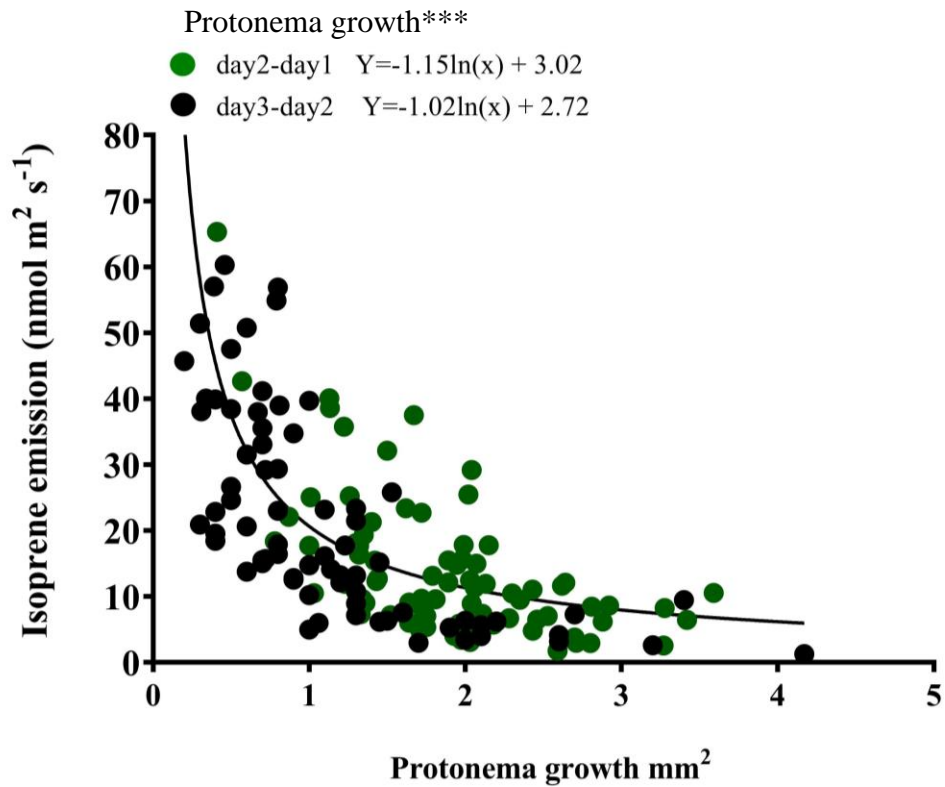


Figure 2.7 *P. juniperinum* % change in protonema growth versus leaf emergence for three individual dates (n=246). Date is plotted on the x-axis. Lines represent % change in protonema growth with leaves present for individual dates. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between % change in protonema growth vs. leaf emergence. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

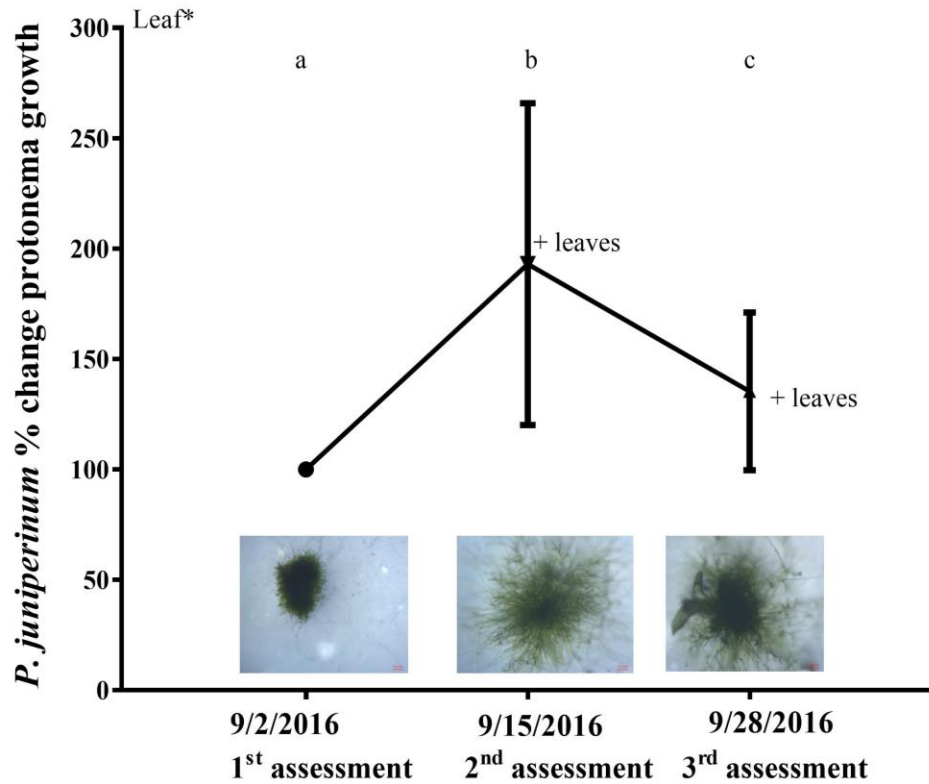


Figure 2.8 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus Chlorophyll Fluorescence Ratio (CFR) in *P. juniperinum* protonema on agar and BCD media (n=52). Chlorophyll Fluorescence Ratio (CFR) is plotted on the x-axis. The line slopes between the two media are statistically significant. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance. Regression line equations are shown for both media source.

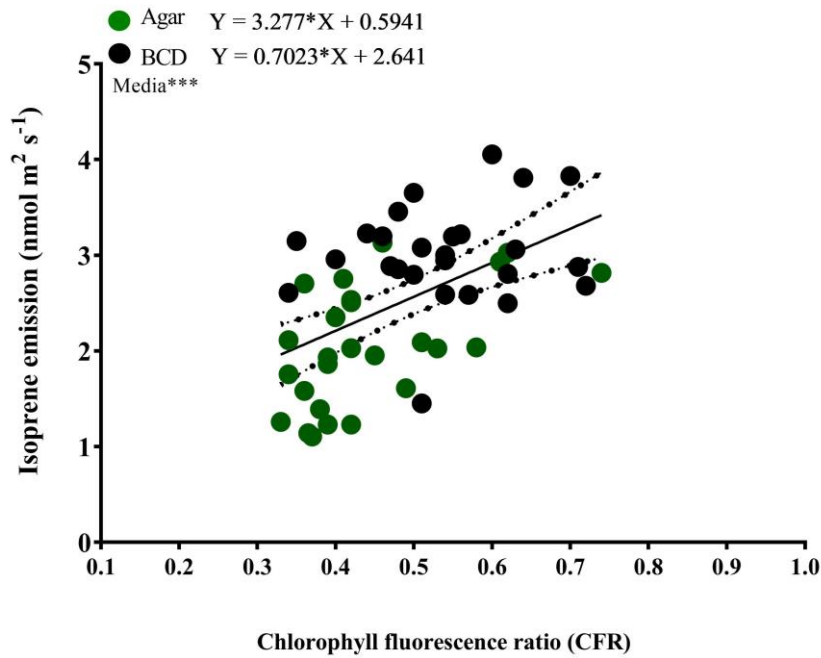


Figure 2.9 Chlorophyll Fluorescence Ratio (CFR) versus *P. juniperinum* protonema on agar and BCD media (n=52). Media is plotted on the x-axis. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

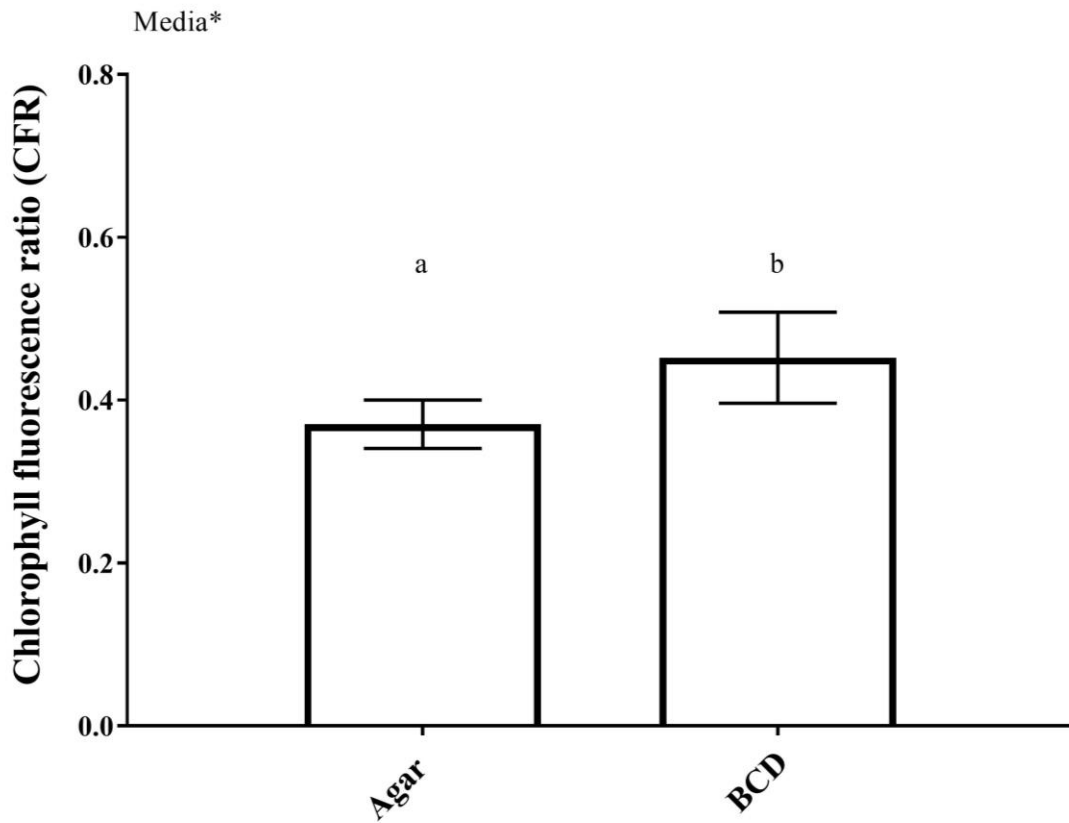


Figure 2.10 Relative Electron Transport Rate (RETR) ($\mu\text{mol m}^{-2} \text{s}^{-1}$) from *P. juniperinum* protonema versus agar and BCD media under low-light setting (0-820 $\mu\text{mol m}^{-2} \text{s}^{-1}$) PPFD (n=20). Photosynthetic Photon Flux Density (PPFD) is plotted on the x-axis. No statistical differences were found between media and RETR. The line slopes between media are not significantly different. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance. Regression line equation shown below for media under RETR versus low-light (PPFD).

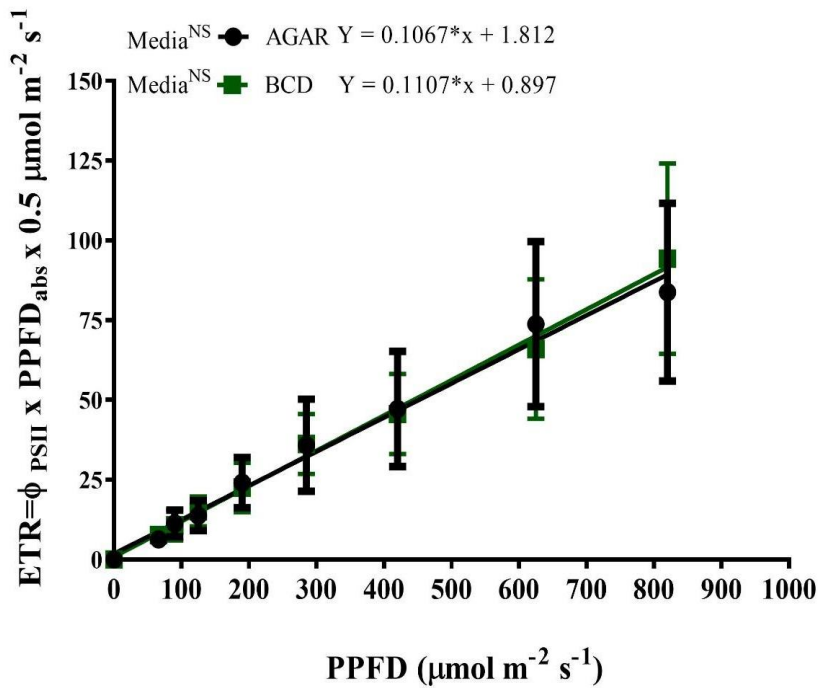


Figure 2.11 Relative Electron Transport Rate (RETR) ($\mu\text{mol m}^{-2} \text{s}^{-1}$) from *P. juniperinum* protonema versus agar and BCD media under high-light setting (0-1150 $\mu\text{mol m}^{-2} \text{s}^{-1}$) PPFD (n=20). Photosynthetic Photon Flux Density (PPFD) is plotted on the x-axis. The line slopes between media are significantly different. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance. Regression line equation shown below for media under RETR versus high-light (PPFD).

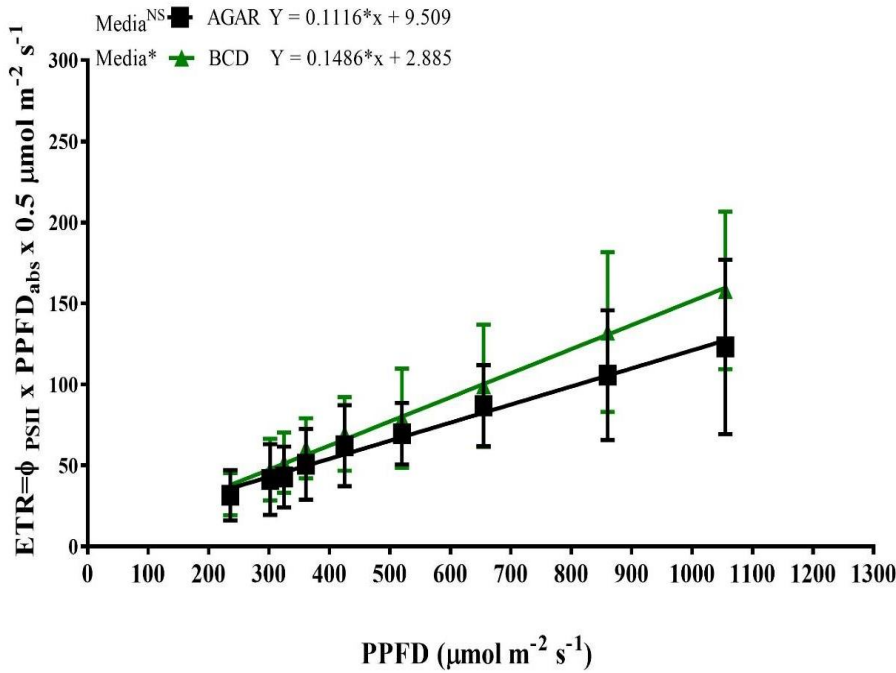


Figure 2.12 Relative Electron Transport Rate (RETR) ($\mu\text{mol m}^{-2} \text{s}^{-1}$) from *P. juniperinum* protonema versus combined agar and BCD media under low-light setting (0-820 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and high-light setting (0-1150 $\mu\text{mol m}^{-2} \text{s}^{-1}$) PPFD (n=40). Photosynthetic Photon Flux Density (PPFD) is plotted on the x-axis. The line slopes between media are significantly different. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance. Regression line equation shown below for media under RETR versus light (PPFD).

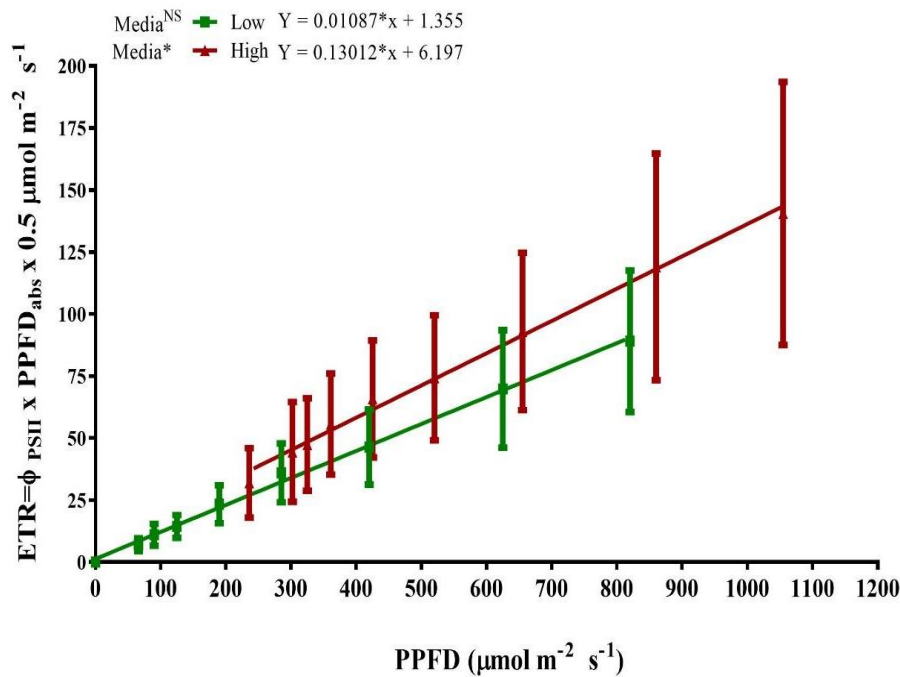


Figure 2.13 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) in *P. juniperinum* protonema versus temperature ($^{\circ}\text{C}$; $n=38$). Temperature is plotted on the x-axis. Regression line is statistically significant. Error bars represent $\pm 1\sigma$ (standard deviation). A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance. Regression line equation shown below for isoprene emission versus temperature.

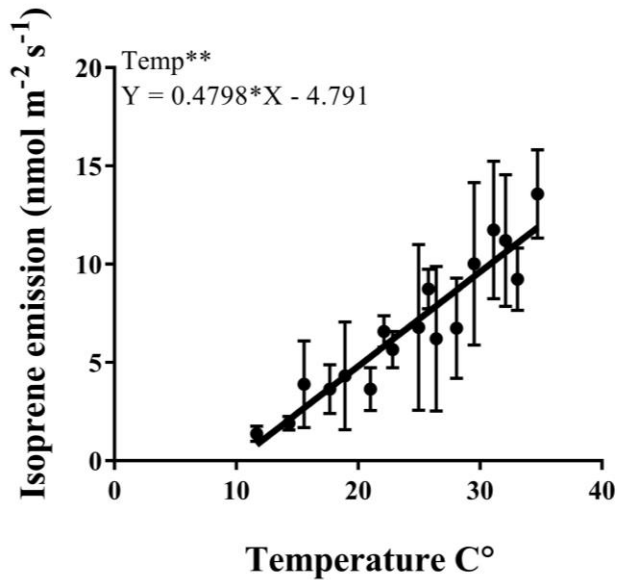
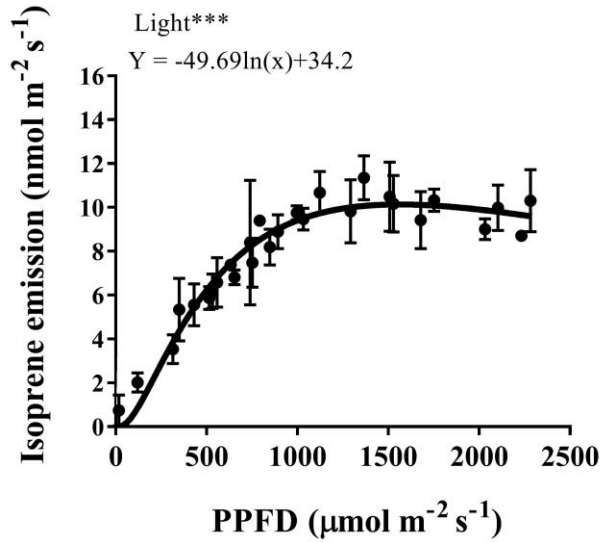


Figure 2.14 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) in *P. juniperinum* protonema versus Photosynthetic Photon Flux Density (PPFD) ($\mu\text{mol m}^{-2} \text{s}^{-1}$) ($n=53$). Photosynthetic Photon Flux Density ($\mu\text{mol m}^{-2} \text{s}^{-1}$) is plotted on the x-axis. Error bars represent $\pm 1\sigma$ (standard deviation). A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance. Regression line equation shown below for isoprene emission versus light (PPFD).



Chapter 3

The role of isoprene in Polytrichaceae sexual systems

Abstract

Mosses are some of Earth's most species-rich, ancient, and stress-tolerant ecosystem engineers. They play key roles in carbon and nitrogen cycling. Emission by mosses of Biological Volatile Organic Compounds (BVOCs), including isoprene, may locally engineer the atmosphere causing changes in the atmospheric hydrological cycle. Isoprene emission is poorly understood in bryophytes, and little is known about variation in emission across species and sexes. In this study, working with mosses in the Polytrichaceae family we (1) investigate the effects of morphological and physiological traits on isoprene emission in *P. juniperinum*, (2) explore the effects of gametangia expression or lack thereof on isoprene emission in *P. juniperinum*, and (3) assess variation in isoprene emission among mosses within the family.

Isoprene emission rate is significantly higher in sexually non-expressing gametophytes than female or male gametophytes. The cosmopolitan *Polytrichum juniperinum* had higher isoprene emission than shade adapted *Atrichum undulatum* and montane *Pogonatum urnigerum*. The highest emission rates were seen from non-sexually expressing gametophytes with significant canopy traits affecting isoprene emission; such as total leaves (TL) per gametophyte, gametophyte width (GW), wet weight (WW) and leaf tip redness (LTR). We are the first to report sexual expression affecting isoprene emission in Polytrichaceae species. We hypothesize that gametophytes with no sexual

expression are the least expensive to maintain as they have a higher investment after sexual organ formation takes place. We also saw great variation in above and belowground traits, along with isoprene emission and physiological measures between species and within sexes. This research uses a combination of physiological and comparative approaches to develop the first comprehensive view of the relationship between isoprene emissions and sexual systems in the mosses.

Keywords: bryophyte, gametophyte, isoprene, life stage, sexual systems

Introduction

Background

Large fractions of atmospheric hydrocarbons are emitted by plants in the form of Biogenic Volatile Organic Compounds (BVOCs). These represent a larger fraction than is emitted by human activities (Purves et al. 2004; Sharkey et al. 2007). Recent data suggest that these emissions have a complex link to climate and contribute to global warming (Peñuelas et al. 2003, 2010; Feng et al. 2019; Guidolotti et al. 2019; Mäki et al. 2019). Increase in temperature can disrupt these chemical signals leading to significant increases in landscape level BVOC emissions with profound implications for both vascular and nonvascular plants (Kivimäenpää et al. 2016). Isoprene, a BVOC, has been shown to increase thermotolerance of photosynthesis in vascular plants (Sharkey et al. 1995) and in nonvascular plants such as mosses (Hanson et al. 1999). As a result, isoprene emission may have been beneficial for the radiation of early plants on land (Hanson et al. 1999). The capacity to emit isoprene was gained and lost multiple times during bryophyte evolution; in addition, it was lost in later divergent plants that have evolved other protective mechanism from light such as heat shock proteins (Waters et al. 1996, Heckathorn et al. 1998). More systematic observations are needed; currently, there are not enough measurements of enzyme activity, substrate pools, costs of alternate protection strategies between emitting and non-emitting species in vascular plants to better understand isoprene and its place in plant evolution (Harrison et al. 2013). No data exists, to our knowledge, concerning the tradeoffs made in the divergence of isoprene emitting species and non-emitting species within families in non-vascular plants.

Isoprene represents a loss of fixed carbon and a significant metabolic cost to plants (Behnke et al. 2012; Ryan et al. 2014). In isoprene emitting plants, emission is controlled through light-dependent regulation of the isoprene synthase enzyme (IspS), which uses dimethylallyl diphosphate (DMADP) as a substrate for isoprene biosynthesis (Potosnak et al. 2014). We know that some of the emitted isoprene can be locally deposited where a number of bacterial and fungal taxa are capable of breaking down and utilizing isoprene as their energy and carbon source (Gray et al. 2015; McGenity et al. 2018; Crombie et al. 2019; Singh et al. 2019). These interactions help regulate soil microbe and fungal communities and affect soil chemical ecology and biogeochemistry.

Isoprene emission fluxes from bryophyte fens have been shown to be comparable to boreal forests (Janson et al. 1998, 1999; Tiiva et al. 2007a,b). Biogenic isoprene has been observed in monitoring stations as early as March and it is speculated to be of bryophytes in origin given that broadleaf trees in these latitudes at this time are bare and unable to produce or emit isoprene (Reimann et al. 2000). Isoprene emissions are highest in the summer influenced primarily by temperature (Tingey et al. 1979; Monson et al. 1994; Schnitzler et al. 1997; Nogues et al. 2018; Meehl et al. 2018). Mosses can emit significant quantities of BVOCs and experience microclimates that can greatly deviate from and broader local conditions (Busby et al. 1978; Hinshiri et al. 1971, Bates, 1979; Bisang et al. 2006; Zotz et al. 2007).

Approximately 60% of mosses have separate male and female individuals (dioecious sexual system) (Villarreal et al. 2013), rather than being hermaphroditic as is more common in vascular plants. In mosses, females generally outnumber males 3:2

(Shaw et al. 1993), even up to 4:1 (Newton 1972; Stark et al. 2001; Baughman et al. 2017). These skewed sex ratios have been hypothesized to be a result of competitive and facilitative interactions among relatives (Hamilton 1967; Clark 1978; Gowaty 1993; McLetchie et al. 2001; Bisang et al. 2008; Korpelainen et al. 2008; Baughman et al. 2017). Most often, it has been found that sterile or undifferentiated moss gametophytes outnumber males and females (Longton et al. 1990), and in mosses such as *Ceratodon purpureus*, the non-reproductive gametophytes make up high percentages of the population (Shaw et al. 1993; Rydgren et al. 2002a,b; Cronberg et al. 2003). Sexual reproduction can be rare for many dioecious mosses; one study found that out of 380 species of dioecious mosses 262 (69%) have been found to have rare sporophytes, which suggests that they are rarely sexually reproductive. Often, this is apparently due to individual females and males living in different patches and not in close proximity (Gemmell 1950; Haig 2016; de Jong et al. 2018).

Isoprene research in mosses has been gaining a foothold in the literature (Hanson et al. 1999, 2001; Tiiva et al. 2007, 2009; Ekberg et al. 2011; Lantz et al. 2015), but studies looking for sex-specific BVOCs in mosses have been rare and more recent (Rosenstiel et al. 2012; McCuaig et al. 2015). Mosses have effective defense in the form of chemical barriers and can serve as model systems for studying land plant evolution, and early chemical defense evolution, as they are sophisticated in ontogeny and have a host of defenses against both abiotic and biotic stress factors. Through isoprene emission, not only do mosses regulate their own system but they appear to exert influence on their surrounding environment. Other local influences exerted by moss emitted BVOC's may

include sex-specific volatile compounds that influence microarthropods mediated fertilization in mosses (Rosenstiel et al. 2012). Further, there are sex-specific fungal communities that affect their ecology (Sharkey et al. 2001; Devika et al. 2012; Kandi et al. 2015, Balkan 2016).

Mosses can influence nearby atmospheric process by emitting isoprene which is reduced by hydroxyl radical (OH) into water droplet nucleation. Secondary Organic Aerosol (SOA) acts as a local form of cloud or fog seeding allowing plants to exert influence on the atmospheric hydrological conditions of its local microclimate. The prospect of microclimate engineering among some mosses is an exciting prospect and bears further study. Bryophytes are key component of many forest landscapes where they serve as components of many diverse ecosystems (Jonsson et al. 1993; Longton et al. 1997; Sedia et al. 2003; Gornall et al. 2011). Mosses also uptake nutrients from the surrounding environment (Wilkinson et al. 2005; Ayres et al. 2006; Gundale et al. 2011), and they provide habitat for micro-invertebrates (Gerson 1982; Sohlberg et al. 1987; Nakamura et al. 1992; Andrew et al. 2003; Korsu et al. 2004; Heino et al. 2005; Jakub et al. 2018). Mosses possess two different terpene synthase genes, unlike vascular plants, which only possess one type of terpene synthase gene. In bryophytes in addition to the plant terpene synthase gene another class called microbial-terpene synthase like genes are also present (Chen et al. 2018). Terpenoids, including isoprene, have important function in biological, ecological and atmospheric processes.

The maintenance of sex is one of great interest in evolutionary biology (Smith 1978; Burt 2000; Vamosi et al. 2003). It has been hypothesized that when sex in a

dioecious species is mate-limited the non-limiting sex will produce the greatest abundance of floral scents (Case et al. 2009), and a similar pattern has been found in bryophytes (Rosenstiel et al. 2012). Mosses have been investigated for their sex-specific interactions (Eppley et al. 2011; Koncz et al. 2012; Balkan, 2015; Bisang et al. 2017; Slate et al. 2017; Maraist et al. 2018), but moss sexual systems and emission profiles are rarely studied together so as to understand these processes.

Purpose of Study

Mosses are some of Earth's most species-rich, ancient, and stress-tolerant "ecosystem engineers" (Jones et al. 1994; Eckstein et al. 2000; Keuper et al. 2011; Jassey et al. 2013). Unfortunately, only 2% of the 20,000 species of mosses have been chemically investigated (Turetsky et al. 2012). Despite their potential evolutionary and ecological significance, sex-specific BVOC emissions are extremely poorly characterized in mosses, creating a fundamental gap in our understanding of the chemical ecology of these ubiquitous plants. The lack of mechanistic understanding of the emission profiles of over ~300 chemical compounds (Rosenstiel et al. 2012) hinders our ability to interpret and predict the responses of mosses will have to anthropogenic drivers such as climate change (Vitousek et al. 1997; Aerts et al. 1999; Bell et al. 2007; Christensen et al. 2007; Erwin 2009; de Vries et al. 2011; Feng et al. 2019).

In this study, we will use a combination of physiological and comparative approaches to develop the first comprehensive view of the relationship between isoprene emissions and sex in mosses. This work will examine the hypothesis that isoprene-BVOC

emissions are related and perhaps contribute to the evolution of sexual systems in the mosses. We aimed to measure the rate of change in sexually expressing individuals in emission capacity within and across species in the Polytrichaceae family. By understanding emission capacity of individual sexes and species-specific profiles we will gain a comprehensive understanding of the variation in isoprene emission within the sexes and enable us to address future hypotheses aimed at addressing fitness costs and benefits associated with sexual reproduction in mosses.

Methods

Study species

We have used three species from the Polytrichaceae family in this study: 1) *Polytrichum juniperinum* (Hedw.), 2) *Atrichum undulatum* (Hedw.), and 3) *Pogonatum urnigerum* (Hedw.). The polytrichaceous mosses reside in the base of the phylogenetic tree, having no close living relatives (Bippus et al. 2018) and contain approximately 200 accepted species (Hyvönen et al. 1998; Bell et al. 2010a,b). The family Polytrichaceae forms a monophyletic group (Hyvönen et al. 2004) and possesses the highest degree of specialization, able to move photosynthate through conducting cells called leptoids, in both the haploid gametophyte and diploid sporophyte. Leptoids acts like protophloem sieve cells in vascular plants (Ligrone et al. 2000). The structure of leaves in Polytrichaceae is, among bryophytes, closest to the function of the leaves of vascular plants with thick cuticularized leaves having differentiated photosynthetic tissue anatomy (Clayton- Greene et al. 1985).

Mosses experience strong limitation of photosynthesis because of the high resistance to CO₂ diffusion through liquid water into the leaf surface and in the cell wall. The evolution of rows of chlorophyll-rich specialized lamellae cells are critical in this moss family for providing increased leaf surface area capable of 6-fold rates of CO₂ uptake per unit area (Proctor 1990, 2005; Marschall et al. 2004). This is a faster rate of diffusion of CO₂ into the plant when compared to other mosses (Krupa 1984). Lamellae also facilitate the absorption of CO₂ by repelling water with the presence of wax coated cells that prevent surface water retention. Air trapped in these lamellae cells minimize water loss (Bayfield 1973) while still allowing free gas exchange. The folding of leaves against the stem, followed by lateral closure appears to minimize water loss from the lamellar space and loss during the progression from leaf arrangements, resembling stomatal closure in vascular plants (Thomas et al. 1996). Poikilohydric plants, such as bryophytes, rely on soil water supply and atmospheric humidity for water uptake and retention (Stuber 2013, Yumol 2016); therefore, growth and weather are closely tied to productivity of the plant. This is a condition left over from the ancestral state (Raven et al. 2002, 2014). *Polytrichum* mosses are able to utilize water vapor in the air (Proctor 2005), giving them an advantage over other mosses. We hypothesize that isoprene emission plays a role in this.

Endohydric *Polytrichum* mosses differ from typical bryophytes in that they have a network of underground rhizomes and efficient internal water and carbohydrate conducting systems with apoplastic sugar loading (Thomas 1990). They are able to translocate photoassimilates to sister shoots suggesting capacity for physiological

integration between connected moss stems (Thomas 1988). This family of mosses also stores starches in their rhizoids, which act as sites for overwinter storage for carbohydrates (Skre et al. 1983). It has been suggested that the inflexed achlorophyllous leaf margins enclosing the photosynthetic lamellae in *P. juniperinum* serve to increase the relative humidity and temperature of the air surrounding the lamellae, and this adaptation can increase the length of the effective growing season (Thomas et al. 1996; Proctor 2005).

P. juniperinum has great desiccation tolerance that is owed to drought evasion strategy enabled by its highly specialized photosynthetic lamellae. It has been hypothesized that early land plants had to be desiccation tolerant in both gametophyte and reproductive states (Renzaglia et al. 2000; Oliver et al. 2000, 2005). New research highlights that a key biochemical pathway to aid moss on land was the development of moss cuticle, wax covered epidermal cells, which predate lignin in vascular plants (Renault et al. 2017).

Polytrichaceae moss collection method

In our first experiment, we wanted to investigate isoprene emission from non-sexually expressing gametophytes to avoid sex-specific differences or maternal effects (Shaw et al. 1999, 2000). In February 2016 we collected *P. juniperinum* (n=45) moss from a single large population in Oregon (Table 3.1). Moss was collected from a forested area with substratum and rhizoidal connections intact and brought to the lab in enclosed containers. From this a number of 5cm by 5cm sectors were selected and were cleaned of

debris and detritus. We identified the moss species using costa cross sections and moss field guides for the Pacific Northwest using Leica microscope (Leitz DME, Leica, Wetzlar, Germany) with an AxioCam 105 Color camera and Zen Blue software (version 1.1.2.0; Carl Zeiss Microscopy). We randomly selected 45 healthy individuals within the containers and immediately made physiological measurements (see below). Once physiological testing concluded gametophytes were labeled and placed individually in PlantCon™ containers (MP Biomedicals, 2017). We proceeded to take both aboveground and belowground morphological measurements (see below).

In our second experiment, we wanted to explore effects of sexual organ (gametangia) expression or lack thereof in isoprene emission under agar a non-nutritive media and BCD a nutritive media. In March of 2016 we collected sexually expressing *P. juniperinum* (n=25) female, male, and undifferentiated (non-sex expressing) gametophytes from six individual locations and grouped them accordingly (Table 3.2). Moss was brought in from the field as described above and was kept together with the rhizoidal connections intact. Gametophytes were selected and randomly distributed between media. Agar and nutritive BCD media was prepared following protocol outlined by Wang et al. (2015). Four females, four males and four non-sexually expressing gametophytes were cleaned of debris and soil and placed in agar media. Five females, four males and four non-sexually expressing gametophytes were cleaned of debris and soil and placed in BCD media. Sixty milliliters of prepared agar media was poured into base of 9.5 cm x 9.5 cm x 3.5 cm PlantCon™ containers (MP Biomedicals, 2017) and sterilized under UV light before each gametophyte was randomly placed in the middle of

a container. The gametophytes were allowed a two-week acclimation period before isoprene emission sampling began.

In our third experiment, we wanted to assess variation in isoprene emission and sexual expression among moss species within Polytrichaceae family. In April 2015, we selected shade adapted *Atrichum undulatum* (n=70) from four locations. We collected cosmopolitan *Polytrichum juniperinum* (n=56) from seven locations, and montane *Pogonatum urnigerum* (n=41) from six locations (Table 3.3). We selected several sites for each moss species as we were investigating species and site-specific differences between related mosses. Mosses were brought in as described from above and immediate testing of isoprene emission began, following morphological assessment of sex.

Aboveground morphological data collection from *P. juniperinum* gametophytes

Our aboveground measurements of *P. juniperinum* gametophytes included twelve canopy structure traits including leaf length (LL, mm) and leaf width (LW, mm) by using a digital caliper (Fowler, Lux Scientific Instrument Corp., Tucson, AZ, USA). Leaf tip redness or the apex of non-photosynthetic tissue (LTR, mm) was measured by examining five leaves from each gametophyte and determining leaf tip redness using a Leica microscope (Leitz DME, Leica, Wetzlar, Germany) with an AxioCam 105 Color camera and Zen Blue software (version 1.1.2.0; Carl Zeiss Microscopy). Lastly, we determined canopy height (CH, mm) of each gametophyte by taking total gametophyte length (GL, mm) measurement and subtracting the rhizoid length (RL, mm).

To count the total number of leaves (TL), gametophytes were viewed under a dissection microscope and individual leaves were counted on each gametophyte (Leica L2, Leica Microsystems Inc.). We obtained leaf mass area (LMA, mg cm^{-2}), which is the ratio between leaf dry weight after 48 hours and leaf area (Villar et al. 2013). We have also calculated plant biomass (B) by dividing dry weight by total leaf area from the moss gametophyte (TLA, mg cm^{-2}).

Water holding capacity measures were estimated using Pypker et al. (2006). Adult *P. juniperinum* gametophytes were saturated with water in a plastic sealed container for 12 hours, after which they were blotted dry, and maximum wet mass was measured using an analytical microbalance (AB104-S/FACT, manufactured by Mettler Toledo Corporation, Tualatin, Oregon). Shoots were air-dried and weighed again at 24 hours, and oven dried at 60°C for 48 hours and then weighed again for dry mass. We determined loss of water weight in gametophyte (LWC, mg) by using dry weight of each gametophyte and subtracting it from the final wet weight of the gametophyte.

We have calculated leaf moisture (LM, mg); in which we took the final dry weight of the gametophyte subtracted final wet weight and divided by wet weight (Xiao et al. 2015; Wang et al. 2006). To measure water content of the gametophyte (WC, mg), we have calculated $\text{WC} = (\text{fresh weight} - \text{dry weight}) / \text{fresh weight} * 100$. Values are represented in milligrams (mg cm^{-2}) and expressed as percentage dry mass (Michel et al. 2013). After all other measurements were completed, we weighed each gametophyte while wet and oven dried each gametophyte at 60°C for 48 hours for the purpose of dry

weight (DW, mg) and wet weight mg (WW, mg) using an analytical microbalance (AB104-S/FACT, manufactured by Mettler Toledo Corporation, Tualatin, Oregon).

In all, we measured seven leaf dimension traits. We placed the gametophyte intact and deconstructed between two transparency sheets and scanned at 1600 dpi to determine leaf area (LA, mm²) and total leaf area by multiplying leaf area with number of leaves (TLA). Once the leaves were removed from each gametophyte, we were able to take five leaves from each gametophyte, use a wet mount each leaf and measure costa length (CL, mm), costa width (CW, mm), costa cross sectional area (CCA, mm²) and lamina thickness, which was measured midway between costa and leaf margin (LT, μm) from all five replicates. We also calculated leaf density (LD, mg m⁻³), by taking the width of cross section of the leaf dividing it by area of the cross section of the leaf, and multiplying it by leaf mass area (LMA, mg cm⁻²) from our canopy trait measurements (Xiong et al. 2016).

To measure two leaf shape traits, we followed protocol from Waite et al. (2000); and calculated perimeter²/leaf area (P2/A, cm⁻³) and length/width of individual gametophytes (L/W, cm). We went a step further and calculated two other measures appropriate for leaf shape traits. To account for total perimeter, we first measured perimeter (P, cm) with the formula $P = 2(\text{width of gametophyte}) + 2(\text{height of gametophyte})$. We then took perimeter and divided it by area (P2/A, cm⁻³) from measuring the leaf surface area. To determine total perimeter/area (TP2/A), we took perimeter we have calculated earlier, and divided it by leaf surface area multiplied by total leaves on each gametophyte. This was calculated from the photograph of each leaf sample under a Leica microscope (Leitz DME, Leica, Wetzlar, Germany) with an

AxioCam 105 Color camera and Zen Blue software (version 1.1.2.0; Carl Zeiss Microscopy).

In addition, we measured ten cell-specific traits where we used enhanced and magnified images of the leaves and mid-lamina cross sections. The average area of an individual cell lumen, cell lumen area (CLA, μm^2) was measured. These were averaged from the five individual leaves from the same gametophyte. To measure, interior cell wall thickness (IWT, μm^2), we followed methods outlined in Waite paper, and measured the lamina cross section centrally between margin and costa (Waite et al. 2010).

Lastly, we supplemented further cell trait measures of our own, such as lamellae number (LN) by counting the number of lamellae cells at five locations within the widest part of the costa cross section from five individual leaves on the same gametophyte (n=25) (Figure 3.1). We also measured lamellae height (CLH, mm) by measuring the widest part of five lamellae stacks within same costa cross section from five individual leaves (n=25) (Figure 3.2). We measured the number of rows of lamellae (NRL) that each costa cross section contained from the five leaves and five costa cross sectional cuts, by measuring how far within each costa cross section the lamellae extends from one end to the other side of the lamina. We also took the number of rows of lamellae (NRL) and multiplied it by lamellae cells we counted (LN) to get an area estimate of lamellae space (LANR) within each leaf costa cross section. Lastly, we quantified how much area was taken up by chloroplast (CHA, μm^2) within lamellae cells from a perpendicular thin layer of leaf section. We used one or two layers of lamellae that was visible and individual cell

size could be measured (ICS, μm), as well as the space that surrounded or was next to the chloroplast (CS, μm) within each cell (Table 3.4).

Belowground morphological data collection from *P. juniperinum* gametophytes

We collected data on belowground rhizoidal function and stem structure to shed light on possible roles of these structures in non-vascular plants and the role they might play in isoprene emission. *P. juniperinum* has been found to have extensive penetrating underground network of rhizoids, which aids in resource acquisition and storage in harsh environments and allows recolonization after fires (Duncan et al. 1982; Viereck et al. 1983). *Polytrichum* mosses are able to inhibit large areas with the aid of a thick tomentum of rhizoidal network as far reaching as 1-2 meters in depth (Smith et al. 2008). We wanted to determine how the rhizoidal network assists in retaining water in *P. juniperinum*, as it is highly variable in some moss species. Some mosses have been reported to have water content potential of up to 20 times the dry weight (Clymo et al. 1982), or the water holding capacity of up to 1400% of their dry mass (Glime 2007; Proctor 2008; Michel et al. 2013).

For our belowground measurements of *P. juniperinum* gametophytes (n=45), we measured fifteen stem related measurements and water related traits. All water related trials were started using 72 hour air dried material that was then soaked until full saturation was achieved after about 24 hours. We measured rhizoidal length (RL, mm) of each gametophyte using a digital caliper (Fowler, Lux Scientific Instrument Corp., Tucson, AZ, USA). We obtained measurements of dry stem area (SAD, mm^2), and dry

diameter of the stem (SSD, mm^{-2}) by first capturing the image under a microscope slide with a Leica microscope (Leitz DME, Leica, Wetzlar, Germany) with an AxioCam 105 Color camera and then tracing the area of the stem or the diameter of costa cross section of the stem (Zen Blue software (version 1.1.2.0; Carl Zeiss Microscopy)).

We placed dry gametophytes in water for 12 hours (WSA12), and 24 hours (WSA24) to measure how much water stems alone absorb without any leaves on the gametophytes. We measured the diameter of the stem at same time intervals. This way we could measure water content in stem area at 12 (SAWC12) and 24 hours (SAWC24) and measured water content within stem diameter at 12 (SDWC12) and 24 hours (SDWC24) (Figure 3.3). We took the final value we obtained from our water measures, as follows; $((24 \text{ hour wet measures} - \text{initial dry measures})/\text{initial dry measures})$ and multiplied by a 100. We calculated total dry stem area (TDSA, mm^{-2}), by taking the dry stem area and multiplying it by the gametophyte length. We proceeded to do the same for wet stem area at 12 hours (TWSA12) and at 24 hours (TWSA24). We also estimated root shoot ratio (RSR, mm^{-2}) by taking the dry area of the root and dividing by dry area of the aboveground gametophyte length (Table 3.5).

Sex determination among mosses within the family of Polytrichaceae

Each gametophyte sexual expression was assessed using forceps and Leica microscope (Leitz DME, Leica, Wetzlar, Germany) with an AxioCam 105 Color camera and Zen Blue software (version 1.1.2.0; Carl Zeiss Microscopy). Species within the Polytrichaceae family were cataloged as either female if sporophyte development was

visible (Hughes 1962) or archegonia was found, in which case the number of archegonia present was documented. Males were categorized based on splash cup presence (Brodie 1951), and all splash cups were examined for viable antheridia containing male sperm. In these species, male splash cups serves as innate markers (Bu 2005); while for females reproduction in terms of fertilization and sporophyte production is fatalistic (Greene 1960; Sarafis 1971; Watson 1975; Callaghan et al. 1978).

Gametophytes were categorized as non-sexually expressing if only sterile paraphyses were found, and no sexual organs were present along the body of the gametophyte (Frederick et al. 1968). Paraphyses are multicellular hair-like structures that can conserve moisture, secrete mucilage and aid in sperm discharge (Goebel 1905). Paraphyses can regenerate into protonema and rhizoids (Brown 1919); hence, they serve an important function in moss life strategy. Sexual expression was assessed for the current year and recorded if previous year sexual expression was visible. Identification and key to each species was accomplished following McCune et al. (2018), with herbarium samples deposited at the Portland State University herbarium.

Isoprene emission among mosses within the family of Polytrichaceae

Polytrichaceae gametophytes were sampled in a randomized fashion on a GC/RGD2 Gas Chromatograph with a RGD2 reduction gas detector (RGD-2, Trace Analytical Instruments, Menlo Park, CA) to avoid species bias. An individual gametophyte was separated from the clump and analyzed for isoprene emission. After moss collection from the field, identification and debris removal, we placed each

gametophytes into a 40 ml glass vial. We proceeded to collect isoprene by closing the vials and placing them under basal conditions of $1,000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ using Dracast LED1000 Pro Daylight light panel, with light-emitting diode array (Photon Systems Instruments) and using a water bath at (30°C). This gives a better spatially realistic profile of emission for sampling volatiles in an ecological context (Geron et al. 2000).

The isoprene collection lasted for a five-minute period, after which a 1 ml Pressure-Lok® Precision Analytical Syringe (VICI Precision Sampling Inc., Baton Rouge, LA, USA) was used to pull the headspace gas from the vial and inject it into a reduction gas detector (GC/RGD2). One ml of the calibration gas mixture was removed with a syringe from a mixing chamber and injected into the GC-RGD2 to create the calibration curve and obtain slope of the equation see (Equation 1) as described in the previous chapter. Isoprene was separated on a stainless steel column (1.3 m long x 2 mm inner diameter i.d.). The column was packed with UNI 8 Beads 3S 60/80 6' x 1/8", 0.085 SS (Alicat Scientific, Inc; Tucson, AZ). The GC-RGD2 was calibrated each day using a standard cylinder containing 71 ppb (v/v) isoprene, references to a National Institute of Standard and Technology (NIST) and helium (He) standard (SRM 1660a; 1 ppm He in N₂, Rochester, NY).

Isoprene gas was mixed with high purity helium using a mass flow controller calibrated to a flow rate of 21 ml/min (Alicat Scientific, Inc; Tucson, AZ). Blanks were run every five samples to ensure quality check and to detect contamination within the column. Isoprene emission measurement was calculated as follows, using Peak Area

Units (PAU) from the standard calibration curve $y= mx+b$, at GC temperature of 298 Kelvin (K), and Atmospheric Pressure (P) of 760 Torr.

$$= \left(\frac{\text{PAU}+20.69}{2.4703} \right) \times \left(\frac{1\text{nmol/L}}{\text{ppb}} \right) \times (.0005) \times \left(\frac{\text{std nl}}{\text{nl}} \right) \times \left(\frac{760 \text{ Torr}}{298\text{K}} \right) \times \left(\frac{378\text{K}}{771.8\text{Torr}} \right) \\ \times \left(\frac{1\text{nmol}}{22.4 \text{ std nl}} \right) \times \left(\frac{\text{volume vial (ml)}}{.5\text{ml}} \right) \times 1 \text{ (factor for loop recovery)} \\ \times \left(\frac{1}{\text{area/m}^2} \right) = (\text{nmol m}^2 \text{ s}^{-1})$$

Equation 1. Formula used to convert isoprene from calibration curve, $y=mx+b$, and PAU to isoprene ($\text{nmol m}^{-2} \text{ s}^{-1}$).

We obtained the volume-mixing ratio of isoprene (ppb) to determine gametophyte emission flux. We determined the area of the gametophyte using AxioCam 105 Color camera, and Zen Blue software, version 1.1.2.0 (Carl Zeiss Microscopy).

Peak identification was performed with the PEAK software (Stein 1999) that integrates raw peak area units and retention times. To identify isoprene, a custom calibration curve was created from isoprene standard (Sigma, Deisenhofen, Germany). For quantification, peak areas of isoprene were determined after baseline correction. Levels of background contamination were determined from blanks. Analytical quality was analyzed by using Standard Reference Material of isoprene (NIST SRM 1515, National Institute of Standards and Technology, Gaithersburg, MD). The standard material was used in the analyses to create calibration curves for quality assurance and quality control purposes.

Both Experiment 1 and Experiment 3 isoprene emission testing involved a snapshot view of emission for non-sexually expressing *P. juniperinum* gametophytes

(n=45), and sexually expressing *A. undulatum*, *P. juniperinum*, and *P. urnigerum* gametophytes (n=167) (Figure 3.4).

Experiment 2 involved repeated measure isoprene emission testing of *P. juniperinum* sexually expressing gametophytes from different locations and under non-nutritive agar and nutritive BCD media (n=25). Isoprene emission was checked three times a month, beginning May of 2016, through August of 2016 totaling eighteen isoprene observations. *P. juniperinum* was kept in PlantCon™ containers under the light source of standardized growth conditions of (150–200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) light intensity and a photoperiod of 16 h light to 8 h dark at $20 \pm 3^\circ\text{C}$ using Adaptis1000 Conviron growth chambers (Pembina, ND). The PlantCon™ containers were allowed to sit in the growth chambers until isoprene emission testing occurred under basal conditions of 30°C and $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$. Each sample was opened daily, and after isoprene emission testing was completed, the individual gametophyte was sprayed with 5 ml tap water to ensure proper hydration. Visual inspection followed, where bacterial growth, brown tissue was recorded and noted if the sample has completely turned brown and showed no visible sign of green leaf tissue. We did have high mortality and out of 70 samples, 25 samples remained to test for continuous isoprene emission. This allowed us to gain basal isoprene emission (Monson et al. 1994) from non-sexually expressing gametophytes, and sexually expressing male gametophytes and female gametophytes from multiple sites.

Laboratory gas exchange measurements of *P. juniperinum* gametophytes

To assess and compare differences between physiological measurements we have subjected gametophytes to the same protocol as was outlined in the previous chapter for gas exchange measurements (see Chapter 2 methods). We have conducted three separate laboratory gas exchange measurements. After isoprene emission testing, gametophyte samples were placed in a glass cuvette containing a moist cotton ball to provide adequate moisture through the trial. All gas exchange measurements in the lab were conducted using protocol outlined by Junior Pulse-amplitude modulated (JUNIOR-PAM) chlorophyll fluorometer and WinControl Software (Walz, Effeltrich, Germany), with a single 100 cm plastic fiber, 1.5 mm in diameter. We measured chlorophyll fluorescence ratio (CFR), as a non-intrusive tool in using it as a proxy to establish information on photosynthetic reactions in the chloroplast (Schreiber et al. 1987). The fluorescence-emission spectra read out two maxima in the 690 nm and the 735 nm region. The fluorescence ratio F_{690}/F_{735} for green leaves is higher (values of 0.8 to 1.1), than in the yellow to red wavelength region (525 to 633 nm), which only yields values for F_{690}/F_{735} of 0.5 to 0.7 (Rinderle et al. 1988). Chlorophyll fluorescence ratio (CFR) was measured with a chlorophyll content meter (OPTI-Sciences model CCM-300, Hudson, USA).

The Junior-PAM (Walz, Germany, <http://www.walz.com/>) was used according to the manufacturer's instructions with the following parameters: light saturation pulse intensity 6, pulse frequency 20 sec, actinic light intensity 8. The effective photochemical quantum yield of photosystem II [$Y(II)$] was calculated as described by Genty *et al.* (1989). Relative Electron Transport Rate (RETR) was calculated with JUNIOR-PAM

chlorophyll fluorometer and WinControl software. Modulated excitation energy and the fluorescence signal were transmitted through a bifurcated fiber-optic cable.

Measurements with the Junior-PAM were conducted under constant fluorescent lighting in the laboratory at mean Photosynthetically Active Radiation (PAR) value of $250 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$. Relative electron transport rate (RETR) can be obtained as $\text{PFD} \times (\Phi)_{\text{PSII}}$ (Bilger et al. 1995). Junior-PAM recorded electron transport rate, photosynthetic yield and PAR light levels ranging from 0-1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Statistical analyses

Graphical and statistical analyses of the data were performed using Prism 7.0 software (GraphPad), R version 3.4.2 (R Studio 2017) and JMP Pro 14.1 (SAS Institute, Cary, North Carolina, USA); with significance determined at $\alpha = 0.05$. We were interested in which independent variables influenced isoprene emission and used Tukey-Kramer HSD Multiple Comparison test (Ellison et al. 2004) to find sources of significant differences between Polytrichaceae species and within sexual expression and non-sexual expression. Violations of normality were diagnosed using distribution plots and normal quantile plots and assessed using Shapiro-Wilk test for normality. Violations of homoscedasticity were assessed using residuals versus predicted values. Violations of independence were assessed using a table or plot of residual autocorrelations. Violations of linearity or additivity were assessed using residuals versus individual independent variables using Tukey 1-df Tests for Nonadditivity (Hair et al. 2009). Violations of unequal variances were estimated using Welch Tests (Rusticus et al. 2014). Violation of

multicollinearity was assessed using nonparametric correlation matrix to determine if any of the variables are highly correlated. As well as we have determined variance inflation factor (VIF), and found low VIFS indicating that our variables did not have multicollinearity (Craney et al. 2002).

For our morphological data for aboveground and belowground, we log transformed our dependent variable, which was isoprene emission because it did not fit a normal distribution and we observed heteroscedasticity after testing for unequal variances with Levene's test using the statistical software JMP Pro 14.1 (SAS Institute, Cary, North Carolina, USA). We wanted to answer if isoprene emission, our dependent variable had significant effects on our independent variables of aboveground and belowground measurements, and what interactions were present among them. To determine the effects of isoprene emission on our aboveground variables, we analyzed the data using the generalized linear models (GLM) procedure in JMP Pro 14.1 (SAS Institute, Cary, North Carolina, USA). To determine the effects of isoprene emission on our belowground variables, we used multiple regression procedure in JMP Pro 14.1 (SAS Institute, Cary, North Carolina, USA). We choose AIC (Akaike's Information Criteria) to evaluate the contribution of subsets of explanatory variables to our response variable. The GLM model assessed variance and bias by controlling for factors causing variability in multiple independent variables (Armstrong 1985). This way we acquired greater statistical power by controlling for differences and correlations between and within groups (Field et al. 2012). We used a multiple regression to determine the influence of our belowground variables on isoprene emissions.

For our species and sex data set within *P. juniperinum*, we ran repeated measure ANOVA (RM ANOVA) to test the effects of sex, media, site, and time on isoprene emission. Isoprene emission was log transformed to meet assumption for analyses. We also used this method to compare data points over time between groups, such as isoprene emission in May 2016, June 2016, July 2016 and August 2016 between and within groups. For data looking at the factors affecting isoprene emission across categorical variables, including leaf number, shoot number, and species, we used ANOVA. Additionally, we used regression analysis to determine how isoprene emission correlated with gas exchange measures.

Results

Experiment 1a:

The effects of aboveground morphological variables on isoprene emission in *P. juniperinum* gametophytes

We found that six variables explain 50% of variability in isoprene emission (GLM; DF=6; $\chi^2=27.325$; $P<0.0001$; Figure 3.5). Isoprene emission among *P. juniperinum* gametophytes was highly variable, with a mean isoprene emission of $16.917 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 1.4183 \text{ SEM}$ (n=45). We found minimum isoprene emission value of $2.917 \text{ nmol m}^{-2} \text{ s}^{-1}$ and a maximum value of $53.460 \text{ nmol m}^{-2} \text{ s}^{-1}$. Four aboveground variables explained 39% of the variability in isoprene emission, and two belowground variables explained 11% of the variability in isoprene emission. The aboveground canopy traits

included: total number of leaves (TL) on a gametophyte, gametophyte width (GW), wet weight (WW), and leaf tip redness (LTR).

Total leaves (TL) mean values were 55.98 ± 2.944 SEM (n=45), with minimum leaves counted as 11, and maximum leaves counted as 103 per gametophyte. We did not see an increase in isoprene emission with increase number of leaves (P=0.06).

Gametophyte width (GW) mean ranges were $10.98 \text{ mm} \pm 0.2494$ SEM (n=45), with minimum gametophyte width of 4.18 mm and maximum value of 13.79 mm. Gametophyte width was shown to be statistically significant when it came to isoprene emission, as it contributed to 19% variability in isoprene emission between gametophytes (ANOVA; DF=1; F=10.4395; P=0.0024). As gametophyte width increased, isoprene emission increased.

We also found that gametophyte leaf length (LL) scaled geometrically with leaf area (LA) resulting in 27% variability due to gametophyte leaf length (LL) among samples (ANOVA; DF=1; F=15.8299; P<0.0003). Unlike Waite and Sack et al. (2010), who found that gametophyte leaf width (LW) scaled geometrically with leaf area (LA), we found that for *P. juniperinum* gametophyte leaf width (LW) did not scale with leaf area (LA) (P=0.081).

Wet weight (WW) mean values were $62.29 \text{ mg} \pm 4.932$ SEM (n=45), with minimum wet weight of 19.6 mg and maximum of 169 mg. As wet weight (WW) increased isoprene emission decreased. Lastly, leaf tip redness (LTR) mean values were $1.004 \text{ mm} \pm 0.03291$ SEM (n=45), with a minimum value of 0.582 mm and a maximum value of 1.58 mm. The more leaf tip redness was present (LTR) the more isoprene

emission decreased. We found no statistical effects for leaf dimension, leaf shape or cell traits on isoprene emission.

Experiment 1b:

The effects of belowground morphological variables on isoprene emission in *P. juniperinum* gametophytes

Two belowground traits contributed to 11% variability in isoprene emission in our final model; stem area dry (SAD), and wet stem area at 24 hours (WSA24). When we logged isoprene emission against stem area dry, we saw that the relationship was statistically significant and as dry stem area increased isoprene emission followed (Regression; $r^2=0.11$; $P=0.0293$; Figure 3.6). Our regression line equation with logged isoprene emission and stem area dry (SAD) was $Y=5.851185*x + 2.2250985$. For our belowground morphological measures, stem area dry (SAD) mean values were $0.0782 \text{ mm}^2 \pm 0.00465 \text{ SEM}$ ($n=45$). For wet stem area at 24 hours (WSA24) mean values were $0.1609 \text{ mm}^2 \pm 0.00626 \text{ SEM}$ ($n=45$).

We also significant correlation between shoot to root ratio (RSR) and logged isoprene emission (Regression; $r^2=0.09$; $F=4.2174$; $P=0.0461$). We were able to establish that as dry stem area (SAD) increased isoprene emission increased. As wet stem area (WSA24) increased isoprene emission decreased. We also saw a strong positive linear correlation between dry stem area (SAD) and wet stem area (WSA24).

Experiment 2:

***P. juniperinum* isoprene emission, sex and morphological variables**

Sex had a significant effect on isoprene emission (RM ANOVA; DF=1,9; F=7.11; P=0.026; Figure 3.7), with males producing higher isoprene emission levels than females. These plants had been previously sexed, but they were not expressing sex when isoprene emission was measured. Female mean isoprene emission value was $8.032 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.642 \text{ SEM}$ (n=27), male mean values were $8.946 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.662 \text{ SEM}$ (n=24). Media did not have a significant effect on isoprene emission (F=0.90; P=0.37), and site also did not have a significant effect on isoprene emission (F=1.42; P=0.30).

The number of leaves on sexually expressing gametophytes was statistically significant and explained 20% variability in isoprene emission (ANOVA; DF=2; F=4.675; P=0.0098). We have found that for females the number of shoots and gametophyte width explained 45% variability in isoprene emission (ANOVA; DF=7; F=5.485; P<0.0001). For males, we found that 49% of variability in isoprene emission could be explained by number of shoots and gametophyte width (ANOVA; DF=12; F=2.790; P=0.0089).

We saw several morphological variables that were influenced by sex of the gametophyte, and invariably affected our isoprene emission measurements. Gametophyte width had statistical significance when it came to the three sexes in our continuous isoprene emission and explained 16% variability between the sexes in gametophyte width (ANOVA; DF=2; F=4.5190; P=0.0160).

Experiment 3:

***A. undulatum*, *P. juniperinum* and *P. urnigerum* isoprene emission, sexual expression and site-specific differences**

Isoprene emission among Polytrichaceae species was statistically significant explaining 42% variability in isoprene emission between species (ANOVA; DF=2; F=59.010; P<0.0001; Figure 3.8). The highest isoprene emission was seen from cosmopolitan *P. juniperinum*, where we saw our maximum value at 59 nmol m⁻² s⁻¹, the highest recorded value for this moss species. *P. juniperinum* mean isoprene emission values were 16.3885 nmol m⁻² s⁻¹ ± 1.902 SEM (n=56). Montane *P. urnigerum* mean isoprene emission were 4.496 nmol m⁻² s⁻¹ ± 0.5981 SEM (n=41). Lastly, our shade adapted *A. undulatum* mean isoprene emission value were 2.4183 nmol m⁻² s⁻¹ ± 0.196 SEM (n=70).

In all three species sexual expression was statistically significant, from our data we can explain 48% variability in isoprene emission by sexual expression nested within species (ANOVA; DF=6; F=24.25; P<0.0001; Figure 3.9). The highest isoprene emission was seen from *P. juniperinum* non-sexually expressing gametophytes. In all three species of Polytrichaceae, species that did not have sexual expression were found to have higher isoprene emission than those that were sexually expressing. We found that sexual expression nested within species contributed 50% variability in isoprene emission between species and sex (ANOVA; DF=2; F=7.334; P=0.0009; Figure 3.10).

We also saw site-specific variation in all three species that explained high variation in isoprene emission. In *A. undulatum* site-specific differences can be attributed

to 18% of variability in isoprene emission (ANOVA; DF=3; F=4.97; P=0.0036; Figure 3.11). In *P. juniperinum*, 27% of variability in isoprene emission can be attributed to site-specific differences (ANOVA; DF=6; F=3.0792; P=0.0123; Figure 3.12). Lastly, in *P. urnigerum*, 30% of variability in isoprene emission can be attributed to site-specific differences (ANOVA; DF=5; F=3.082; P=0.0208; Figure 3.13).

Laboratory gas exchange measurements

Experiment 1

We found isoprene emission positively correlated with CFR across species (Regression, $r^2=0.85$; $P<0.0001$; Figure 3.14). Our regression line equation with CFR was $Y= 36.22*x-16.12$. Mean CFR values were 0.9264 ± 0.011 SEM (n=45), with minimum value of 0.698 and maximum value of 1.074.

We found statistical differences between the forty-five non-sexually expressing gametophytes we tested for RETR in low photosynthetic photon flux density (PPFD) $\mu\text{mol m}^{-2} \text{s}^{-1}$ and high photosynthetic photon flux density (PPFD) $\mu\text{mol m}^{-2} \text{s}^{-1}$ (ANOVA; DF=1; F=18.343; $P<0.0001$). Our low PPFD $\mu\text{mol m}^{-2} \text{s}^{-1}$ setting values between the 45 non-sexually expressing gametophytes explained 16% variation between samples, and under high PPFD setting we saw 24% variation in RETR values.

Experiment 2

We found a significant positive correlation between CFR and isoprene emission (Regression; $r^2=0.07$; $P<0.0001$; Figure 3.15). Our regression line equation for isoprene

emission vs. CFR measures were $Y=2.820*x + 0.1808$. Mean CFR values were 0.703 ± 0.0094 SEM (n=25). Also, we saw sex-specific differences in CFR values, with the highest CFR values in males (ANOVA; DF=2; F=5.446; P=0.0046; Figure 3.16). Under female sexual expression, mean CFR value were 0.678 ± 0.0105 SEM (n=15). Under male sexual expression, mean CFR value were 0.723 ± 0.008 SEM (n=12). Lastly, under non-sexually expressing gametophytes, mean CFR value were 0.693 ± 0.010 SEM (n=12).

The sexes were significantly different in (RETR); however, these effects depended on light conditions and the type of media the plants were growing on. Under low PPFD $\mu\text{mol m}^{-2} \text{s}^{-1}$ we found significant differences in RETR curves between sexes for plants growing on BCD media. Our regression line equation for males on BCD media was $Y=0.0447*x + 4.912$ (Figure 3.17). Male RETR values were higher than female or non-sexually expressing gametophyte values. At neither low nor high PPFD $\mu\text{mol m}^{-2} \text{s}^{-1}$ we found no statistical differences in RETR curves between sexes for plants growing on agar media (P=0.51; P=0.40). Only under high PPFD $\mu\text{mol m}^{-2} \text{s}^{-1}$ growing on BCD media did we find significant differences in RETR curves between sexes (ANOVA; DF=1; F=9.0432 P<0.0001).

Experiment 3

Isoprene emission had a positive correlation with CFR, and was statistically significant in *A. undulatum* (Regression; $r^2=0.41$; P=0.0123; Figure 3.18). Our regression

line equation with CFR was $Y = 9.9136x - 17.1921$. We found no statistical significance for isoprene emission vs. CFR values in *P. juniperinum* ($P = 0.445$).

CFR values explained 24% of variability among species in the Polytrichaceae family (ANOVA; $DF = 1$; $F = 8.4475$; $P < 0.0074$; Figure 3.19). *P. juniperinum* had higher CFR values than *A. undulatum*. Mean CFR value for *A. undulatum* was 0.73 ± 0.015 SEM ($n = 15$). Mean CFR value for *P. juniperinum* was 0.79 ± 0.026 SEM ($n = 13$).

Species differences among RETR values were high, explaining 45% of variability in RETR values among species (ANOVA; $DF = 1$; $F = 21.2391$; $P < 0.0001$; Figure 3.20). Cosmopolitan *P. juniperinum* had five fold higher values in RETR measures than did shade adapted *A. undulatum*.

We accounted for nested sex within species, and used RETR as our response variable and found statistical significance between sexes (ANOVA; $DF = 1$; $F = 4.4601$; $P = 0.0357$; Figure 3.21). *A. undulatum* under PPFD $\mu\text{mol m}^{-2} \text{s}^{-1}$ mean values were $18.6 \mu\text{mol m}^{-2} \text{s}^{-1} \pm 0.72$ SEM ($n = 15$). *P. juniperinum* under low PPFD $\mu\text{mol m}^{-2} \text{s}^{-1}$ mean values were $29.2 \mu\text{mol m}^{-2} \text{s}^{-1} \pm 1.32$ SEM ($n = 13$). We could not analyze isoprene emission versus RETR, as each sample contained eight RETR measures, and we have sampled one isoprene emission value at basal isoprene emission ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 30°C).

Discussion

Aboveground morphological variables and isoprene emission

A single moss gametophyte is made up of many leaves and each leaf contributes to isoprene emission (Wildermuth et al. 1996; Velikova et al. 2011, 2012; Schnitzler et al. 2005). Even within the same population of *P. juniperinum*, we saw a high degree of variation of isoprene emission all within the range of what has already been previously reported (Hanson et al. 1999) and even exceeding previously reported values. We found that the four most significant variables, canopy traits, contributed 39% of variation in isoprene emission. We saw high variation in isoprene emission from the forty-five *P. juniperinum* gametophytes of a single population. Within the canopy, multivariate traits were found to have a positive correlation between total leaves (TL), dry weight (DW), and canopy height (CH), and negative statistical significance was found between leaf tip redness (LTR) and total leaves (TL) (Figure 3.5). Leaf number contributed to 11% variation in isoprene emission among gametophytes and gametophyte width contributed to 19% variation in isoprene emission revealing that a larger area will contribute to more isoprene emission and chloroplast area.

Like Waite and Sack (2010), we have found high correlation between leaf length (LL) and gametophyte length/gametophyte width (GL/GW) which support taller gametophyte structure. High allocation to structural support with longer gametophytes need more support for more leaves. We found gametophyte leaf length (GL) scaled geometrically with leaf area (LA), as it has been shown in mosses by Waite (2010). We also show that non-sexually expressing gametophytes had significantly higher dry weight compared with males and females. This suggests that nutrient requirement and allocation

pattern of resources differ in investment strategies between gametophytes that are sexually reproducing versus gametophytes that are non-sexual.

Belowground morphological variables and isoprene emission

Many internal factors (genetics and biochemical) and external factors (abiotic and biotic influences) control BVOC emissions (Lehning et al. 1999; Peñuelas et al. 2001, Geron et al. 2001; Sasaki et al. 2005; Sharkey et al. 2007; Monson et al. 2012; Sharkey et al. 2017). We were able to show a high rate of variation in isoprene emission out of which two belowground morphological variables were extracted for use in our final emissions model. These were stem area dry (SAD) and wet stem area at 24 hours (WSA24). These together contributed to 11% variation in isoprene emission in our model (Figure 3.6).

Isoprene synthesis requires energy and photosynthetic carbon (Grinspoon et al. 1991; Rasulov et al. 2009; Melis et al. 2013; Unger et al. 2013; Jiang et al. 2013). When conditions limit photosynthesis, alternate carbon sources have been found to be used in vascular plants. Besides recently photosynthetically fixed CO₂, alternate carbon sources include xylem transported carbohydrates, and leaf internal carbon pools which can be used for isoprene production (Schnitzler et al. 2004). Given vascular plants can utilize alternate source of carbon for isoprene production, such as starch from xylem transported carbon, we hypothesize that our model moss *P. juniperinum* might also use alternate carbon sources, as we know that starch is stored in its underground rhizoids and these acts as sites for overwinter storage of carbohydrates (Skre et al. 1983).

Species and isoprene emission

In this study, we tested isoprene emission from three species of the Polytrichaceae family, which we collected from a range of altitude from 4 meters to 88 meters above sea level and from a variety of substrates (Table 3.3). The phenological stage of the mosses at the time of collection could have played a part in the high degree of variation we saw in isoprene emission rates between and within species. We saw low isoprene emission values for our deep shade adapted *A. undulatum* which is always found in forest understory and does not live in direct sunlight. While, our sun loving *P. juniperinum* was able to emit high amounts of isoprene which can be comparable to levels found in isoprene emitting vascular plants. This is not entirely surprising as this moss has the ability to survive in insolation values exceeding equatorial noon sunlight (Marschall et al. 2004; Proctor et al. 2005), and it is accustomed to high temperature regimes (Monson et al. 1994; Geron et al. 2001).

In vascular plants, it has been noted that isoprene emission rates vary greatly among species and isoprene emission ability may or may not be shared by member of the same family or genus with no clear evolutionary link between emitters (Harley et al. 1999; Sharkey et al. 2001, 2007; Dani et al. 2014; Loreto et al. 2015). Given basal emission rates of the three species tested, we saw varying isoprene emission rates due to environmental and genetic factors. Further work is required to evaluate independently how these factors affect the instantaneous isoprene emission rate against basal isoprene emission rate.

Sex and isoprene emission

Over 60% of moss species have separate sexes (Wyatt et al. 1984; Hedenäs et al. 2011), while sexual differentiation only occurs in 5% of seed plants (Richards 1997). Sexual reproduction in mosses is an energy and resource intensive process (Glime 1984). Sex is costly and is subject to resource limitations in the Polytrichaceae family (Koncz et al. 2012). The “cost of sex hypothesis” predicts that the most expensive sex should be the rarest sex (Stark et al. 2000) and suggests that the costly sex would have few resources to allocate elsewhere, including isoprene production. In *P. juniperinum*, we found that male and female plants, that are not currently expressing sexual organs, differ in isoprene emission, with males emitting more isoprene than female counterparts (Figure 3.7). However, in our experiment using all three species, in which the males and females were expressing sex organs, males and females did not differ in isoprene emission (Figure 3.9, 3.10). Our results indicate that there are sex-specific differences in isoprene emission between non-sexually expressing male and female moss plants, but these may lessen when plants are paying the cost of producing sexual organs. Further study is required, as sexual organ formation takes up considerable resources and space in moss gametophytes. The difference we saw in male and female isoprene emission could be due to a number of both environmental and genetic factors.

Also, in our study, we found that non-sexually expressing gametophytes of all three species had significantly higher isoprene emission rates compared to sexually expressing moss, suggesting resource limitation does exist in mosses. Previously, reproductive effort has been investigated in mosses by assessing sporophyte abortion

rates, morphological differences between the sexes in terms of size, growth, branching and number of sexual organs in each sex (Glime 1984; Bowker et al. 2000). We are the first to report that sexual expression affects isoprene emission in moss, and presents a reproductive cost as sexually expressing females and males had much lower isoprene emission rates than non-sexually expressing gametophytes. Isoprene emission in each sex is a resource that is limited, perhaps affects reproductive performance, and contributes to reproductive cost in these species. To our knowledge, this study is the first to examine potential trade-offs in resource allocation among female, male and non-sexually expressing gametophytes. Future investigation should differentiate between trade-offs associated with isoprene emission in sexually expressing and non-sexually expressing gametophytes and consider the cost and benefit associated with these emissions in conjunction with biomass and nutrient content. We propose that further study of these isoprene-emitting mosses should help resolve some fundamental unanswered questions in resource allocation between species and between sexes, and even fundamental questions such as land plant evolution of the sexes. Much more work is needed to understand the evolutionary pressures and environmental factors that influence isoprene emission from mosses and all major group of land plants.

Changes in resource allocation associated with isoprene production might play a role in directing and constraining sexual reproduction in mosses under stress (Eppley et al. 2011, Huttunen 2003; Kis-Papo et al. 2003; Stark et al. 2005). Changing stressors associated with global warming, particularly in high latitude environments may exacerbate these effects. The factors influencing maintenance of sex are of great interest

in evolutionary biology (Vamosi et al. 2003). While sex-specific BVOC emissions are poorly characterized in mosses, we do know they are sex-specific and mediate microarthropod fertilization in mosses (Rosenstiel et al. 2012). Sexual expression explained 38% variability in isoprene emission.

In many bryophytes, a large part of the population is made up of sterile plants, with males and females absent (Stark 2002; Rydgren et al. 2002a,b) and many interdependent factors affect reproductive success (Longton et al. 1983, 1990; Mishler et al. 1988; Shaw 1991a,b; Bisang et al. 2004; McLetchie 2001; Silva et al. 2012). We saw the highest isoprene emission from all three species in non-sexually expressing gametophytes (Figure 3.9). In turn, *A. undulatum* explained 14% variability in isoprene emission within its sexes while *P. urnigerum* explained 18% variability in isoprene emission in sexes. In *P. juniperinum*, we saw the least amounts of variability, accounting for only 11% variation within sexes. We had a total of 65 gametophytes that did not express sex, and 35 gametophytes that expressed sexual female and male characteristic, with males emitting higher isoprene values than females (Figure 3.10). Large moss populations contain non-sexual gametophytes, and the key to understanding isoprene fluxes from moss populations will be to understand the control on the development of sex organs in mosses (Zaugol'nova 1971; Watson 1974; Callaghan et al. 1978; Glime 2017). Life history for male and female gametophytes must be assessed independently, as the sexes are dimorphic in size, maturation rates, and reproductive output (Shaw et al. 1999).

Laboratory gas exchange measurements

Most mosses have saturating light curve levels that are close to $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ Photosynthetic Photon Flux Density (PPFD), while species in the Polytrichaceae family attain the highest light saturating curves. For *A. undulatum* light saturation have been found to be $\sim 648 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD (Marschall et al. 2004). This low light level saturation helps explain the low isoprene emission we saw from this species typically found in deep shade near water sources. *P. urnigerum* light saturation have been found to be $\sim 957 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD (Marschall et al. 2004). This is a high elevation montane species that is often exposed to more sunlight than shade adapted *A. undulatum* and has a thicker leaf composition than the one-cell layer leaves of *A. undulatum*. Lastly, the cosmopolitan *P. juniperinum*, the highest isoprene emitter we have documented, has been shown to have light saturation curves comparable to vascular ‘sun plants’ reaching values as high as $2175 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD (Proctor et al. 2002; Marschall et al. 2004; Proctor 2005). We saw this species emit the highest amount of isoprene, but further study is needed to look at how habitat and environment influences isoprene emission. In general, we saw high within species variation in isoprene emission as well, which was potentially due to the fact that we had a wide range of environmental variation including altitude and substrate differences.

We measured the basal isoprene emission under controlled condition as a function of temperature of 30°C and as a function of light at $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD. This measure depends on substrate availability and photosynthetic machinery from the plant; this measure is often taken as the inherent capacity of isoprene emission (Monson et al. 1994; Sharkey et al. 1999; Geron et al. 2000). A second level of isoprene emission rate

called the “instantaneous emission rate’ needs further investigation in non-vascular plants. This measure is set against environmental condition on basal emissions (Monson et al. 1994). Future work should focus on understanding how environmental drivers affect isoprene emission in mosses.

Isoprene production rate and chlorophyll content in marine phytoplankton shows a “simple and robust” relationship (Shaw et al. 2003; Palmer et al. 2005), with peak isoprene emission correlating strongly to chlorophyll fluorescence (Bonsang et al. 1992, 2010; Baker et al. 2000). We used chlorophyll fluorescence ratio (CFR) as a non-intrusive tool as a proxy to establish information on photosynthetic reactions in the chloroplast and stress level in plants (Schreiber et al. 1987). We saw chlorophyll fluorescence ratio highly correlating to isoprene emission. We found a positive correlation between CFR and isoprene emission (Figure 3.14; Figure 3.15). We also saw sex-specific CFR value differences between species, owing to cost of sexual reproduction, with males having higher CFR values than females or non-sexually expressing gametophytes (Figure 3.16). High light dwelling *P. juniperinum* had higher CFR values than shade adapted *A. undulatum* with species difference explaining 24% variability in CFR measures (Figure 3.19).

Since the light dependent isoprene emission is related to electron transport rate in vascular plants (Dani et al. 2014; Niinemets et al. 1999, 2015), we looked at electron transport rates within our samples to determine if we can establish a relationship between the two. RETR was measured independently using fluorescence and was not coupled

with LiCor measurements and should only be taken as indicative and not as absolute measures (Dani et al. 2014).

We have observed species and sex-specific RETR curves (Figure 3.17). We analyzed if we could find species differences in RETR values, and showed that sun tolerant *P. juniperinum* had five fold higher RETR values than shade adapted *A. undulatum* (Figure 3.20). Lastly, we showed sexual differences in RETR between the species (Figure 3.21), with non-sexual gametophyte having higher values of RETR than sexually expressing gametophytes.

Mosses vary widely in their photosynthetic light response, but *Polytrichum* species were shown to have the highest light saturating curves of 39 species of mosses tested (Marschall et al. 2004). This moss species is unique given their lamellae that forms a ventilated photosynthetic tissue (Marschall et al. 2004). The photosynthetic lamellae aids in lowering leaf resistance to CO₂ uptake, allowing the utilization of direct sunlight (Proctor 2005). With our RETR curves, we did not reach full saturation, as some *Polytrichaceae* species reach saturation at 2549 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Marschall et al. 2004). Our highest value was measured consistently at 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and in further experiments; a higher saturation PPFD would allow us to measure RETR saturation curves for this species in conjunction with isoprene emission. We aim to create electron transport rate curves by taking isoprene emission values at each level of a full light saturation curve. This will allow us to confer what has been found with vascular plants and determine if electron transport rate is positively correlated with isoprene emission in mosses as it is in vascular plants (Niinemets et al. 1999).

Under drought conditions, it has been shown that while electron transport values are still favorable, isoprene emission can decrease which highly suggest carbon limitation (Dreyer et al. 2001; Dani et al. 2014; Darbah et al. 2008). It is hypothesized that leaf temperature determines the amount of isoprene in the membrane, when the temperature goes up isoprene emission ameliorate the stresses on the membrane, but once cooling it can dissipate as a safety valve (Rosenstiel et al. 2004; Singsaas et al. 1998).

Lower isoprene emission was shown to exist with young leaves compared to mature leaves. Isoprene emission from young leaves was twice what was observed in older leaves. The saturating light for isoprene emission could positively correlate with photosynthetic electron transport rate in mosses as it has been observed in vascular plants (Niinemets et al. 1999; Rasulov et al. 2009; Morfopoulos et al. 2014). We can speculate that, as with vascular plants, we would see electron transport rate scale with isoprene emission. Further research and methodological development is necessary. Our experimental setup and data collection would have had to take an isoprene emission sample at each light interval to determine if this holds true. From preliminary data collection we were able to confirm that this is the case at least in what we were able to observe. RETR values we observed are less than half the values we have seen in our previous experiments, possibly due to the fact that the gametophytes were planted in March and checked for RETR measures at the end of the experiment as they were embedded in media by this time, with many shoots sprouting out. We believe that these values reflect a low photosynthetic rate given exposure to low light levels throughout the trial.

Tables and Figures

Table 3.1 Summary table of *P. juniperinum* populations used for non-sexually expressing isoprene emission (n=45).

Altitude (meters)	Latitude	Longitude	Substrate
241	45°47'17"N	122°56'16.00"W	Clay soil, hillside road cut in forest

Table 3.2 Summary table of *P. juniperinum* populations used for sexually expressing isoprene emission (n=25).

Site	Altitude (meters)	Latitude	Longitude	Substrate
1	15	45°41'38.34"N	122°52'14.23"W	Gravelly soil, on hillside near highway
2	21	45°38'7.45"N	122°49'45.09"W	Gravelly soil, steep hillside next to road
3	145	45°41'50.54"N	122°52'40.83"W	Gravelly soil, steep hillside next to road
4	315	45°46' 58.78" N	123°22'14.14" W	Clay soil, hillside road cut
5	412	45°44'6.80"N	122°59'30.62"W	Clay soil, hillside road cut in forest
6	241	45°47'17"N	122°56'16.00"W	Clay soil, hillside road cut in forest

Table 3.3 Summary table of *Atrichum undulatum*, *Polytrichum juniperinum* and *Pogonatum urnigerum* populations used for sexually expressing isoprene emission (n=167). Abbreviations: MC-Multnomah county, CC-Columbia county, CL-Clatsop county MC-Marion country, COC-Cowlitz county, WC-Washington county, TC-Tillamook county.

Moss	ID	Alt. (m)	Latitude	Longitude	County	Substrate
<i>A. undulatum</i> (n=9)	U1	8	45°8'1.55"N	122°0'57.57"W	MC	gravelly soil
<i>A. undulatum</i> (n=24)	U2	20	45°44'43.95"N	122°57'32.99"W	CC	loamy soil
<i>A. undulatum</i> (n=22)	U3	76	45°43'34.68"N	122°59'3.91"W	CC	decayed tree stump
<i>A. undulatum</i> (n=15)	U4	31	45°44'24.32"N	122°57'37.19"W	CC	clay soil
<i>P. juniperinum</i> (n=3)	J1	21	45°44'46.89"N	122°57'37.54"W	CC	loamy soil
<i>P. juniperinum</i> (n=5)	J2	9	45°13'38.97"N	122°52'5.00"W	MC	gravelly soil
<i>P. juniperinum</i> (n=5)	J3	56	46°20'37.13"N	122°43'41.10"W	COC	gravelly soil
<i>P. juniperinum</i> (n=13)	J4	39	45°45'40.19"N	123°18'25.48"W	WC	gravel
<i>P. juniperinum</i> (n=14)	J5	4	45°38'7.44"N	122°49'44.95"W	MC	gravel
<i>P. juniperinum</i> (n=12)	J6	9	45°41'38.75"N	122°52'14.18"W	MC	gravelly soil
<i>P. juniperinum</i> (n=4)	J7	83	45°57'55.06"N	123°40'46.24"W	CL	loamy soil
<i>P. urnigerum</i> (n=4)	PU1	83	45°57'55.06"N	123°40'46.24"W	CL	loamy soil
<i>P. urnigerum</i> (n=11)	U2	72	45°43'47.10"N	122°59'22.03"W	CC	loamy soil
<i>P. urnigeium</i> (n=4)	U3	71	45°57'37.98"N	123°41'6.02"W	CL	loamy soil

<i>P. urnigerum</i> (n=9)	U4	88	45°57'44.30"N	123°41'1.16"W	CL	loamy soil
<i>P. urnigerum</i> (n=7)	U5	21	45° 57'39.31"N	123°40'58.62"W	TC	loamy soil
<i>P. urnigerum</i> (n=6)	U6	37	45°57'46.89"N	123°41'1.49"W	TC	loamy soil

Table 3.4 Summary table for aboveground morphological trait measurements from *P. juniperinum* gametophytes to assess discernable characteristics and contributions to isoprene emission (n=45).

Canopy structure	Leaf mass area (LMA) (mg cm⁻²) Leaf surface area (LSA) (cm⁻²) Biomass (B) (mg cm⁻²) Leaf length (LL) (mm) Leaf width (LW) (mm) Total leaves (TL) Leaf tip red (LTR) (mm) Wet weight (WW) (mg) Dry weight (DW) (mg) Canopy Height (CH) (mm) Leaf moisture (LM) (mg) Water content of gametophyte (WC) % Loss of water weight (LWC) (mg)
Cell traits	Cell lumen area (CLA) (mm⁻²) Cell lumen length (CLL) (μm) Interior cell wall thickness (IWT) (μm) Cell lamellae height (CLH) (μm) Lamellae number (LN) Number of rows of lamellae (NRL) Lamellae number * rows of lamellae (LANR) Chloroplast area (CHA) (μm⁻²) Cell space (CS) (μm) Individual cell size (ICS) (μm)
Leaf dimension traits	Leaf area (LA) (mm⁻²) Leaf density (LD) (mg m⁻³) Total leaf area (TLA) (mm⁻²) Costa length (CL) (mm) Costa width (CW) (mm) Costa cross sectional area (CCA) (mm⁻²) Lamina thickness (LT) (μm)
Leaf shape traits	Perimeter (P) (cm) Perimeter² /area (TP2/A) (cm⁻³) Total Perimeter² /area (TP2/A) (cm⁻³) Length/width (L/W) (cm)

Table 3.5 Summary table for belowground morphological trait measurements from *P. juniperinum* gametophytes to assess discernable characteristics and contributions to isoprene emission (n=45).

<p>Rhizoidal network and Stem structure</p>	<p>Rhizoid length (RL) (mm) Root shoot ratio (RSR) (mm⁻²) Stem area dry (SAD) (mm⁻²) Total area dry stem (TDSA) (mm⁻²) Total wet stem area (TWSA12) (mm⁻²) Total wet stem area (TWSA24) (mm⁻²) Wet stem area 12 hr. (WSA12) (mm⁻²) Wet stem area 24 hr. (WSA24) (mm⁻²) Water content in stem area 12hr (SAWC12) Water content in stem area 24hr (SAWC24) Stem dry diameter (SDD) (mm-2) Wet diameter of the stem 12 hr. (WDS12) (mm⁻²) Wet diameter of the stem 24 hr. (WDS12) (mm⁻²) Water content in stem diameter 12hr (SDWC12) Water content in stem diameter 24hr (SDWC24)</p>
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Figure 3.1 Illustration of *P. juniperinum* costa cross section with number of lamellae counts (LN) measurement at the widest part of the costa cross section. Each costa cross section was taken from five leaves with five replicate and at the widest part number of lamellae was counted using a Leica microscope (Leitz DME, Leica, Wetzlar, Germany) with a AxioCam 105 Color camera and Zen Blue software (version 1.1.2.0; Carl Zeiss Microscopy).

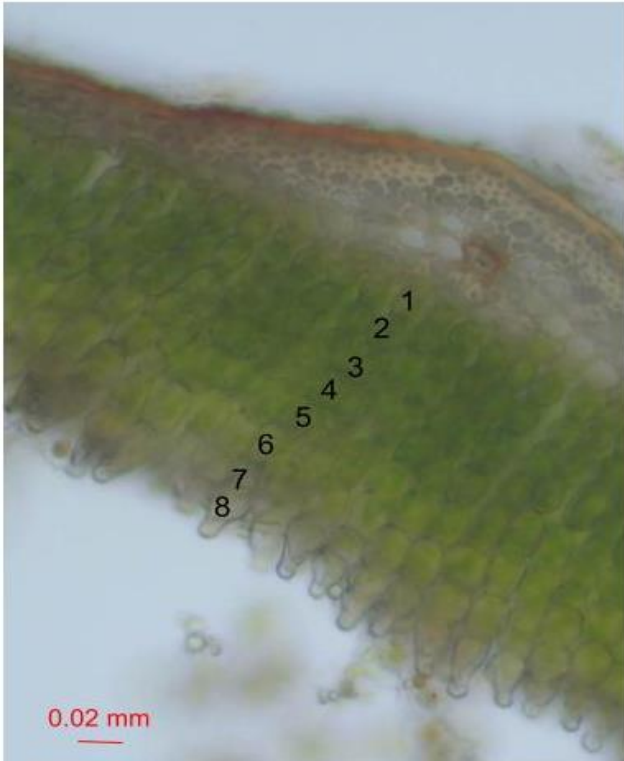


Figure 3.2 Illustration on *P. juniperinum* costa cross section with five lamellae height (CLH) measurement at the widest part of the costa cross section. This was accomplished by using a Leica microscope (Leitz DME, Leica, Wetzlar, Germany) with a AxioCam 105 Color camera and Zen Blue software (version 1.1.2.0; Carl Zeiss Microscopy).

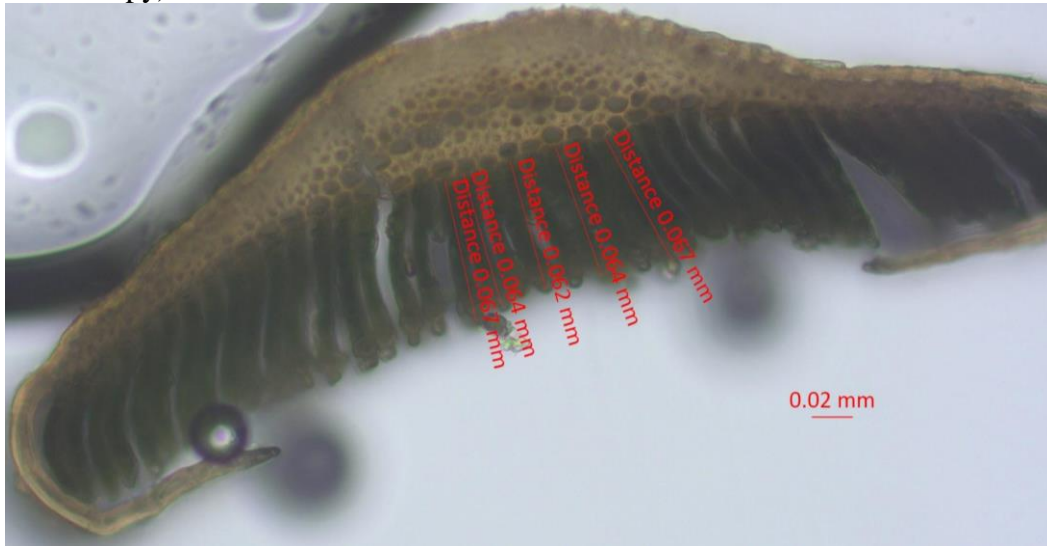


Figure 3.3 Illustration on *P. juniperinum* costa cross section of gametophyte stem after leaf removal. Stem area was measured at 12 hour water logged, following 24 hours water logged and dry state to approximate area and swelling of the stem. Water capacity assessment was estimated using a Leica microscope (Leitz DME, Leica, Wetzlar, Germany) with a AxioCam 105 Color camera and Zen Blue software (version 1.1.2.0; Carl Zeiss Microscopy).

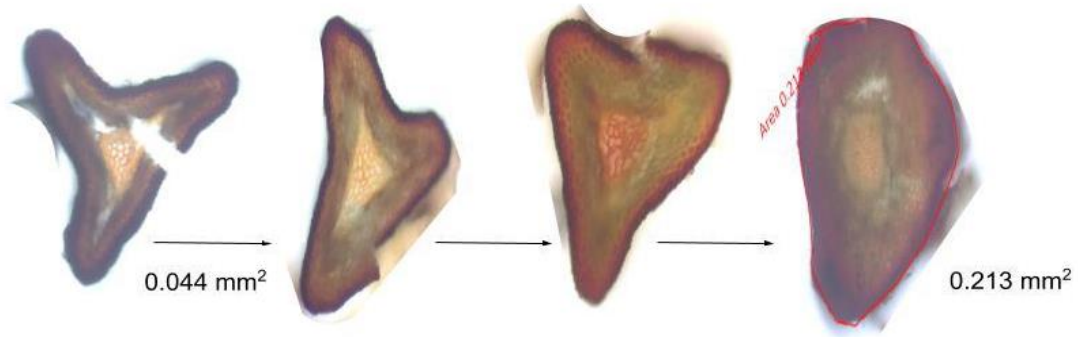


Figure 3.4 Setup for isoprene emission assessment for individual gametophyte emission using Dracast LED1000 Pro Daylight light panel, with light-emitting diode array (Photon Systems Instruments). *Polytrichum* species with rhizoids were pulled through a septa and inverted in glass vial. The rhizoid was then placed into a test tube filled with tap water.

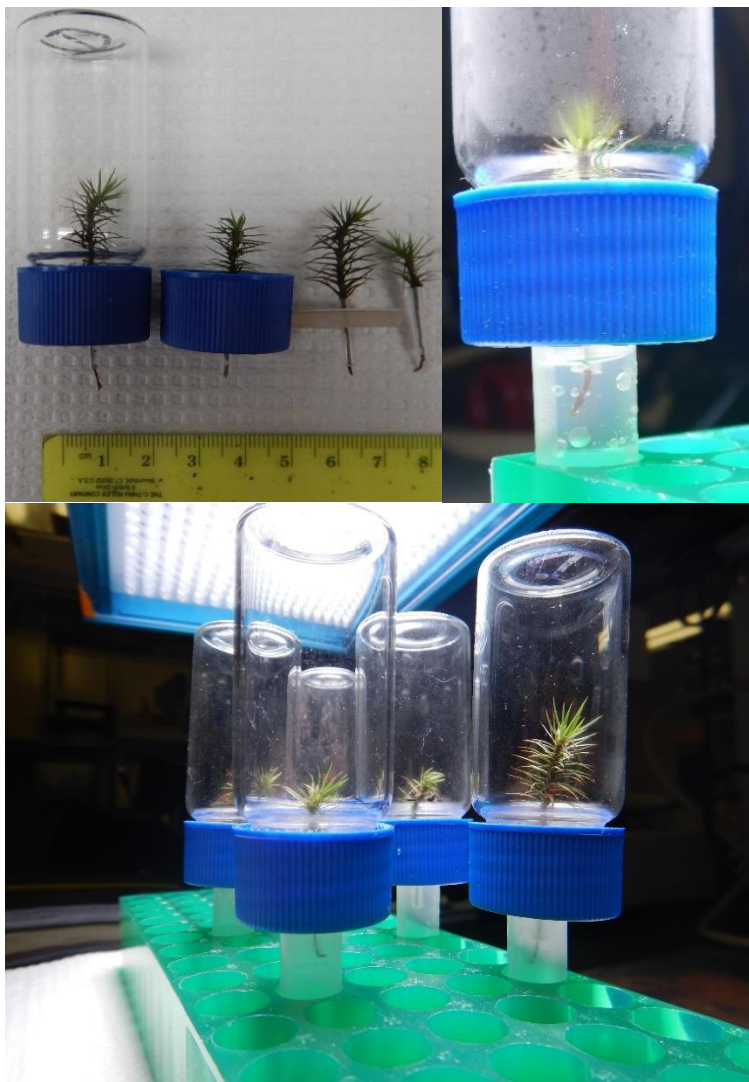


Figure 3.5 Logged isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus above and belowground morphological variables in non-sexually expressing *P. juniperinum* gametophytes (n=45). Above and belowground variables are plotted on the x axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between logged isoprene emission vs. canopy traits: total leaves (TL), wet weight (WW), gametophyte width (GW), leaf tip redness (LTR), vs. belowground traits: steam area dry (SAD), and wet stem area at 24 hours (WSA24). Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

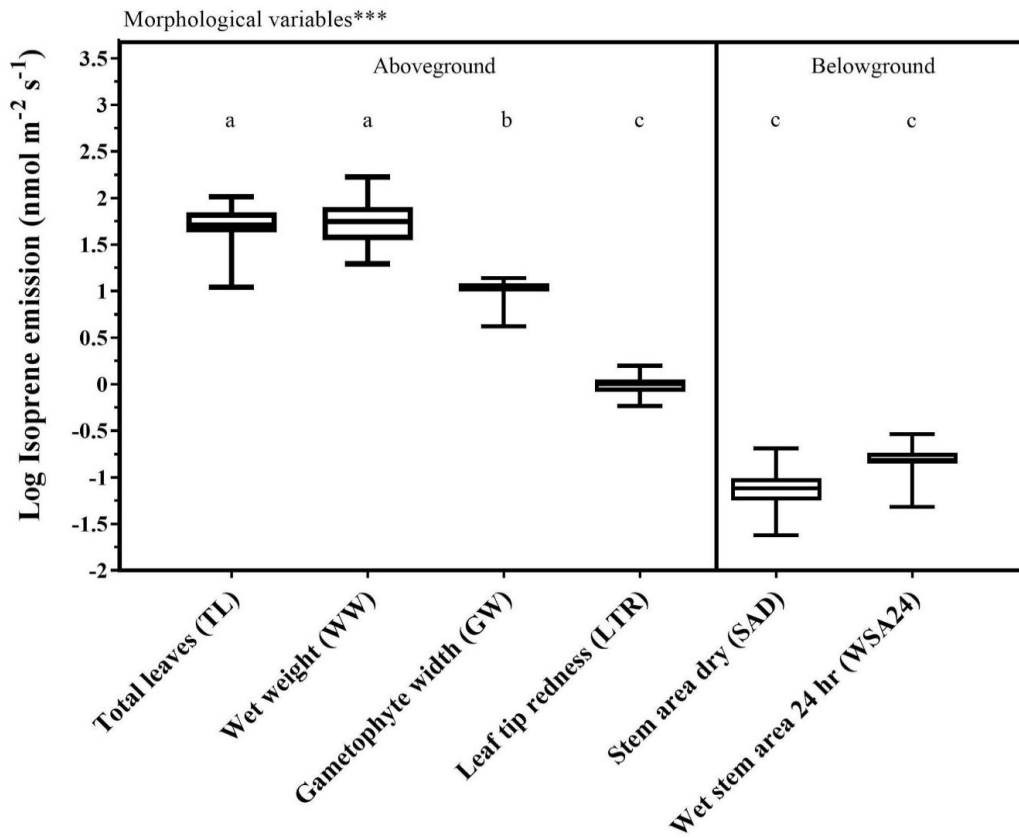


Figure 3.6 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) best fit regression line versus stem area dry (SAD) mm^2 in *P. juniperinum* gametophytes (n=45). Stem area dry (SAD) mm^2 is plotted on the x axis. Regression line is statistically significant. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance. Regression line equation shown below for isoprene emission and stem area dry (SAD).

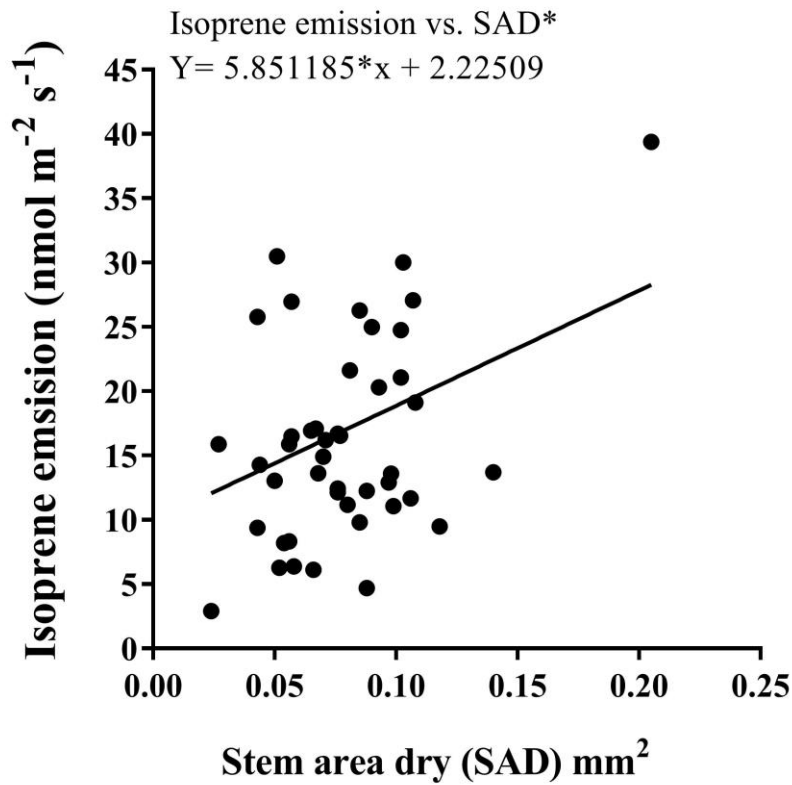


Figure 3.7 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus plant sex in *P. juniperinum* gametophytes that were not expressing sex during the experiment ($n=300$). Sex is plotted on the x-axis. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

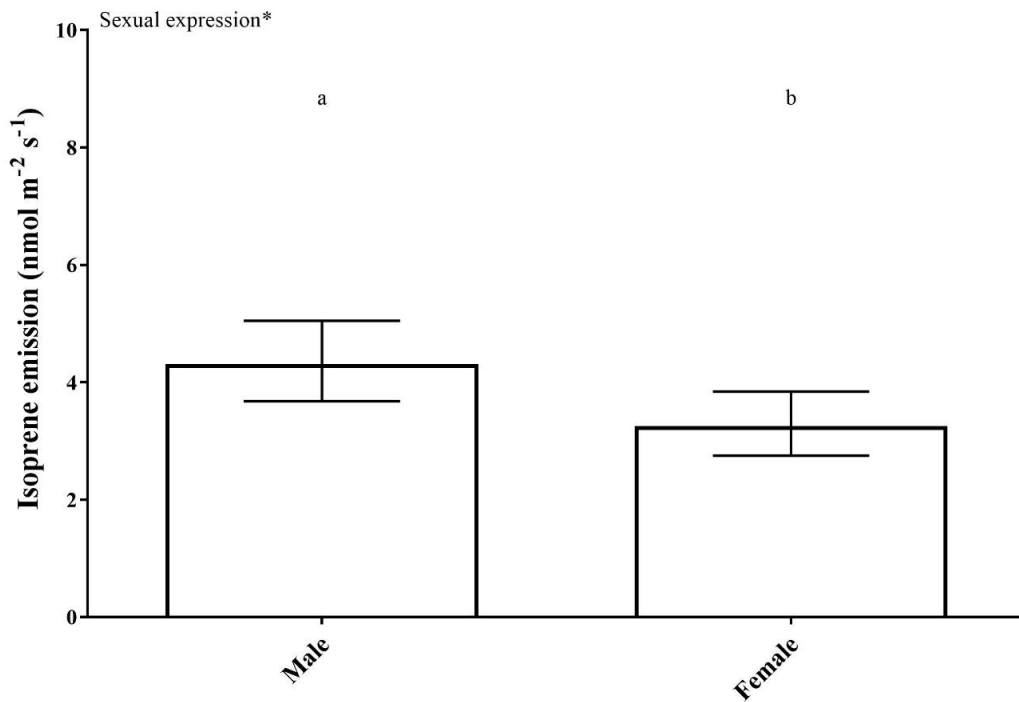


Figure 3.8 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus species: *Atrichum undulatum*, *Pogonatum urnigerum* and *Polytrichum juniperinum* gametophytes (n=167). Species are plotted on the x-axis. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

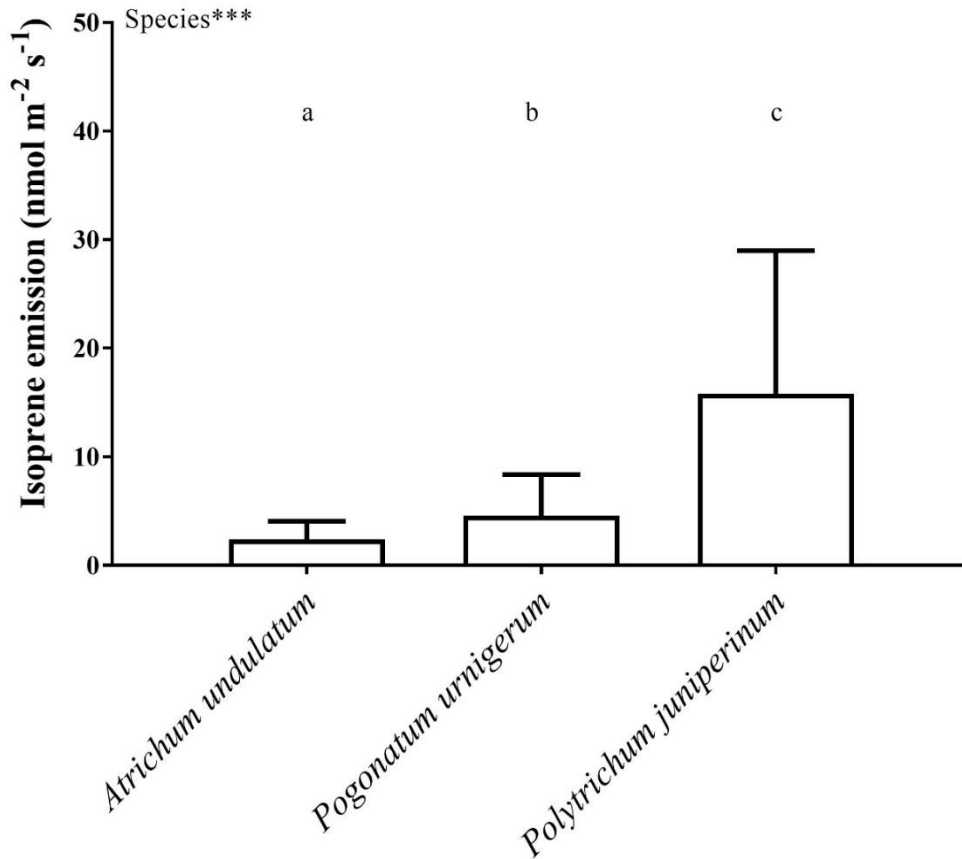


Figure 3.9 Logged isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus sex between species of *A. undulatum*, *P. urnigerum* and *P. juniperinum* gametophytes (n=167). Species and sex are plotted on the x-axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) vs. species and sex. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

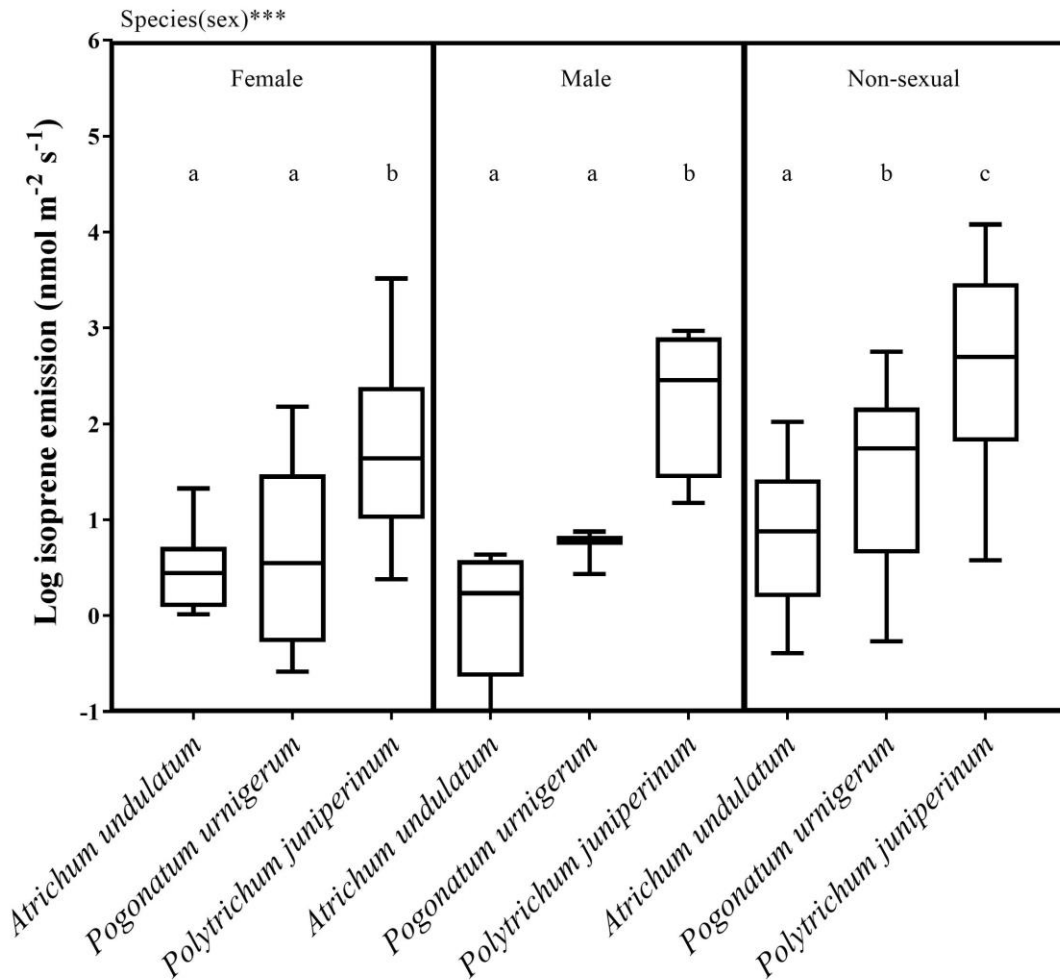


Figure 3.10 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus sexes in Polytrichaceae gametophytes (n=167). Sexual expression, female, male and non-sexual expressions are plotted on the x-axis. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

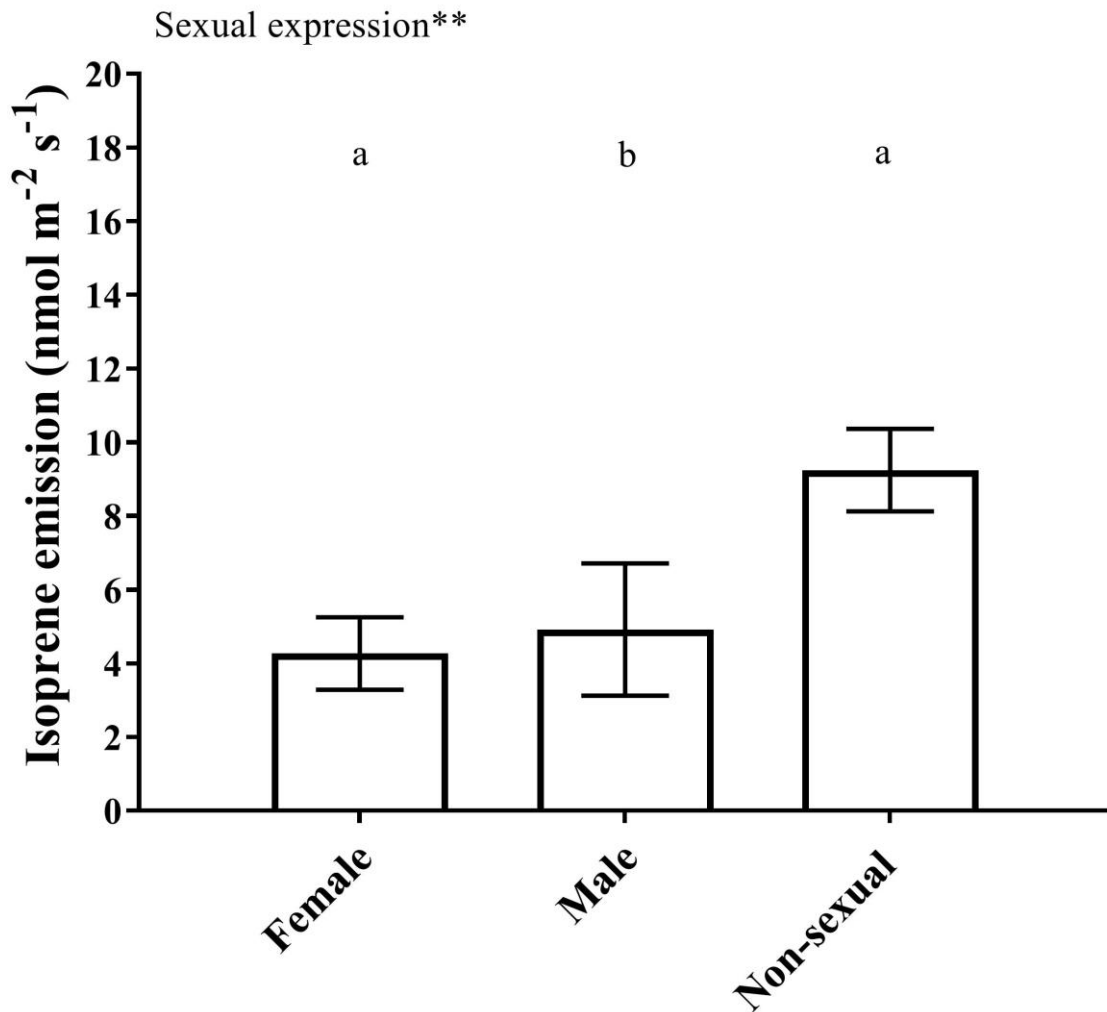


Figure 3.11 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus site in *A. undulatum* gametophytes ($n=70$). Altitude is plotted on the x- axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) vs. site. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

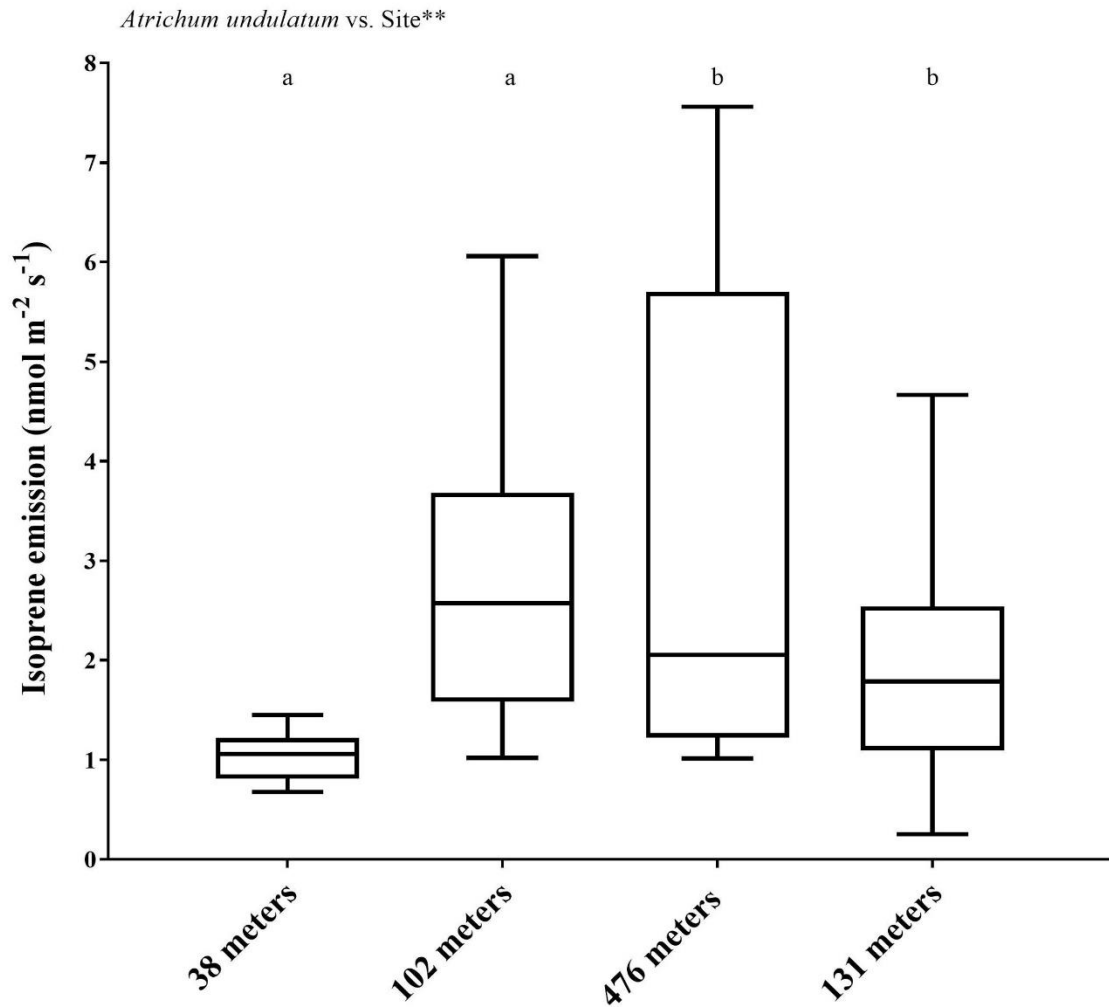


Figure 3.12 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus site in *P. juniperinum* gametophytes (n=56). Altitude is plotted on the x- axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) vs. site. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

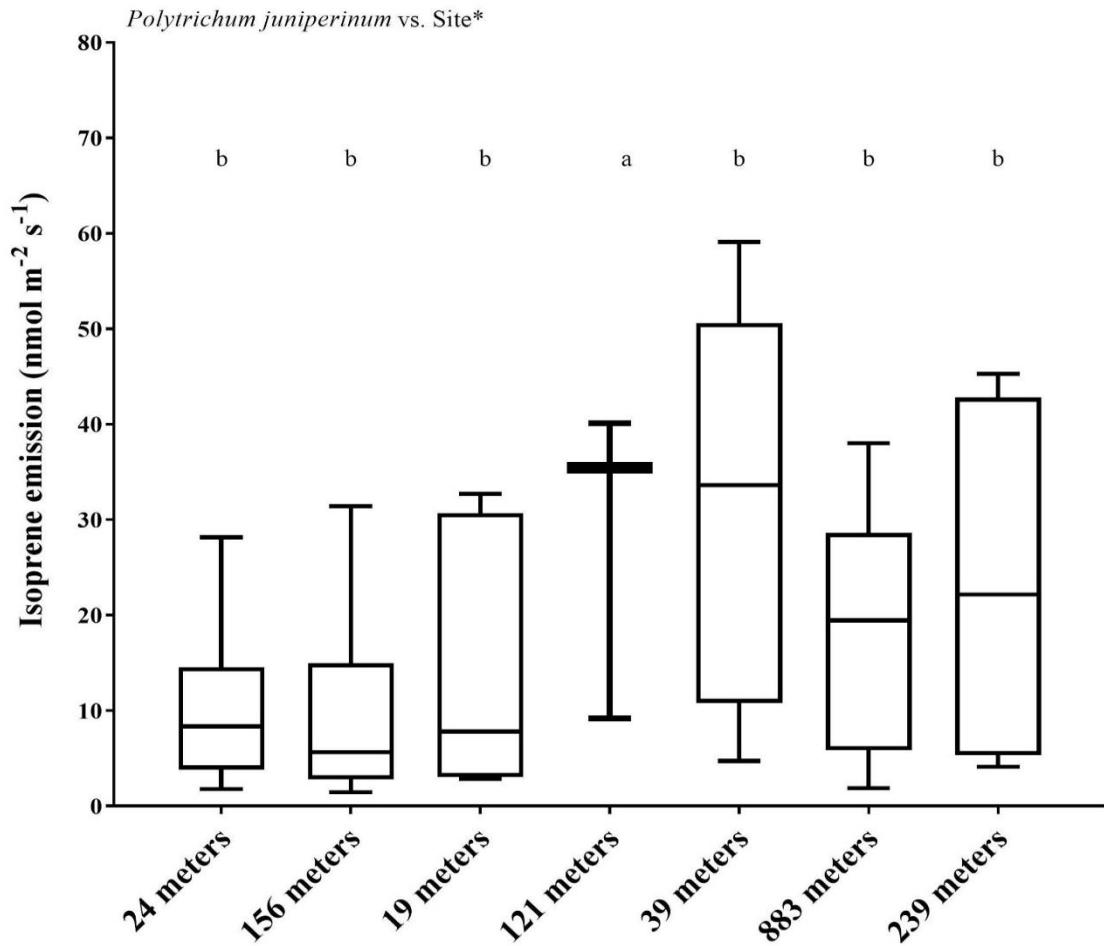


Figure 3.13 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus site in *P. urnigerum* gametophytes ($n=41$). Altitude is plotted on the x- axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) vs. site. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

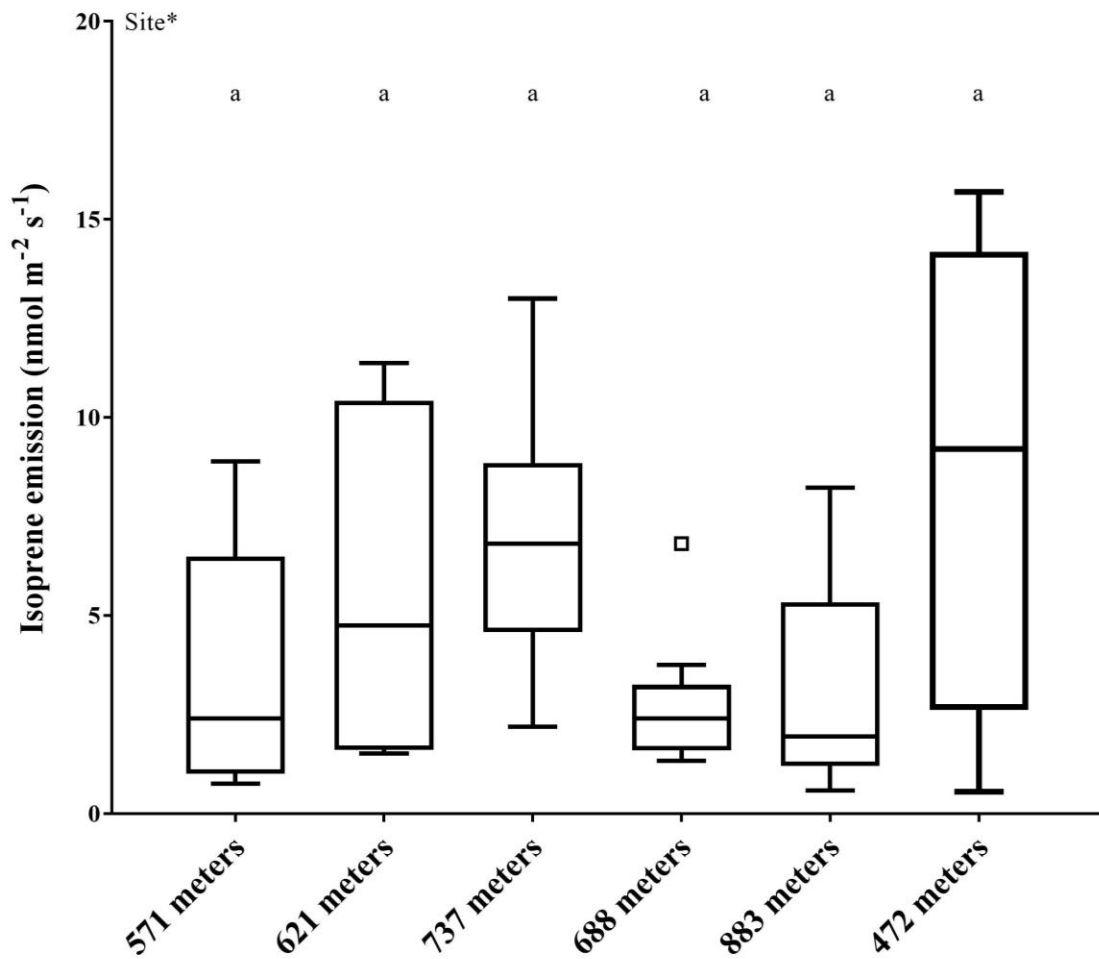


Figure 3.14 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) best fit regression line versus Chlorophyll Fluorescence Ratio (CFR) in *P. juniperinum* gametophytes (n=45). CFR is plotted on the x-axis. Regression line is statistically significant. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance. Regression line equation shown below for isoprene emission and CFR.

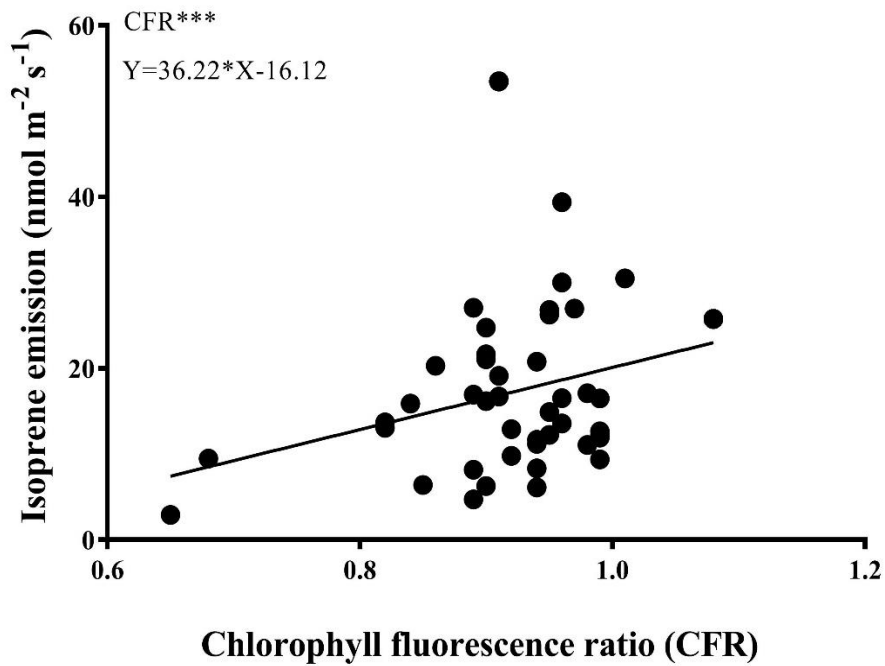


Figure 3.15 Logged isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) best fit regression line versus Chlorophyll Fluorescence Ratio (CFR) in *P. juniperinum* gametophytes (n=28). CFR is plotted on the x-axis. The nonlinear regression line is statistically significant in *A. undulatum*. The regression line in *P. juniperinum* is not statistically significant. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance. Regression line equation shown below for isoprene emission vs. CFR for *A. undulatum*.

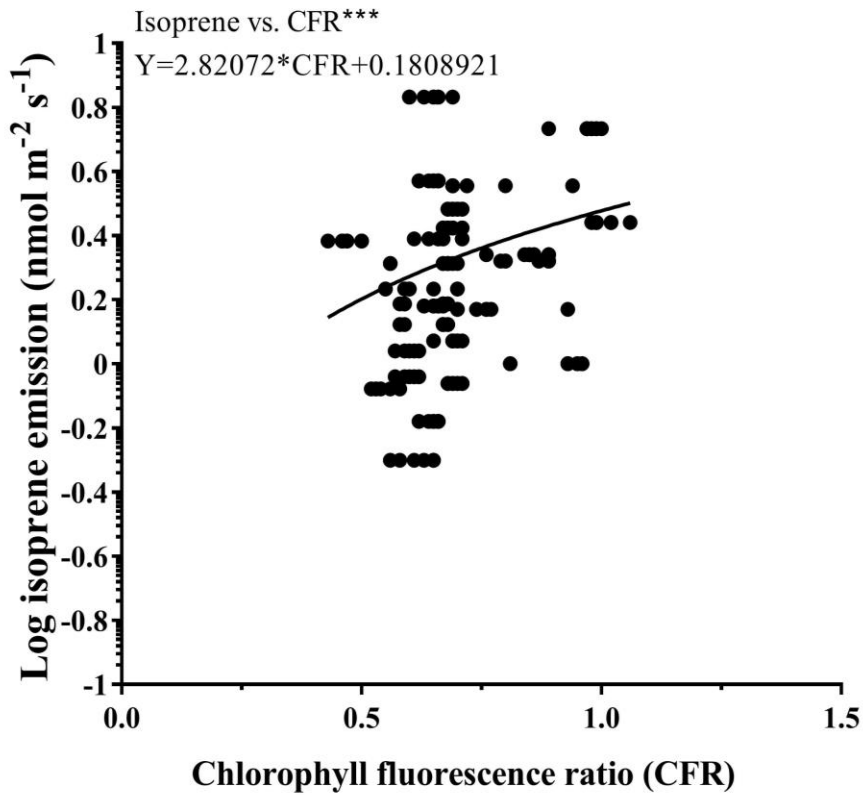


Figure 3.16 Chlorophyll Fluorescence Ratio (CFR) versus sex in *P. juniperinum* (n=25). Sex is plotted on the x-axis. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

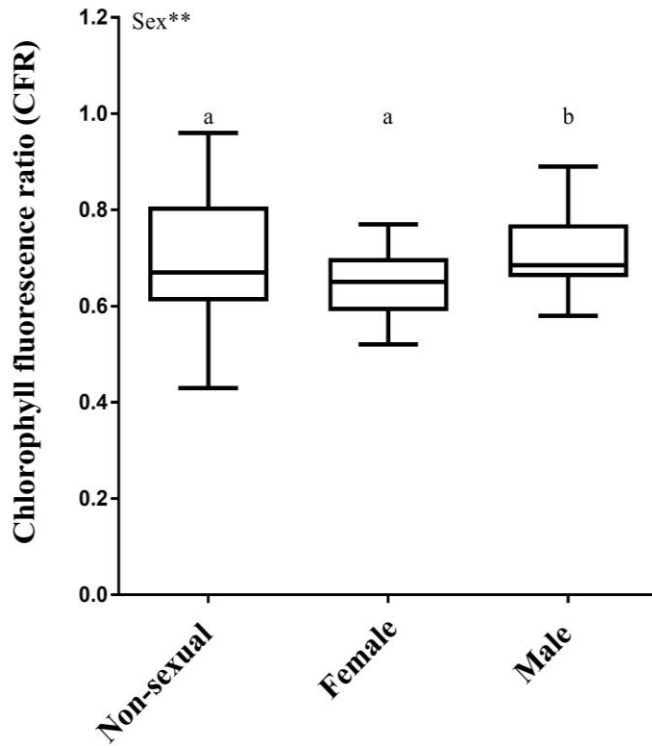


Figure 3.17 Relative Electron Transport Rate (RETR) ($\mu\text{mol m}^{-2} \text{s}^{-1}$) versus sex on BCD media in *P. juniperinum* and *A. undulatum* gametophytes (n=28). Photosynthetic Photon Flux Density (PPFD) ($\mu\text{mol m}^{-2} \text{s}^{-1}$) is plotted on the x-axis. The regression line is statistically significant in male *A. undulatum*. The regression line in *P. juniperinum* is not statistically significant. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance. Regression line equation shown below for RETR vs. PPFD for *A. undulatum*.

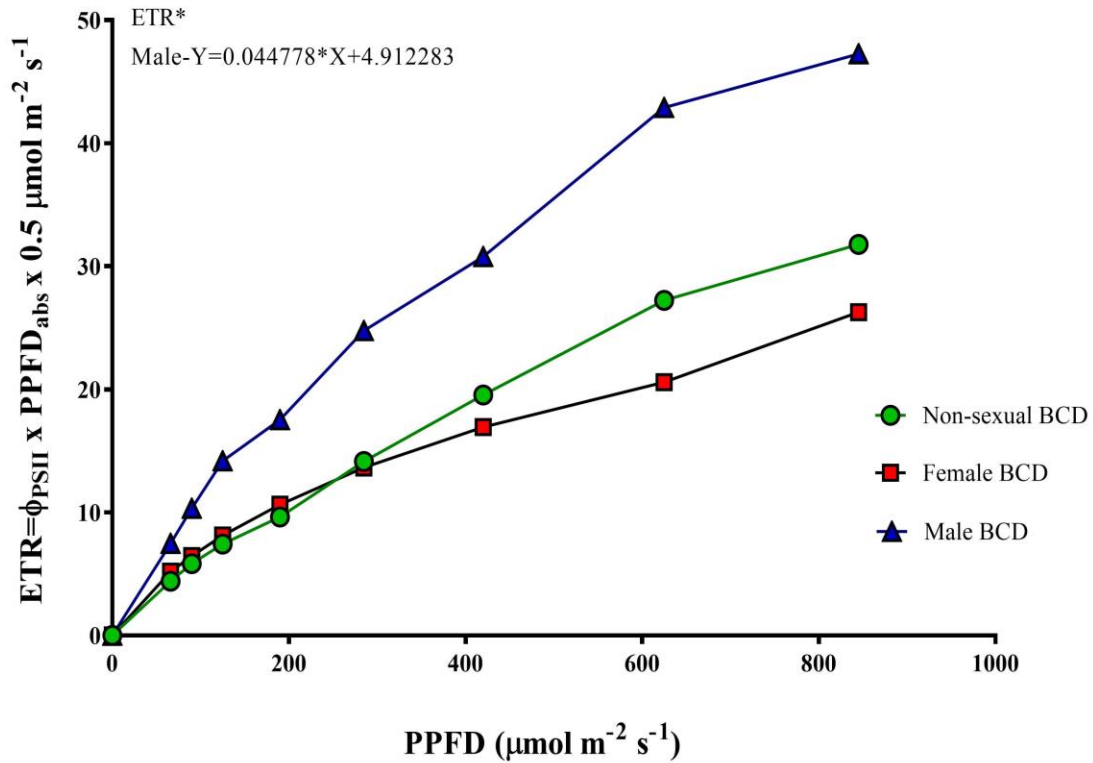


Figure 3.18 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) best fit regression line versus Chlorophyll Fluorescence Ratio (CFR) in *P. juniperinum* gametophytes (n=28). Chlorophyll Fluorescence Ratio (CFR) is plotted on the x-axis. The regression line in *P. juniperinum* is not statistically significant. The regression line in *A. undulatum* is statistically significant. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance. Regression line equation shown below for isoprene emission vs. CFR for *A. undulatum*.

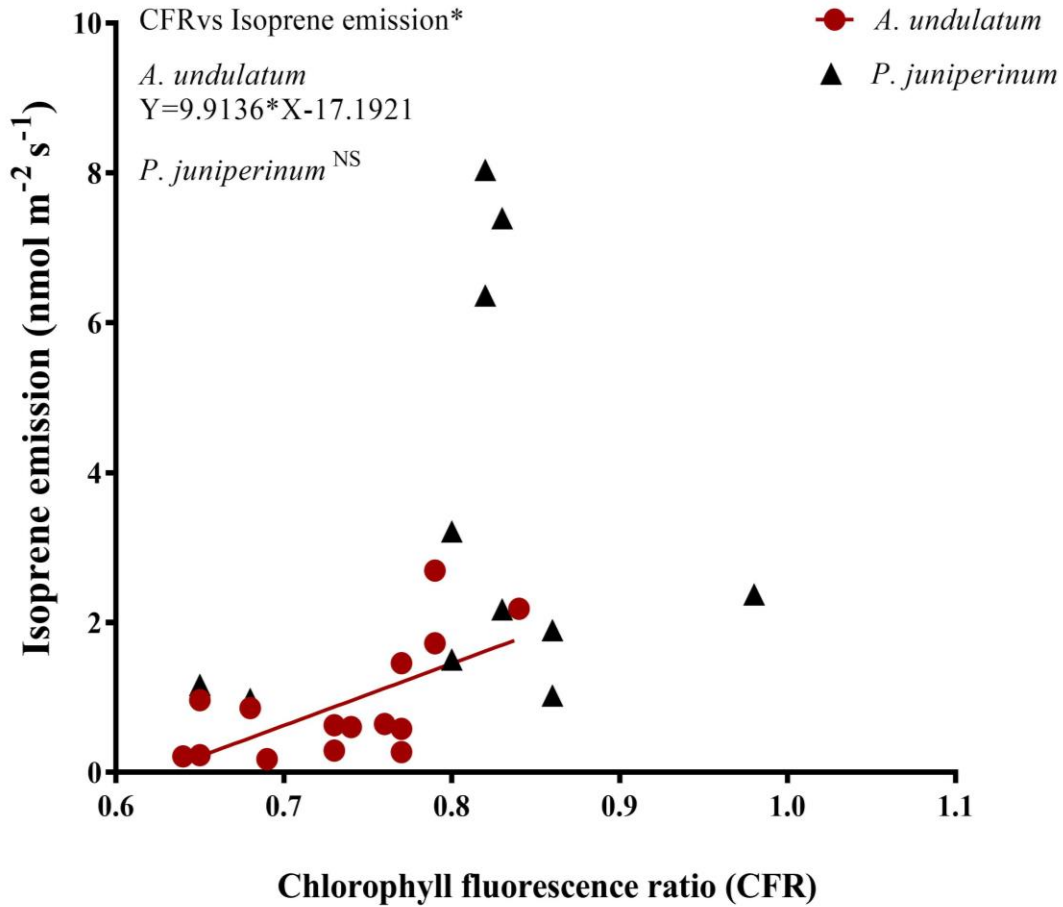


Figure 3.19 Chlorophyll Fluorescence Ratio (CFR) versus species: *P. juniperinum* and *A. undulatum* gametophytes (n=28). Species is plotted on the x-axis. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

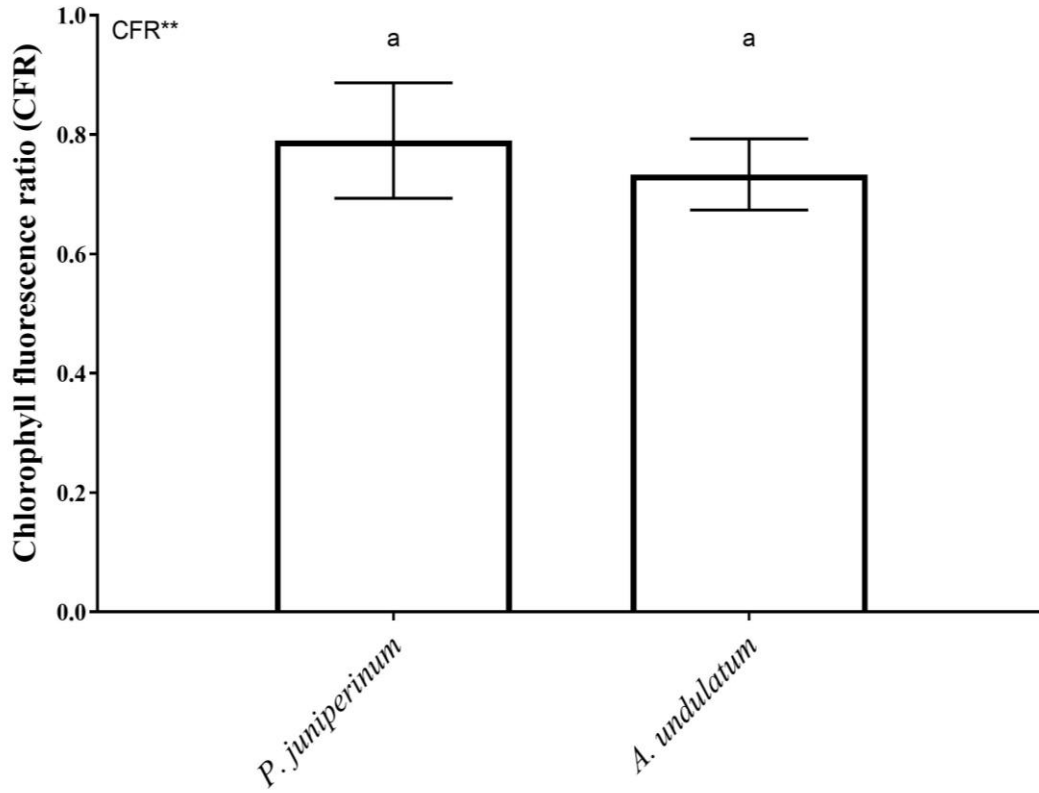


Figure 3.20 Relative Electron Transport Rate (RETR) ($\mu\text{mol m}^{-2} \text{s}^{-1}$) versus Photosynthetic Photon Flux Density (PPFD) ($\mu\text{mol m}^{-2} \text{s}^{-1}$) in *P. juniperinum* and *A. undulatum* gametophytes (n=28). PPFD is plotted on the x-axis. The regression line for both species is statistically significant. Error bars represent $\pm 1\sigma$ (standard deviation). A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance. Regression line equation shown below for RETR vs. PPFD for *P. juniperinum* and *A. undulatum*.

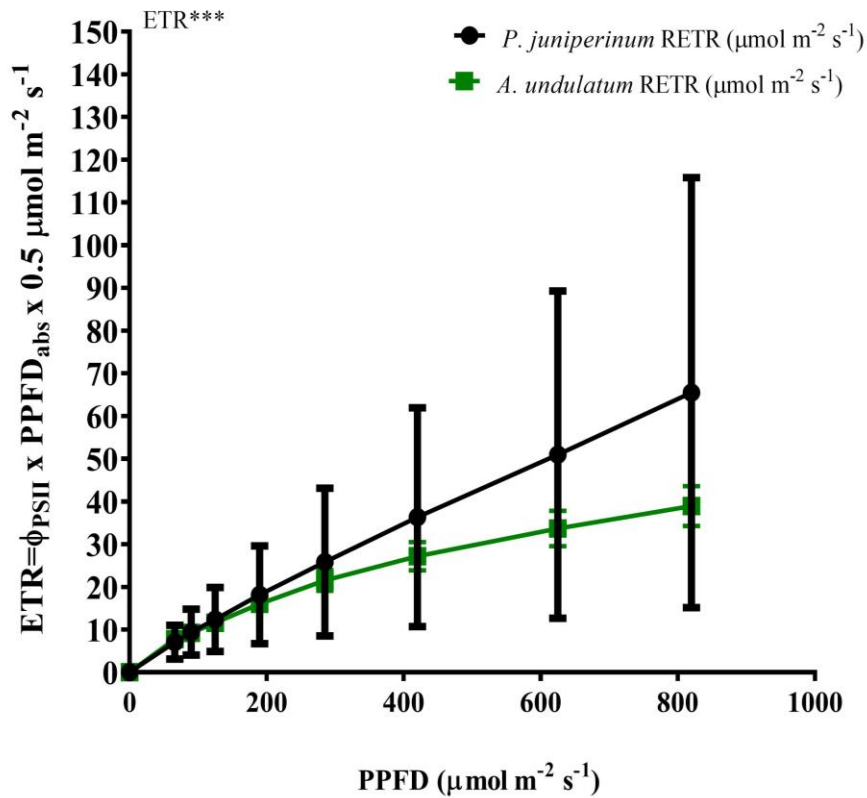
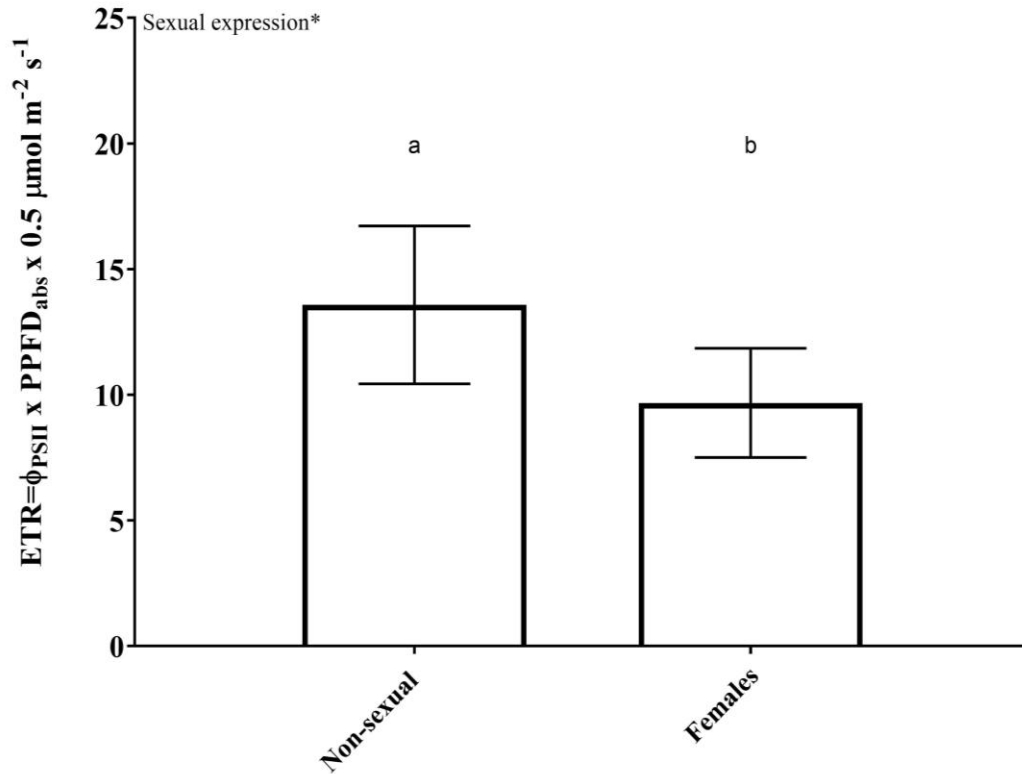


Figure 3.21 Relative Electron Transport Rate (RETR) ($\mu\text{mols m}^{-2} \text{s}^{-1}$) versus sex in *P. juniperinum* and *A. undulatum* gametophytes (n=28). Sex is plotted on the x-axis. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.



Chapter 4

The effect of nitrogen addition on moss morphology, sexual reproduction, and isoprene production

Abstract

Isoprene (2-methyl-1, 3-butadiene) accounts for approximately one third of all global BVOC emission and has been shown to protect plants from both abiotic and biotic stress with species and sex-specific preferences. As anthropomorphic nitrogen (N) increases in altered ecosystems, understanding the influences of N on morphology, physiology and reproductive effort is important and has not been well-studied in bryophytes. In this study, we set up a four-year common garden experiment in the moss *Polytrichum juniperinum* to test the effects of nitrogen addition on plant morphology, sexual reproduction and isoprene emission. Morphological, sexual expression and physiological measures varied significantly across the six populations from which we collected plants. Nitrogen addition significantly affected gametophyte length, isoprene emission and chlorophyll fluorescence ratio (CFR). We are the first to report increased isoprene emission under nitrogen addition in *P. juniperinum*. The high variation in morphology and physiology makes mosses ideal study systems as they are highly adaptable to their environment. The role of isoprene emission in mosses has gone nearly unnoticed, BVOC in vegetation have been demonstrated to have both regional and global atmospheric chemistry and climate impacts, while estimating emission fluxes from mosses is poorly understood.

Keywords: Ammonium nitrate (NH_4NO_3), bryophyte, isoprene emission, *Polytrichum juniperinum*, sexual reproduction

Introduction

Background nitrogen deposition

Tracking nitrogen deposition patterns is important for understanding how anthropogenic sources of nitrogen affect both natural habitats and urban environments (Pujadas et al. 2000; Raciti et al. 2008; Kaushal et al. 2014; Jones et al. 2018; Chávez et al. 2018). The global deposition of anthropogenic N now surpasses those from natural sources, with a four-fold increase in the amount of N available to organisms in less than a century (Vitousek et al. 1997; Fields 2004; Luo et al. 2004; Bobbink et al. 2010; Schulte-Uebbing et al. 2018; Taboada et al. 2018; Calvo-Fernández et al. 2018). To quantify N deposition is a complex task, with a wide range of compounds in the gas phase, in aerosols, and in precipitation (Krusche et al. 2003; Miller et al. 2003; Mahowald et al. 2011; Guo et al. 2018; Zheng et al. 2018; Cook et al. 2018; Lu et al. 2018; Xu et al. 2018). It is now well established that the level of N introduced to the atmosphere is increasing due to human activities (Vitousek et al. 1997; Galloway 1998, 2008; Bobbink et al. 2010; Erisman et al. 2013; Mogollón et al. 2018), and the process of climate as drivers of nitrogen are difficult to predict (Hess et al. 2018). Nitrogen deposition models forecast that by the year 2100 nitrogen deposition will be the third largest threat to biodiversity with climate change remaining the leading continuing threat (Galloway et al. 2004; Gruber et al. 2008; Sala et al. 2010; Wankel et al. 2010; Erisman et al. 2013; Stevens et al. 2018).

Anthropogenic bio-available N sources are driven by emission of nitrogen oxide species, such as nitrogen oxides: such as nitric oxide and nitrogen dioxide ($\text{NO}_x = \text{NO} + \text{NO}_2$) and ammonia (NH_3), in the atmosphere (Crutzen 1970, 1971; Liang et al. 1998; Glarborg et al. 2018). The oxidized forms of N, such as N_2O and NO_x , are predominantly emitted as a byproduct of transport, industry and energy production which helps explain regional differences in N deposition, with transportation contributing 70% of the emissions of oxidized compounds (Patterson et al. 1994; Clapp et al. 2001; Carslaw 2005; Yuan et al. 2005; Hoekman et al. 2012; Chaichan et al. 2018; Souri et al. 2018; Teixeira et al. 2018). Once released to the atmosphere, N reactive compounds, such as NO_x , oxidize to nitric acid (HNO_3) in a relatively short period of time, while the oxidation to organic nitrates occurs in less than a day (Zhang et al. 2012). Nitric acid (HNO_3) or nitrate (NO_3^-) is the dominant sink for reactive nitrogen oxides in the atmosphere (Fang et al. 2011). Nitric acid (HNO_3) and nitrate (NO_3^-) are considered to be the principal compounds in nitrogen deposition (Phillips et al. 2006). In wet deposition, most of the nitrogen is in the form of nitrate (NO_3^-) and ammonium (NH_4^+). In dry deposition, nitric acid (HNO_3), ammonia (NH_3), and nitrogen dioxide (NO_2) dominate (Poikolainen et al. 2009).

The largest source of nitrogen comes from fuel NO_x from vehicles (Widory 2007). NO_x contributes to the formation of ozone (O_3) and aerosol particulate matter and is thus an important factor affecting regional air quality and radiative balance due to smog production (Ehhalt et al. 2001). Since the lifetime of fixed nitrogen is short, most of the N deposition is near its source and is local in origin (Ollinger et al. 1993; Asman et al.

1998; Munger et al. 1998; Aneja et al. 2001; Kirchner et al. 2005, 2014; Moomaw et al. 2002; Zhang et al. 2012; Redling et al. 2013; Xu et al. 2018). Nitrogen deposition in North America is damaging sensitive ecosystems, even with efforts to reduce nitrogen oxide emissions (Li et al. 2006) and further steps are needed to reduce nitrogen deposition (Ellis et al. 2013). In the Pacific Northwest nitrogen deposition spans the range of 0.15 kg N ha⁻¹ yr⁻¹ to 39 kg N ha⁻¹ yr⁻¹ (Root et al. 2013). In and around urban areas, N deposition from anthropogenic sources can be many times the natural sources, significantly changing the behavior of the nitrogen cycle (Skeffington et al. 1988; Lovett 1994; Fenn et al. 1998, 2003; Kim et al. 2011; Pardo et al. 2011; Zhang et al. 2012; Benedict et al. 2018; Kharol et al. 2018).

An increasingly large body of evidence suggests that traits of plants adapted to nutrient-poor or nutrient-rich habitats may have feedback effects on a number of aboveground and belowground systems and communities (Aerts et al. 1989; Hobbie 1992; Nadelhoffer et al. 2000; Aber et al. 2003; Kou et al. 2018; Hou et al. 2018; Xia et al. 2018). Many biological and physiological processes, including photosynthesis, respiration, and carbon allocation due to increased nitrogen deposition have been used to analyze and detect environmental stress in plants (Darrall 1989; Townsend et al. 1996; Nellesmann et al. 2001; Clark et al. 2008; Bobbink et al. 2010; Smithwick et al. 2013; Fusaro et al. 2017; Damgaard et al. 2017; Hess et al. 2018).

An increase in nitrogen in some cases can have a stabilizing effect on ecosystem development (Kristensen et al. 1998; Aerts et al. 1999; Neff et al. 2002; Dijkstra et al. 2004; Zechmeister et al. 2008; Kou et al. 2018; Ibáñez et al. 2018), but in most cases

increased nitrogen deposition alters leaf and root composition (Xia et al. 2018), and alters growth responses of trees by increasing sensitivity to increased temperatures (Hess et al. 2018).

Even at low rates, N enrichment causes community shifts (Payne et al. 2017), affects greenhouse gas emissions (Song et al. 2017), causes key shifts in ecosystems such as widespread loss of species (Stevens et al. 2004; Maskell et al. 2010; Duprè et al. 2010) increase carbon sequestration (McKinley et al. 2008; de Vries et al. 2009; Liu et al. 2010), and promotes microbial competition in both subsoil and topsoil (Jones et al. 2018). Nitrogen increase can adversely affect seed productivity and flowering (Phoenix et al. 2012; Bogdziewicz et al. 2017; Wolf et al. 2017), increase competition and plant density among plant species, and influence decomposition and ecosystem nutrient turnover (Price et al. 1980; Steer et al. 1986; Muchow 1988; Vitousek et al. 1997; Cox et al. 2001; Vos et al. 2005; Kosola et al. 2018; Lemaire et al. 2018). There is also strong evidence to suggest that current rates of N deposition are altering many biogeochemical processes, which are influenced by climatic conditions (Southon et al. 2013; Neumann et al. 2018) and already cause reduction in soil respiration (Janssens et al. 2010), and alter photosynthetic capacity, foliar nutrient status and nitrogen metabolism (Mao et al. 2018).

Nitrogen deposition and bryophytes

The distribution pattern of mosses in urban centers can be related to the degree and extent of pollution (LeBlanc et al. 1971; Rao 1982). The major source of nitrogen input for mosses comes from atmospheric wet deposition as precipitation, throughfall and

dry deposition of fixed N; this allows for a correlation between the N content of moss tissue and local N deposition (Pitcairn et al. 1995; Solga et al. 2006; Wilson et al. 2009; Harmens et al. 2011; Kosonen et al. 2018). Mosses are readily available and can be used as an inexpensive tool to monitor nitrogen levels. While the N content in mosses provides no direct quantitative measurement of atmospheric deposition, the data obtained can give an indication of spatial patterns and temporal trends of N deposition from the atmosphere (Schröder et al. 2010, 2014; Kosonen et al. 2018). The use of moss nitrogen content and $\delta^{15}\text{N}$ can be used to acquire nitrogen signal strength and origin (Pearce et al. 2003; Pitcairn et al. 2003; Turetsky 2003; Paulissen et al. 2004; Bragazza et al. 2005; Solga et al. 2005, 2006; Pesch et al. 2008; Arróniz-Crespo et al. 2008; Harmens et al. 2011; Varela et al. 2013; Schröder et al. 2010, 2014; Erisman et al. 2014; Meyer et al. 2015; Izquieta-Rojano et al. 2016; Song et al. 2016; Felix et al. 2016; Shi et al. 2017; Kosonen et al. 2018; Carballeira et al. 2018; Olmstead et al. 2018).

Cryptogamic covers, such as cyanobacteria, algae, lichens, bryophytes, fungi, and archaea fix approximately 50 million tons of nitrogen per year and take up approximately 14 billion tons of carbon dioxide per year (Elbert et al. 2012). Mosses can adjust their N assimilating regime in response to anthropogenic N deposition (Bakken et al. 1995; Granath et al. 2009; Wiedermann et al. 2009; Waite et al. 2010; Lee et al. 2018). As a result, mosses can be vulnerable to increased N uptake. NO, NO₂ and NH₃ is readily deposited on moist leaf surfaces, where it is reduced through nitrate reductase to nitrite (Cape et al. 2004). Mosses are known as competitive scavengers of N and are known to reduce N availability for higher plants (Svensson 1995). They play an important role in N

cycling within the environment (Turetsky 2003) for both past and present atmospheric conditions shifting over time (Shetekauri et al. 2018; Xu et al. 2018; Olmstead et al. 2018).

High concentrations of nitrogen deposition has also been directly linked to negative effects on bryophyte communities leading to losses in moss cover and productivity (Pearce et al. 2003; Koranda et al. 2006; Arróniz-Crespo et al. 2008; Britton et al. 2018; Xing et al. 2019), losses in biodiversity (Alatalo et al. 2018), declines in nitrate reductase activity of plant tissue (Pearce et al. 2002; Gordon et al. 2001), and reduction in shoot length (Bell 1992, Salemaa et al. 2008). Without a vascular nutrient transport system and in the absence of roots, mosses are perennial primary producers that accumulate mineral cations and anionic nutrients through wet and dry deposition. High surface area to volume ratio in moss in addition to high cationic exchange capacity (Clymo et al. 1963; Little et al. 1974; Wells et al. 1990; Büscher et al. 1990; Turetsky et al. 1993) favor the accumulation of atmospherically available nutrients from which fixed N is obtained (Gerdol et al. 2002).

Purpose of Study

While the interaction between nitrogen deposition and mosses are well-studied in some respects, the effects of nitrogen deposition on volatile organic compounds (BVOCs), such as isoprene, is largely unknown in mosses. Only some plant species produce isoprene, and it has also been hypothesized that the isoprene-producing plants both protect themselves against ozone and, in NO_x-rich environments, increase the ozone

stress on their non-isoprene-producing neighbors (Arneth et al. 2007). This suggests a physiological role of isoprene emission being more related to its putative function as an antioxidant (Holopainen 2013). In high N environments, where reactive N species such as NO_x dominate near major roadways, plants are able to utilize the excess nitrogen and isoprene can oxidize in the presence of NO_x creating organic nitrates (Tuazon et al. 1990; O'Brien et al. 1995).

We theorize that nitrogen will have a significant influence on bryophyte isoprene production, as a positive significant relationship has been found between isoprene emission rate and leaf nitrogen concentration in both sun and shade leaves of oak trees (Litvak et al. 1996). Understanding how nitrogen availability influences BVOC productivity and potentially their life history, including sexual reproduction, in mosses is key to understanding how these species are functioning, particularly in urban areas with high N input. Recently, sex-specific BVOC emissions have been shown to influence the reproductive success of *C. purpureus* (Rosenstiel et al. 2012), a cosmopolitan moss, suggesting a previously unrecognized role for these products in the moss sexual life cycle. Moss sex-specific isoprene/BVOC emissions are an important and poorly characterized piece of the chemical diversity that exists in these early land plants, and how gametangia expression changes with increased nitrogen availability creates a fundamental gap in our understanding of the chemical ecology of these ubiquitous plants. To test the effects of nitrogen on *Polytrichum juniperinum* populations, a common garden experiment was set up at Portland State University (PSU), which contained male-majority and female-majority intact moss patches from six populations. Ammonium nitrate was

administered monthly for four years with treatments of 0 kg N ha⁻¹ yr⁻¹, 10 kg N ha⁻¹ yr⁻¹, and 20 kg N ha⁻¹ yr⁻¹. We measured morphological, reproductive, and physiological traits, including isoprene emissions. Using our data, we are able to test the hypothesis that increased nitrogen will influence bryophyte 1) morphology, 2) reproduction, and 3) isoprene production.

Materials and Methods

Study species

Cosmopolitan, endohydric *Polytrichum juniperinum* mosses differ from typical bryophytes in that they have a network of underground rhizomes, and efficient internal water and carbohydrate conducting systems with apoplastic sugar loading (Thomas et al. 1990). They are able to translocate photoassimilates to sister shoots suggesting capacity for physiological integration between connected moss stems (Thomas et al. 1988; Eckstein et al. 1999). This family of mosses also stores starch in their rhizoids which act as sites for overwinter storage for carbohydrates (Skre et al. 1983). The family Polytrichaceae forms a monophyletic group (Hyvönen et al. 2004) and possesses the highest degree of specialization in being able to move photosynthate through conducting cells called leptoids, in both the haploid gametophyte and diploid sporophyte. The structure of leaves in Polytrichaceae is closest to the function of the leaves of vascular plants of all the mosses (Clayton-Greene et al. 1985; Potter et al. 1995), with thick cuticularized leaves that possess differentiated photosynthetic tissue anatomy.

Polytrichum has a “high octane physiology” (Dani et al. 2014), and its highly complex anatomy facilitates high isoprene emission rates (Hanson et al. 1999). *Polytrichum* mosses possess many morphological, anatomical, and physiological properties that affect the capacity to accumulate nutrients on their surfaces and affect their uptake of nitrogen intracellularly (Zechmeister et al. 2003). *P. juniperinum* is equipped with a species-specific unique photosynthetic lamellae, a “proto phloem” like leptoid rich plasmodesmata, translocation of photoassimilates to sister gametophytes (shoots) through its rhizoidal system that allows for storage of starches not seen in other moss families (Thomas et al. 1988; Shotyk et al. 1998; Fernández et al. 2002; Limpens et al. 2003). It has been suggested that the inflexed achlorophyllous leaf margins enclosing the photosynthetic lamellae in *P. juniperinum* serve to increase the relative humidity and temperature of the air surrounding the lamellae, this adaptation can increase the effective growing season of the moss (Bazzaz et al. 1970; Bayfield et al. 1973; Krupa et al. 1977; Sveinbjörnsson et al. 1981). *Polytrichum* mosses have great desiccation tolerance that is a strategy for drought resistance and is enabled, at least in part, by its photosynthetic lamellae (Proctor et al. 2005, 2006). The negative effect of similarly increased lamellae temperature during summer months could be offset by its lower dark respiration rates (Herbst 1975), which could aid in attaining higher rate of new stem production, turf densities and higher rate of decomposition. The available reserve photosynthate present in the adult moss shoot could be transferred into new gametophytes via the extensive underground rhizoid system (Bowen et al. 1931; Wigglesworth et al. 1947; Collins et al. 1974; Trachtenberg et al. 1978; Schofield et al. 1981; Reinhart et al. 1981; Döbbeler et al.

1987; Kodner et al. 2001; Groeneveld et al. 2007; Elumeeva et al. 2011; Tuba et al. 2011).

Since mosses experience strong limitation of photosynthesis because of the high resistance to CO₂ diffusion through liquid water on the leaf surface and in the cell wall, the evolution of specialized lamellae cells are critical in this moss family to help reduce this resistance (Thomas et al. 1988; Barsig et al. 1998), providing larger and dry area for CO₂ uptake (Proctor 2005; Marschall et al. 2004) and facilitate the absorption of CO₂ by repelling water with cuticular waxes (Proctor 1979; Haas 1982; Neinhuis et al. 1995). The folding of leaves against the stem, followed by lateral closure appears to minimize water loss from the lamellae space and loss during the progression from wet to dry conditions (Bayfield 1973; Callaghan et al. 1978; Penny et al. 1982) and resembles stomatal closure in vascular plants (Thomas et al. 1996). *Polytrichum* populations that have been exposed to nitrogen have shown greater gametophyte height increase compared to rural sites, but at a cost of 36% less new shoot growth and 56% less old shoots with new growth (Bell et al. 1992). This suggests that in rural site with low nitrogen deposition, moss is able to utilize and benefit from extra nitrogen by growing upward toward light (Marschall et al. 2004). It is good to keep in mind that *Polytrichum* species incorporate about 23% photosynthetic assimilates to its new structural material aboveground, and 77% of assimilates are either respired or allocated belowground, but from this experiment did not link isoprene emission to allocation (Skre et al. 1983). Bryophytes are the closest living relatives to the first land plants, they are very suitable models for understanding the evolution of isoprene emission, and perhaps untangle the

biological processes involved in BVOC/isoprene emission function and its implications from an evolutionary perspective.

***P. juniperinum* moss collection method along a nitrogen gradient**

To establish a stable population of *P. juniperinum* we collected plants from six populations of *P. juniperinum* along a nitrogen gradient of low to high N in the winter of 2014 in Oregon. Each site was chosen to represent differing geographically isolated populations, environmental and altitudinal gradients. We established a common garden experiment at Portland State University Research greenhouse facility. *P. juniperinum* was collected from two sites near high traffic density, labeled as Site 1 and Site 2; two populations from mid traffic density, labeled as Site 3 and Site 4; and two were collected from low traffic density areas, labeled as Site 5 and Site 6 (Table 4.1). We have also recorded environmental variables associated with each site (Table 4.2).

P. juniperinum was identified and specimens were placed in the Portland State University Herbarium (HPSU). Each population contained male, female and non-expressing gametophytes (shoots). Plants were removed with all of the substrate down to the rock outcrop below the soil. This ensured the least disruption to the thick rhizoidal network holding the moss mats together as higher mortality have been reported when this species is disturbed by severing the rhizoidal network (Watson 1979). The moss mats were placed in 39.37 cm diameter pots, 50 cm deep holding 38 liters of substrate of 2:1 propagation grade sand and soil. Approximately 30 majority-male pots and 30 pots majority-female pots were planted from each site. Each 39.37 cm diameter pot in which

the moss patch was placed contained approximately 1500 moss gametophytes, yielding ~90,000 individual gametophytes. In addition, two set of control pots from each population and treatment containing only sand/soil mix pot was placed in between the rest of the pots. These were all randomly rotated every second week along with the 60 pots to ameliorate environmental conditions. Each pot was set 1.2 meters away from adjacent pots on all sides.

To obtain an idea of population differences, fifty random gametophytes from each pot were randomly collected and assessed for age category (Table 4.3). *Polytrichum* species possess morphological cues in their aboveground gametophyte, which can serve in identifying age characteristics and life history, and give a spectrum of age distribution observed to be initiated annually (Longton et al. 1967; Longton 1970, 1972, 1979; Collins 1976; Callaghan et al. 1978). All samples were cleaned of debris, such as other plant species, lichen and detritus, and all sporophytes were cut. Mosses were allowed to acclimate in a ‘common garden’ setting to reduce variation as a result of previous environmental conditions and maternal effects (Shaw 1986). Weeds were manually removed regularly, and pots were monitored on an ongoing basis.

Sex determination from six populations of *P. juniperinum*

To measure sex-specific characteristics, we assessed each population on a number of morphological and physiological characters. Annual growth increments can be observed to have regular variation in leaf length, where stem elongation continues throughout a season, but longer leaves are created during the middle of the growing

season and are conspicuous (Héban 1973). In male-majority pots, splash cups are visible discs on tops of the gametophytes, and new growth from the tip of the splash cups can be seen in early spring. In female-majority pots, after sporophyte production which develop for about a year after fertilization (Innes 1990), the gametophyte stem ceases to exist (Greene 1960; Sarafis 1971; Watson 1975; Callaghan et al. 1978), or shoots may innovate from below the perichaetium and continue to grow and produce sporophytes (Hedderson et al. 2008). All gametophytes may produce branches from shoot bases or on rhizoidal wicks (Wigglesworth 1947). Female *P. juniperinum* gametophytes therefore start new each time reproduction ensues, terminating with sporophyte maturation and spore release (Hughes 1962; Watson 1975; Hughes 1990).

We classified reproductive effort by counting sporophytes from females, gametophyte containing splash cups from males, and we counted non-sexually expressing gametophytes from each site and year. Non sexual gametophytes were counted and classified. Individual stems were chosen at random, gametophyte shoots from each sex were selected from each pot and marked with gold wire to follow sexual expression and morphological measurements (Watson 1975). We used gold-coated 0.31 mm 28 gauge soft wire as it is chemically inert. Once banded baseline measurements were taken, the gold wire was placed 5 mm below the last leaf on the gametophyte. In female-majority pots, 6 females were randomly chosen, 2 males and 2 undifferentiated gametophytes were marked and measured with digital caliper. For male-majority pots, 6 males, 2 females and 2 undifferentiated gametophytes were marked and measured. Three measurements were

taken from each gametophyte, the total height, lowest leaf to apex of shoot, and diameter of each shoot. Individual shoots were scored by age category (Callaghan et al. 1978).

Reproductive development followed an index described by Greene (1960). A total of 600 individual gametophytes were scored and studied. For male gametophytes, antheridia splash cups were counted, and for females, sporophytes were counted. When the study began, a majority of female stems had sporophytes. The sporophytes were collected in the late operculum intact stage of development (Greene 1960; Longton et al. 1967) and were cut, counted and stored once maturity was reached to avoid cross contamination among populations. Growth for males was measured from the base of the gametophyte 2-5 mm from the soil to the top of apex of the shoot, where usually antheridia resided. Females were measured from 3-5 mm from the soil surface to the top of the leafy apex of the shoot, not including sporophyte length. Vegetative stems were measured 3-5 mm from the soil surface to the top of the apex of the shoot. Growth of stems was followed from time of collection to termination of nitrogen addition experiment.

Morphological data collection from six populations of *P. juniperinum*

Morphological measurements were obtained from the populations before and after nitrogen addition. The collection involved intact *in situ* gametophyte measurements. Additionally, morphological characteristics were observed to ascertain population and leaf level differences between additions. We assessed gametophyte length (GL, mm), gametophyte width (GW, mm), gametophyte rhizoid length (GRL, mm), and

gametophyte canopy area (GCA, m²) using a digital Fowler caliper (Lux Scientific Instrument Corp., Tucson, AZ, USA). Total bryophyte cover in each pot was assessed, and percent cover green shoots and percent cover brown shoots were estimated.

Each pot was photographed ~60 cm above the canopy, and images were transferred to ArcMap GIS software (Version 10.4.1, ESRI Inc., ESRI, Los Angeles, USA). We estimated percent green and percent brown cover by using the Classification Tool in the Spatial Analyst package in ArcMap. We ran a Maximum Likelihood Classification (MLC), which is based on maximum likelihood probability theory. It assigns each pixel from a jpeg image to classes based on means and variances of the class signature, created precisely to look for specific pixel ratios (which is a technique that mathematically extracts factors from a set of variables (Shukla et al. 2017)). The jpeg was transformed into a multispectral image and was categorized and assigned into 50 classes. We ran unsupervised classification, where clusters were created from the statistical properties of the pixels. Pixels with similar properties were grouped to form individual clusters (ArcGis Desktop, 10.4.1, ESRI Inc., Los Angeles, USA).

In addition, sex-specific counts, live, brown shoots, and total number of shoots for each population and each pot were counted and monitored before and after the nitrogen experiment. All other morphological and anatomical observations were counted and recorded. Dead shoots were classified as no longer viable when the apex of the shoot was entirely brown (Potter et al. 1995). A detailed list can be found on summary of morphological and physiological measures (Table 4.4).

Ammonium nitrate (NH₄NO₃) addition method to six population of *P. juniperinum*

P. juniperinum moss patches were allowed to acclimate for a six-month period and ammonium nitrate additions began in the winter of 2015. All six sites and sex-specific pots were randomly distributed between the additions so that 10 female-majority pots and 10 male-majority pots from the three nitrogen level gradient were distributed evenly across the three N treatment additions (Table 4.5). The nitrogen additions imposed were as follows: the 0 kg N ha⁻¹ yr⁻¹ group (control pots or No N) with a total of 20 pots, the 10 kg N ha⁻¹ yr⁻¹ group (Med N) with a total of 20 pots, and the 20 kg N ha⁻¹ yr⁻¹ group (High N) with a total of 20 pots. Nitrogen in the form of granular ammonium nitrate was mixed with water to create an aqueous solution of ammonium nitrate NH₄NO₃ which was applied at the end of each month for four consecutive years starting in the winter of 2015 until winter of 2019.

Ammonium nitrate was measured out with analytical microbalance (AB104-S/FACT, manufactured by Mettler Toledo Corporation, Tualatin, Oregon). The measured compound was placed in a 500 ml Erlenmeyer flask and vortexed with 200 ml of tap water until crystals dissolved completely. The NH₄NO₃ liquid was then transferred into a 1 Liter hand held pressure pump mister sprayer (Solo INC., Model 418, Newport News, VA). The same volume of water was added to the control pots at same frequency. The sprayer was held 20 cm above each pot and sprayed in clockwise circular motion beginning in the middle of the pot to the edge of the pot (Figure 4.1). Additional water was added to remove the fertilizer solution from the moss canopy and to minimize any potential damage to the plants as described in (Potter et al. 1995).

The inside diameter of each pot was measured to be = 41.91 cm, with a radius of 20.995 cm. We then calculated area of the pot, by $A = \pi * r^2$, area of inside the pot equals 1379.48 cm^2 or 0.137948 m^2 per pot. We then followed by taking 1 hectare converted to square meters, which equals to 10000 m^2 . We have then taken our inside pot radius of 20.995 cm and divided it by 1 hectare, yielding $0.0000137948 = 1.37948 * 10^{-5}$ hectares. We then took our desired addition of 20 kg (High N) and multiplied it by 1.37948^{-5} hectares. This yielded 0.000276 kg/pot , which we converted into grams as 0.276 grams per pot of NH_4NO_3 for equivalent of $20 \text{ kg N ha}^{-1} \text{ m}^{-1}$. Each month 0.276 grams of NH_4NO_3 was sprayed into each pot in the High N addition, giving an annual N addition rate of $240 \text{ kg N ha}^{-1} \text{ year}^{-1}$. Next, we took our (Low N) of 10 kg N and multiplied it by 1.37948^{-5} hectares. This yielded us with 0.000138 kg/pot , which we converted into grams as 0.138 grams per pot of NH_4NO_3 for equivalent of $10 \text{ kg N ha}^{-1} \text{ m}^{-1}$. Each month 0.138 grams of NH_4NO_3 was sprayed into each pot in the Low N addition, giving an annual N addition rate of $120 \text{ kg N ha}^{-1} \text{ year}^{-1}$.

Physiological measurement of six populations of *P. juniperinum*

All gametophytic leaf level isoprene emission samples were taken non-destructively by leaving *P. juniperinum* gametophyte intact. All six populations survived the transplant process. We collected isoprene emission data from moss canopy level from each intact population of *P. juniperinum*, because mechanical damage (burning, cutting, wind, rubbing) of a leaf, or nearby leaves, decreases isoprene emission (Loreto et al. 1993). Therefore, we sampled intact moss ramets for this experiment as this method with

basal emission rate, as it gives a more spatially realistic profile of emission for sampling volatiles in an ecological context as it was more feasible to leave moss tissue intact for a multi-year experiment involving the addition of anthropogenic nitrogen (N) addition when trying to correlate isoprene emission to morphological and physiological traits.

All sixty pots not including six control pots (two per N addition regime) were tested using static accumulation chambers, and samples were collected between 9 am and 10 am to reduce diurnal variation. Measurements were taken after testing and utilizing a lab constructed 77mm × 77mm × 97 mm static chamber (Magenta™ vessel) made of polypropylene closure (Sigma-Aldrich Co., 2017) which, we have outfitted with thermocouple and Swagelok® and a small fan for proper mixing of static air within the chamber. Magenta vessels™ were designed and inverted to best fit our experimental design. Leaf tissue was gently inserted into the magenta vessel when inverted, and carefully measured as to given an accurate representation of actual moss gametophytic canopy area (CA) under the magenta vessel (Figure 4.2). We have added a drilled hole to the side of the vessel equipped with a septa and Swagelok® to be able to take measurements from the side of the vessel. Measurement was taken by inserting a 2 ml Pressure-Lok® Precision Analytical Syringes (VICI Precision Sampling Inc., Baton Rouge, LA, USA) after 10 minutes of incubation. Canopy moss temperature was measured with a shaded thermocouple (maintained at 30°C, ±3C) in order to collect precise dynamic isoprene emission measurements.

An acclimation period was allowed before measurements were taken and temperatures were monitored with thermocouples and reported. Experiments were carried

out under standard conditions (30°C , $1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density at leaf level (PPFD), unless otherwise noted. Light was provided with a Dracast LED1000 Pro Daylight light panel, with light-emitting diode array (Photon Systems Instruments). Sexually expressing gametophytes patches were chosen, with no other debris and only photosynthetic active green tissue was sampled. Sampling order was randomized throughout the two trials in order to ensure that all sixty pots were sampled in a random matter. From each pot, four measurements were taken, each area consisting of about 16 cm^2 , resulting in four replicates per pot.

After 10 minutes, 1mL of air was removed via Pressure-Lok® precision analytical syringe (VICI Precision Sampling Inc., Baton Rouge, LA, USA) and injected into a reduction gas detector (RGD2, Trace Analytical, Menlo Park, CA). Isoprene was separated on a stainless steel column (1.3 m long x 2 mm inner diameter i.d.). The column was packed with UNI 8 Beads 3S 60/80 6' x 1/8", 0.085 SS (Alicat Scientific, Inc; Tucson, AZ). Peak times and areas were recorded and transformed to ppm of isoprene. The GC-RGD2 was calibrated each day using a standard cylinder containing 71 ppb (v/v) isoprene, references to a National Institute of Standard and Technology (NIST) Helium (HE) standard (SRM 1660a; 1ppm He in N₂, Rochester, NY), (Praxair Inc., Specialty Gases, Portland, OR) with a ten point standard dilution of isoprene and He as a carrier gas. Blanks were run every four samples to ensure quality check and to look for contamination within the column. Isoprene gas was mixed with high purity helium using a mass flow controller calibrated to a flow rate of 21 ml/min. (Alicat Scientific, Inc; Tucson, AZ). One mL of the calibration gas mixture was removed with a syringe from a

mixing chamber and injected into the GC-RGD2 to create the calibration curve and obtain slope of the equation see (Equation 1.) Isoprene emission measurement was calculated as follows; using Peak Area Units (PAU) from the standard calibration curve $y= mx+b$, at GC temperature of 298 Kelvin (K), and Atmospheric Pressure (P) of 760 Torr.

$$\begin{aligned}
 &= \left(\frac{\text{PAU}+20.69}{2.4703} \right) \times \left(\frac{1\text{nmol/L}}{\text{ppb}} \right) \times (.0005) \times \left(\frac{\text{std nl}}{\text{nl}} \right) \times \left(\frac{760 \text{ Torr}}{298\text{K}} \right) \times \left(\frac{378\text{K}}{771.8\text{Torr}} \right) \\
 &\times \left(\frac{1\text{nmol}}{22.4 \text{ std nl}} \right) \times \left(\frac{\text{volume vial (ml)}}{.5\text{ml}} \right) \times 1 \text{ (factor for loop recovery)} \\
 &\times \left(\frac{1}{\text{area/m}^2} \right) = (\text{nmol m}^2 \text{ *s}^{-1})
 \end{aligned}$$

Equation 1. Formula used to convert isoprene from calibration curve, $y=mx+b$, and PAU to isoprene ($\text{nmol m}^{-2} \text{ s}^{-1}$).

We obtained the volume mixing ratio of isoprene (ppb) we used Equation 1 to determine gametophyte emission flux. We determined the area of the gametophyte using a ruler. Peak identification was performed with the PEAK software (Stein 1999) that integrates raw peak area units and retention times. To identify isoprene, a custom calibration curve was created from isoprene standard (Sigma, Deisenhofen, Germany). For quantification, peak areas of isoprene were determined after baseline correction. Levels of background contamination were determined from blanks. Analytical quality was analyzed by using Standard Reference Material of isoprene (NIST SRM 1515, National Institute of Standards and Technology, Gaithersburg, USA).

Isoprene emission measures were achieved twice during the nitrogen addition, before N addition in 2015 and after N addition in 2016. We calculated that we can fit ~9

magenta vesselsTM into each ~40cm diameter container (Figure 4.3). The portable Gas Chromatograph with the Reduced Gas Detector (GC/RGD2) was utilized in multiple field sites at the time of this study, and monthly isoprene emission collection was not possible. Isoprene emission measures were taken in the winter of 2015 and in the summer of 2016, but due to seasonal differences it was not possible at this time to compare isoprene emission measures before and after nitrogen addition.

Canopy level isoprene emission was measured using a MagentaTM vessel, but to establish area of moss under the vessel, we have used displacement by volume of moss tissue to calculate accurate headspace in the vessel from which isoprene measurement was taken. Where appropriate dry weight measurements were taken to assess initial moss selection. The volume displacement method was standardized by taking 10 point measurements of 16 cm² moss with differing heights under the vessel and placing it into a cylinder to accrue water displacement. Moss ramets are highly varied in their structure, unlike vascular plants in which a flat area can easily be calculated. We felt given mosses 3D structure this method is better suited than what is currently used for vascular plant isoprene emission assessment.

Our second physiology measure was chlorophyll fluorescence ratio, as a non-intrusive tool in using it as a proxy to establish information on photosynthetic reactions in the chloroplast (Schreiber et al. 1987). The fluorescence-emission spectra read out two maxima in the 690 nm and the 735 nm region. The fluorescence ratio F₆₉₀/F₇₃₅ for green leaves is higher (values of 0.8 to 1.1) than in the yellow to red wavelength region (525 to 633 nm) which only yields values for F₆₉₀/F₇₃₅ of 0.5 to 0.7 (Rinderle et al.

1988). Chlorophyll fluorescence ratio (CFR) was measured with a chlorophyll content meter (OPTI-Sciences model CCM-300, Hudson, USA).

Statistical Analyses

We used repeated measures ANOVA to determine the effect of site, nitrogen treatment and pot majority-sex on gametophyte length and gametophyte width for two years after nitrogen treatment (2016 and 2017). We used repeated measures ANOVA to determine the effect of site, nitrogen treatment, and pot majority-sex on reproductive effort (sporophytes) for three years after nitrogen treatment (2016, 2017, and 2018). We used ANOVA to determine the effect of site on isoprene production before we applied the nitrogen treatments, and we used ANOVA to determine the effect of site, nitrogen treatment, and pot majority-sex on isoprene emission after the treatment was applied. We used a similar ANOVA to determine the effect of site, nitrogen treatment and pot majority-sex on CFR after the nitrogen treatments were applied. We transformed our data when necessary to meet the assumptions of ANOVA. We also used Tukey-Kramer HSD Multiple Comparison test (Ellison et al. 2004) to find sources of significant differences between sites and nitrogen additions. JMP Pro 14.1 (SAS Institute, Cary, North Carolina, USA) was used for all analyses.

Results

Gametophyte length

We found significant differences among sites in gametophyte length (GL) (RM ANOVA; DF=5,591; F= 15.97; P <0.0001; Figure 4.4). After nitrogen addition, mean gametophyte length was highest from our Site 4 with mean values of 1.823 cm \pm 0.046 SEM (n=200). Our Site 5, had the smallest gametophyte length with mean values of 1.621 cm \pm 0.0371 SEM (n=200). We also found a significant site by time interaction where 11% of gametophyte length (GL) variation could be explained by time (RM ANOVA; DF=5,591; F= 45.71; P <0.0001), with site differences becoming less pronounced, and sites resembling each other as the experiment progressed.

Gametophyte length (GL) was significant different among nitrogen addition treatments, explaining 21% of variation in gametophyte length (GL) (RM ANOVA; DF=2,591; F=7.723; P=0.0005). The longest gametophyte length (GL) was in the 20 N kg ha⁻¹ yr⁻¹ addition with mean values of 2.259 cm \pm 0.101 SEM (n=200), and lowest in the 0 N kg ha⁻¹ yr⁻¹ addition with mean values of 1.186 cm \pm 0.042 SEM (n=200). In the 10 N kg ha⁻¹ yr⁻¹ addition we saw intermediate mean values of 1.886 cm \pm 0.078 SEM (n=200). We also found a significant nitrogen treatment by time interaction (RM ANOVA; DF=2,591; F=14.32; P<0.0001), where we saw gametophyte length mean values closer to each other in all three treatments.

Gametophyte length (GL) differed significantly between the male- and female-majority pots, with gametophyte length in female-majority pots longer than gametophyte length in male-majority pots (RM ANOVA; DF=1,591; F=19.65; P <0.0001). We also found a significant difference for the interaction between nitrogen treatment and pot majority sex (RM ANOVA; DF=2,591; F=5.4261; P = 0.0046; Figure 4.5). Gametophyte

length in female-majority pots was longest in the 20 N kg ha⁻¹ yr⁻¹ addition with mean values of 2.403 cm ± 0.132 SEM (n=200), and lowest in the 0 N kg ha⁻¹ yr⁻¹ addition with mean values of 1.344 cm ± 0.052 SEM (n=200). In the 10 N kg ha⁻¹ yr⁻¹ addition we saw intermediate mean values of 1.965 cm ± 0.126 SEM (n=200). Gametophyte length in male-majority pots was longest in the 20 N kg ha⁻¹ yr⁻¹ addition with mean values of 2.065 cm ± 0.162 SEM (n=200), and lowest in the 0 N kg ha⁻¹ yr⁻¹ addition with mean values of 1.175 cm ± 0.0437 SEM (n=200). In the 10 N kg ha⁻¹ yr⁻¹ addition we saw intermediate mean values of 1.437 cm ± 0.0404 SEM (n=200). The interaction between pot majority sex and time was not significant (RM ANOVA; DF=1,591; F=2.75; P = 0.09), as the differences in gametophyte length between male- and female-majority pots did not change during the course of the experiment.

Gametophyte width

We also found statistical significance in site differences for gametophyte width (GW) (RM ANOVA; DF=5,591; F= 13.8676; P <0.0001; Figure 4.6). The widest gametophyte width (GW) was found in Site 5 with mean values of 1.184 cm ± 0.030 SEM (n=200). Site 3 had the least wide gametophyte width cm (GW) with mean values of 1.083 cm ± 0.010 SEM (n=200). We also found a significant site by time interactions (RM ANOVA; DF=5,591; F= 22.27; P <0.0001), with sites becoming significantly less different in gametophyte width as the experiment progressed.

Gametophyte width (GW) was not significant between treatments (RM ANOVA; DF=2,591; F=1.28; P=0.28). Gametophyte width was also not significantly different

between male- and female-majority pots (RM ANOVA; DF=1,591; F=0.71; P=0.40). However, for gametophyte width the interaction between nitrogen additions and pot majority sex was significant (RM ANOVA; DF=2,591; F= 32.5288; P <0.0001; Figure 4.7). Gametophyte width in majority female pots was widest in the 20 N kg ha⁻¹ yr⁻¹ addition with mean values of 2.403 cm ± 0.139 SEM (n=200), and least wide in the 0 N kg ha⁻¹ yr⁻¹ addition with mean values of 1.343 cm ± 0.056 SEM (n=200). In the 10 N kg ha⁻¹ yr⁻¹ addition we saw intermediate mean values of 1.192 cm ± 0.0232 SEM (n=200). Gametophyte width in male-majority pots was widest in the 10 N kg ha⁻¹ yr⁻¹ addition with mean value of 1.634 cm ± 0.136 SEM (n=200), and least wide in the 0 N kg ha⁻¹ yr⁻¹ addition with mean values of 0.910 cm ± 0.0253 SEM (n=200). In the 20 N kg ha⁻¹ yr⁻¹ addition we saw intermediate mean values of 1.192 cm ± 0.0232 SEM (n=200).

We also found a significant interaction among pot majority sex, treatment and time (RM ANOVA; DF=2,591; F= 16.14; P <0.0001). Gametophyte width decreased in both male- and female-majority pots over the course of the nitrogen addition, because of moss rapid turnover rate. While gametophyte width in female-majority pots did not change over time, gametophyte width in male-majority pots changed during our nitrogen addition, and the widest mean values were found in the 20 N kg ha⁻¹ yr⁻¹ addition with mean value of 0.921 ± 0.0191 SEM (n=200). Our least wide gametophyte width in male-majority pots remained in the 0 N kg ha⁻¹ yr⁻¹ addition, with mean values of 0.765 cm ± 0.0143 SEM (n=200).

Reproductive effort

Female reproductive effort in terms of sporophyte count was statistically significant among sites (RM ANOVA; DF=5; F=5, 49; P =0.0002; Figure 4.8). The highest count of sporophytes was from Site 6 with 1848 sporophytes (n=20), and we counted the lowest sporophytes in Site 3 with 945 (n=20). Some sites increased sporophyte output between the years and treatment, while other sites remained stable across time.

We found that treatment did not significantly affect sporophyte count (RM ANOVA; DF=2,49; F=2.6403; P = 0.08). Reproductive effort in sporophyte count was statistically significant between male-majority and female-majority pots, with plants in female-majority pots producing more sporophytes than plants in male-majority pots (RM ANOVA; DF=1,449; F=13.4107; P=0.0006). We did not find majority-sex by treatment interaction for sporophyte count (RM ANOVA; DF=2,49; F=2.6559; P = 0.08). Lastly, we saw a significant time and majority-sex interaction (RM ANOVA; DF=2,48; F=4.1298; P=0.0221). Sporophyte count was similar in both male-majority and female-majority pots for the first two years of nitrogen addition, with very low count of sporophytes in male-majority pots. For our last year of nitrogen addition, sporophyte count was much higher in female-majority pots than male-majority pots.

Isoprene emission before nitrogen addition

Site-specific differences alone explained 22% variability of isoprene emission (ANOVA; DF=5; F=13.1835; P <0.0001; Figure 4.9). Within each site, we saw even more variation in isoprene emission than between sites (Figure 4.10). Overall, we saw

mean isoprene emission values of $6.787 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.218 \text{ SEM}$ (n=240). The highest isoprene emission was seen from Site 1 with a mean values of $10.337 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.281 \text{ SEM}$ (n=40). We saw Site 6 mean values of $7.123 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.114 \text{ SEM}$ (n=40). Site 5 mean values of $7.118 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.856 \text{ SEM}$ (n=40). Site 3 mean values of $6.742 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.186 \text{ SEM}$ (n=40). Site 4 mean values of $5.534 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.081 \text{ SEM}$ (n=40). Lastly, Site 2 had the smallest isoprene emission with a mean values of $3.867 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.134 \text{ SEM}$ (n=40).

We also saw sex-specific differences in isoprene emission with female-majority pots having a larger mean emission than male-majority pots (ANOVA; DF=1; F=10.2925; P <0.0015; Figure 4.11). We saw that Site 1, Site 2, Site 4 and Site 6 female-majority pots had higher isoprene emission values than male-majority pots. Only in Site 3, and Site 5, we saw male-majority pots having higher isoprene emission than female plots. In Site 1, we saw the highest isoprene emission from female-majority plots with mean values of $13.735 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.472 \text{ SEM}$ (n=40). Our lowest isoprene emission from majority-female plots was seen from Site 2 with mean values of $4.231 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 1.154 \text{ SEM}$ (n=40). We saw the highest isoprene emission from male-majority pots from Site 1 with mean values of $6.932 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 1.79 \text{ SEM}$ (n=40), and the lowest isoprene emission from Site 2 with mean values of $3.508 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.812 \text{ SEM}$ (n=40).

Isoprene emission after nitrogen addition

We continued to see site-specific differences which explained 12% variability of isoprene emission between sites after nitrogen addition (ANOVA; DF=11; F=7.3488; P <0.0001; Figure 4.12; Figure 4.13). After nitrogen addition we saw isoprene emission mean values of $28.562 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.710 \text{ SEM}$ (n=240). Isoprene emission was highest for Site 1 with mean values of $38.801 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.793 \text{ SEM}$ (n=40). Site 2 mean values were $35.321 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.894 \text{ SEM}$ (n=40). Site 3 mean values were $24.678 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.679 \text{ SEM}$ (n=40). Site 5 mean values were $27.298 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.663 \text{ SEM}$ (n=40). Site 6 mean values were $24.173 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.521 \text{ SEM}$ (n=40). Our lowest isoprene emission was seen from Site 4 with mean values of $21.093 \text{ nmol m}^{-2} \text{ s}^{-1} \pm 0.512 \text{ SEM}$ (n=40).

We did not observe a statistically significant difference between male- and female-majority pots in isoprene emission after N addition (P=0.89). We did however, observe a significant interaction between treatment and sexual expression in isoprene emission in the $10 \text{ N kg ha}^{-1} \text{ yr}^{-1} \text{ N}$ treatment (ANOVA; DF=5; F=23.63; P < 0.0001; Figure 4.14). Female and male isoprene emissions were highest in the $10 \text{ N kg ha}^{-1} \text{ yr}^{-1} \text{ N}$ treatments. Male mean values were higher than female values $2.341 \pm 0.023 \text{ nmol m}^{-2} \text{ s}^{-1} \text{ SEM}$ (n=44). Female mean values were $2.214 \pm 0.181 \text{ nmol m}^{-2} \text{ s}^{-1} \text{ SEM}$ (n=36). In the $20 \text{ N kg ha}^{-1} \text{ yr}^{-1}$ females values were similar to male values, female mean values were $1.514 \pm 0.035 \text{ nmol m}^{-2} \text{ s}^{-1} \text{ SEM}$ (n=52), and male mean values were $1.560 \pm 0.105 \text{ nmol m}^{-2} \text{ s}^{-1} \text{ SEM}$ (n=32). Lastly, in the $0 \text{ N kg ha}^{-1} \text{ yr}^{-1}$ treatment female values were higher than male values, with female values of $1.625 \pm 0.141 \text{ nmol m}^{-2} \text{ s}^{-1} \text{ SEM}$ (n=32), and male values of $1.389 \pm 0.015 \text{ nmol m}^{-2} \text{ s}^{-1} \text{ SEM}$ (n=48).

We also saw treatment specific differences which explained 32% variation in isoprene emission (ANOVA; DF=2; F=56.09; P <0.0001; Figure 4.15). Our 10 kg N ha⁻¹ yr⁻¹ treatment had the highest isoprene emission with mean values of 2.284 nmol m⁻² s⁻¹ ± 0.071 SEM (n=80). The 20 kg N ha⁻¹ yr⁻¹ treatment had mean values of 1.533 nmol m⁻² s⁻¹ ± 0.033 SEM (n=84). Lastly, we saw our lowest isoprene emission with our 0 kg N ha⁻¹ yr⁻¹ treatment with mean values of 1.484 nmol m⁻² s⁻¹ ± 0.041 SEM (n=80).

Physiological measurements

We saw site-specific differences in CFR values (ANOVA; DF=5; F=8.1600; P <0.0001; Figure 4.16). Our highest CFR values were seen from Site 6, with mean values of 0.9222 ± 0.006 SEM (n=484). Our lowest CFR values were found in Site 4, with mean values of 0.85 ± 0.0190 SEM (n=484).

Nitrogen additions significantly affected CFR (ANOVA; DF=2; F=3.8437; P <0.0228; Figure 4.17). Our 0 kg N ha⁻¹ yr⁻¹ addition, was significantly lower (0.998 ± 0.0124 SEM; n=484) in CFR than our 20 kg N ha⁻¹ yr⁻¹ addition (1.007 ± 0.009 SEM; n=484). Our 10 kg N ha⁻¹ yr⁻¹ addition was not significantly different from either other addition. We did not find sex-specific differences in our CFR values (P=0.097).

Discussion

We collected moss samples from six locations around Portland Oregon, and we have established a large common garden experiment where we used Ammonium nitrate (NH₄NO₃) to evaluate the effects of nitrogen addition on morphology, reproductive

effort, and physiology in *P. juniperinum*. We used nitrogen values of 0 kg N ha⁻¹ yr⁻¹, 10 kg N ha⁻¹ yr⁻¹, 20 kg N ha⁻¹ yr⁻¹ as for the Pacific Northwest nitrogen deposition spans in this range of 0.15 kg N ha⁻¹ yr⁻¹ to 39 kg N ha⁻¹ yr⁻¹ (Root et al. 2013). We found significant effects of nitrogen addition and majority-sex of populations on gametophyte morphology. We also found significant effects of the nitrogen treatment on isoprene emissions and chlorophyll fluorescence ratio. We discuss our results below.

Gametophyte morphology

Gametophyte length (GL) and gametophyte width (GW) had high variation between sites (Figure 4.4; Figure 4.6). We saw the dynamics of moss communities going through cyclic changes as evidenced by rapid turnover of all moss populations. We found that both gametophyte length and gametophyte width were significantly affected by our six sites. However, for both morphological traits, the differences among sites diminished across time.

We saw gametophyte length (GL) affected by nitrogen addition (Figure 4.5), but not gametophyte width (GW) (Figure 4.7). We saw 21% variation in gametophyte length be attributable to nitrogen addition (Figure 4.5). *P. juniperinum* could have used the added N to grow longer as it established optimal growth and the benefit of extra nitrogen was utilized either as respired carbon or allocated into belowground growth; as extensive rhizoidal system exist in this species (Wigglesworth 1947). In *Polytrichum* species, photosynthetic assimilates can be added to form new structural material belowground, or they can be respired or allocated to aboveground structures (Skre et al. 1983). Work from

previous studies shows that nitrogen in *Polytrichum* ecosystems is retained in the soil and dead moss tissue (Bowden 1991). Further work will be required to assess belowground biomass to fully comprehend nitrogen treatment affects both aboveground and belowground where it could be utilized by rhizoids or stored in the soil.

Sex-specific resource allocation was evident. Gametophyte length (GL) was affected by sex, with majority-female pots having longer lengths than males (Figure 4.5), and this difference was significantly affected by the nitrogen treatments. The sex-specific difference was not found in the control treatment or in the high nitrogen treatment, but only in the 10 kg N ha⁻¹ year⁻¹ treatment.

We know that *Polytrichum* species have the very low caloric values, with very little energy fixed in tissue of the moss (Rastorfer 1976), suggesting that they spend all their acquired photosynthetic carbon on either allocation or respiration. Other studies have found that in *Polytrichum* species, variations in N addition of 20 vs. 10 kg N ha⁻¹ year⁻¹ did not affect growth (Van der Wal et al. 2005). While others, have shown that *Polytrichum* populations that have been exposed to nitrogen addition have greater height increase at a cost of 36% less new shoots (Bell et al. 1992), but we do not know if these new shoots were sexually expressing.

Reproductive effort

Nitrogen treatment had no significant effect on reproductive effect. However, reproductive effort in majority-female and majority-male pots was statistically different when it came to sporophyte reproduction, which was higher than male splash cup counts,

as expected. Sporophyte count was highly variable among sites. Sporophytes production was most likely a result of inter-gametophyte self-fertilization (Stark *et al.* 2009; Koncz *et al.* 2012). We saw that non-sexually expressing gametophytes outnumbered female and male gametophyte counts (Figure 4.8). In bryophytes, sex expression is reduced mainly because sporophyte maturation is resource-limited (Stark *et al.* 2009). Variation among populations in viability and germination capability of the spores refers to different levels of effective sexual reproduction. Populations with high viability and germination capability of spores indicate effective realized sexual reproduction (Longton 1988, Innes 1990). Denser populations as compared to sparser ones have a higher allocation to sexual reproduction (Wyatt and Derda 1997, Cronberg *et al.* 2006). We saw extremely dense populations produce more sporophytes than less dense populations.

Physiology measurements

In this study, nitrogen addition influenced isoprene emission in *P. juniperinum* populations. We are the first to report isoprene emission increasing under nitrogen addition in any moss species, and we are the first to report isoprene emission increasing under nitrogen addition in *P. juniperinum*. We found significant effects of the nitrogen treatment on isoprene emission in this species. Isoprene emission variation was statistically significant between sites and explained 30% variation in winter of 2015 (Figure 4.9), and within sites (Figure 4.10). We also saw isoprene emission differences between the sexes before nitrogen addition, suggesting sex-specific resource allocation at this time of year, with female isoprene emission higher than male isoprene emission

(Figure 4.11). After nitrogen addition, we continued to see site-specific differences in the summer of 2016 explaining 12% variability between sites (Figure 4.12) and within sites (Figure 4.13). Isoprene emission rates vary among vascular plant species from near zero to over 50 nmol m⁻² s⁻¹ (Harley et al. 1999), and here we are the first to show large variation in isoprene emission from *P. juniperinum* under nitrogen addition.

In our experimental study, the medium level nitrogen treatment resulted in the highest isoprene emission level with almost twice the isoprene emission as the control treatment with no additional nitrogen, and significantly more than the high nitrogen treatment (Figure 4.14; Figure 4.15). The availability of nitrogen affects the concentration of secondary compounds such as isoprene in a predictable manner according to the carbon/ nutrient balance hypothesis (Bryant et al. 1983). If a plant is growing in nitrogen poor environments, the plant can use the carbon from photosynthesis for isoprene emission instead of growth allowing it to have increased secondary compounds (Bryant et al. 1983, Herms et al. 1992, Jones et al. 1999). A previous study assessing nitrogen in *P. juniperinum* population has found that belowground biomass of both live and dead tissue had higher nitrogen concentrations than aboveground tissue (Bowden 1991). This suggest that *P. juniperinum* under high nitrogen addition may not utilize all of available nitrogen.

Nitrogen uptake response in mosses involves several steps (Heijmans et al. 2001, Xing et al. 2010). Initially, mosses can utilize nitrogen and benefit from nitrogen addition (Jauhainen et al. 1994; Williams et al. 1997). In many moss populations, nitrogen is limiting (Bowden 1991; Bakken 1995; Eckstein et al. 1999, 2000; Aldous 2002; Ayres et al. 2006; Koranda et al. 2006; Arróniz-Crespo et al. 2008; Du et al. 2014; Juutinen et al.

2015; Felix et al. 2016; Izquieta-Rojano et al. 2016; Becker et al. 2017; Kosonen et al. 2018), and thus additions of nitrogen result in increasing photosynthesis which can be used for additional growth, reproduction, or production of secondary compounds. The first phase of utilizing the nitrogen, when plants are limited in nitrogen, was seen in *P. juniperinum* in the difference between our control and our medium nitrogen addition, when isoprene emission increased significantly with nitrogen addition. This reflects similar findings in vascular plants, where nitrogen addition increases isoprene emission rate and photosynthesis (Harley et al. 1994).

Eventually, in the final phase of nitrogen uptake response in mosses, a maximum nitrogen content is reached and nitrogen leaches into the soil or fungal hyphae in the rhizome mat (Bowden 1991; Bragazza et al. 2005). Once maximum nitrogen content is reached, which we saw in our highest nitrogen addition, the added nitrogen may have been leached into the rhizoidal mat or soil reflecting the low isoprene emission we saw in our high nitrogen addition group. At this stage, the added nitrogen was potentially harmful as we saw these values were close to isoprene emission values from our no nitrogen, control pots. Nutrient translocation and retention from older tissue to young tissue is a key mechanism mosses use to avoid nutrient loss and nitrogen utilization peaks during highest growth (Clymo et al. 1982; Aerts et al. 1999). Under our highest nitrogen addition, carbon allocation could have been used for peak growth and additional nitrogen was not utilized resulting in lower isoprene emission, as all the resources were utilized in either above or belowground growth. The highest nitrogen addition we applied could have represented an environmental shift in available nitrogen to *P. juniperinum*, and this

may have hindered the mosses ability to uptake this form of nitrogen and utilize it. We know that vascular plants can utilize many forms of nitrogen, and we could have seen a decrease in isoprene emission in our high nitrogen addition because of competition for carbon skeletons between the supply and demand of isoprenoid precursor dimethylallyl pyrophosphate (DMAPP) (Rosenstiel et al. 2004).

Mosses can also utilize different forms of nitrogen, and mosses access to nitrogen depends on direct bioavailable nitrogen coming from atmospheric deposition, internal nitrogen cycling by mineralization, and lastly species dependent nitrogen translocation (Aldous 2002; Bowden 1991). Future research should focus on determining the role of nitrogen from the demand side process including analyses of aboveground and belowground tissue concentrations, fungal biomass retention of nitrogen, and soil nitrogen content. Additional insights could be gained from work to understand what carbon allocation strategies *P. juniperinum* uses and what the consequences for ecosystem level carbon cycling under enhanced nitrogen deposition.

We also saw significant site-specific chlorophyll fluorescence ratio (Figure 4.16). Our highest CFR values were seen from forest Site 5, and Site 6, indicating reduced stress compared to the other sites. We found our highest CFR values in the highest nitrogen treatment (Figure 4.17), which we used as a proxy to establish information on photosynthetic reactions in the chloroplast (Schreiber et al. 1987). Stress in plants induces lower chlorophyll content, and lower rate of photosynthesis, because of degradation of chlorophyll content due to many factors, including excessive nitrogen

addition (Rinderle et al. 1988). We did not see this pattern, as only healthy green gametophytes were sampled for chlorophyll content.

Conclusion

We have shown that moderate nitrogen addition significantly increases isoprene emission in moss populations. These results have important implications for understanding isoprene emissions from bryophyte communities as anthropogenic nitrogen levels increase. Our data also suggest that nitrogen male and female of *P. juniperinum* will respond differentially to nitrogen in terms of morphology, but this may not result in differential response in isoprene emission at the population level. Future work should focus on understanding the effect of additional community interactions, including the microbiome, on isoprene production in mosses across nitrogen gradients.

Tables and Figures

Table 4.1 Summary table of *Polytrichum juniperinum* from six populations (n=60).

Site	Location	Expected Nitrogen background	Latitude	Longitude	Elevation (meters)
1	Highway	High N	45°41'38.34"N	122°52'14.23"W	15
2	Highway	High N	45°38'7.45"N	122°49'45.09"W	21
3	Intermediary	Medium N	45°41'50.54"N	122°52'40.83"W	145
4	Intermediary	Medium N	45°46' 58.78" N	123°22'14.14" W	315
5	Forest	Low N	45°44'6.80"N	122°59'30.62"W	412
6	Forest	Low N	45°47'17"N	122°56'16.00"W	241

Table 4.2 Site characteristics of *Polytrichum* moss collected in the winter of 2015. Soil type, slope, depth to water table and available water in storage profile data was assessed from Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture. Web Soil Survey. Available online. Accessed (7/13/2018)<https://websoilsurvey.sc.egov.usda.gov>.

Site	Location	Location	Soil type	Slope	Depth to water table (cm)	Available water storage in profile (mm)
1	Highway	High N	gravelly loam, un-weathered bedrock	30-70 %	203 +	Low (83)
2	Highway	High N	sandy loam, gravelly loamy sand	40-60 %	91-183	Moderate (216)
3	Intermediary	Medium N	silt loam, clay loam	60-70 %	63-99	Moderate (198)
4	Intermediary	Medium N	silt loam, clay loam, weathered bedrock	30-60 %	203+	Moderate (216)
5	Forest	Low N	silt loam, clay loam	12-20 %	163	Moderate (193)
6	Forest	Low N	silt loam	15-30 %	64-252	Moderate (191)

Table 4.3 Age classification rubric of *P. juniperinum* populations.

Age category	Classification
Year 1	(0.0-2.0 cm) (n=50)
Year 2	(2.0-4.0 cm) (n=50)
Year 3	(4.0-6.0 cm) (n=50)
Year 4	(6.0-8.0 cm) (n=50)
Year 5	(8.0-10.0 cm) (n=50)
Year 6	(10.0-12.0 cm) (n=50)
Year 7	(12.0-14.0+ cm) (n=50)

Table 4.4 Summary of all morphological measurements taken from *P. juniperinum* populations.

12/01/2015 Before N	06/05/2016 After N	01/10/2017 After N	06/05/2018 After N
G. Length (mm)	G. Length (mm)	G. Length (mm)	G. Length (mm)
G. Width (mm)	G. Width (mm)	G. Width (mm)	G. Width (mm)
G. Canopy area (mm)	G. Canopy area (mm)	G. Canopy area (mm)	G. Canopy area (mm)
G. Rhizoid length (mm)			
Damage by larvae	Damage by larvae	Damage by larvae	Damage by larvae
% cover green	% cover green	% cover green	% cover green
% cover brown	% cover brown	% cover brown	% cover brown
CFR		CFR	
CFR by sex		CFR by sex	
Isoprene emission (nmol m⁻² s⁻¹)		Isoprene emission (nmol m⁻² s⁻¹)	
Canopy area (m ²) for Isoprene		Canopy area (m ²) for Isoprene	
Total sporophyte count	Total sporophyte count	Total sporophyte count	Total sporophyte count
Last year sporophyte count	Last year sporophyte count	Last year sporophyte count	Last year sporophyte count
New year sporophyte count	New year sporophyte count	New year sporophyte count	New year sporophyte count
Total splash cup count	Total splash cup count	Total splash cup count	Total splash cup count
Last year splash cup	Last year splash cup	Last year splash cup	Last year splash cup
New year splash cup	New year splash cup	New year splash cup	New year splash cup

Non-sexual gametophytes	Non-sexual gametophytes	G. Canopy area (mm)	Non-sexual gametophytes
Total gametophyte	Total gametophytes	Total gametophyte	Total gametophyte
Age category	Age category	Age category	Age category
		Pots with less than 50 shoots	Pots with less than 50 shoots

Table 4.5 Experimental design set up showing three nitrogen additions. Within each block there are twelve pots. The three additions contain six control pots, two control pots in each addition and 20 pots in each addition.

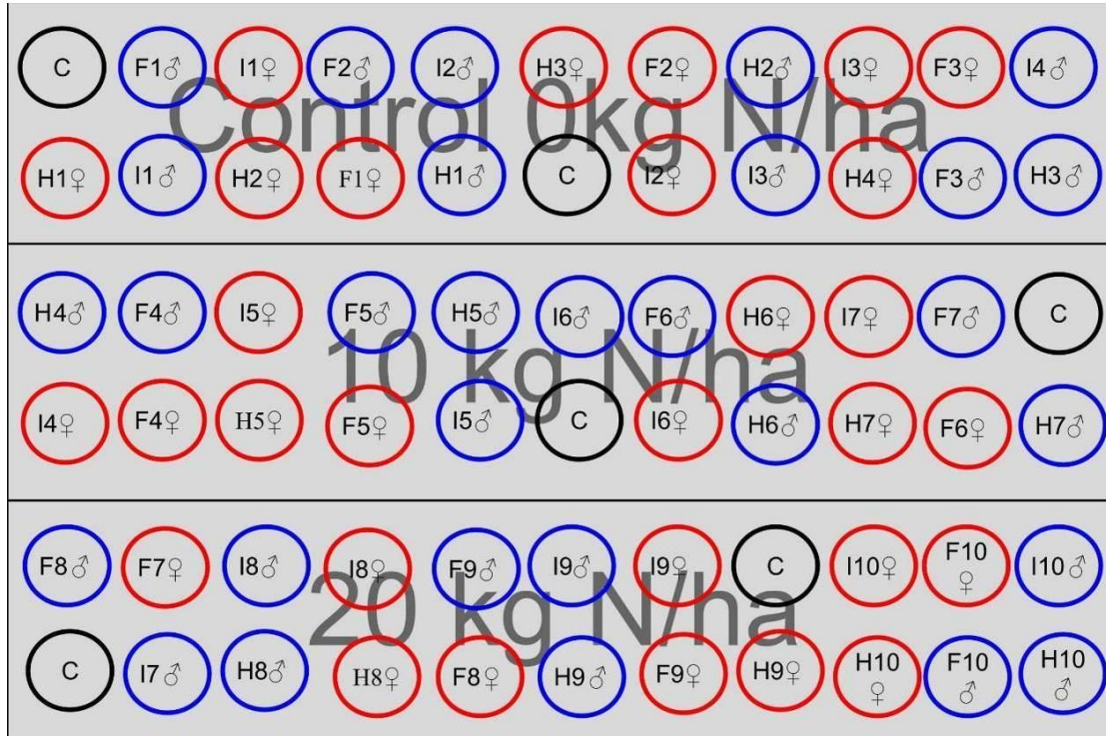


Figure 4.1 Ammonium nitrate addition to *P. juniperinum* moss via 1-Liter (L) hand held pump mister sprayer (Solo INC., Model 418, Newport News, VA).



Figure 4.2 Small 77mm × 77 mm × 97mm static chamber (magenta jar) created for headspace analyses using (Magenta vessel™). Equipped with battery-operated fan to ensure equal mixing of air in the chamber and avoid settling.



Figure 4.3 Magenta vessel™ used to collect isoprene emission data from *P. juniperinum* populations before and after nitrogen addition. Seen here inverted with Pressure-Lok® precision analytical syringe (VICI Precision Sampling Inc., Baton Rouge, LA, USA).



Figure 4.4 Gametophyte length (GL) cm versus sites (1-6) in *P. juniperinum* (n=3596). Site is plotted on the x-axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between gametophyte length and site. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

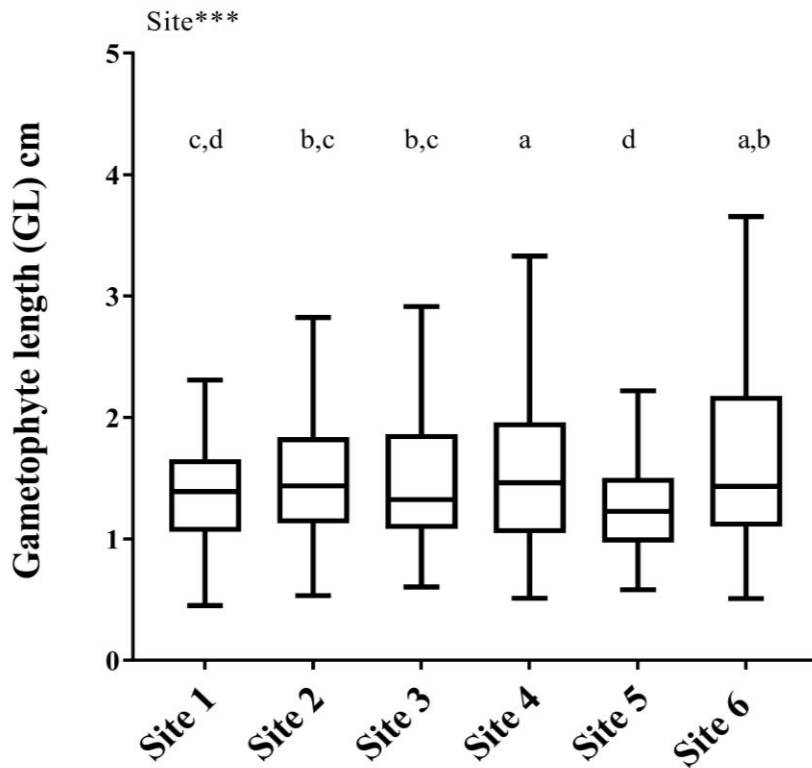


Figure 4.5 Gametophyte length (GL) cm versus sex and nitrogen treatment in *P. juniperinum* (n=3596). Nitrogen treatment is plotted on the x-axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between gametophyte length vs. nitrogen treatment and sex. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

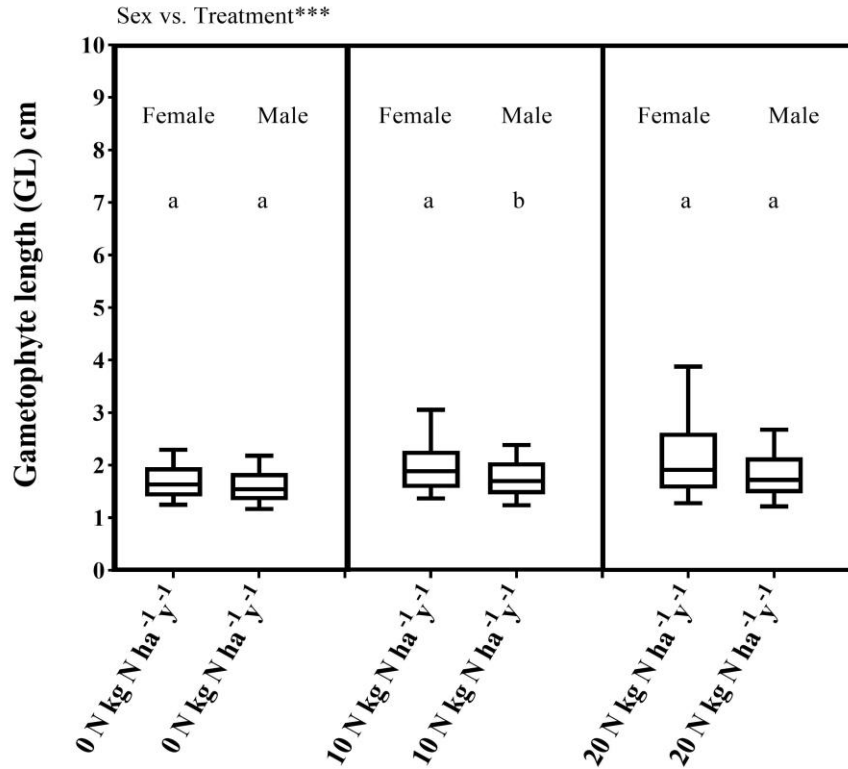


Figure 4.6 Gametophyte width (GW) cm versus sites (1-6) in *P. juniperinum* (n=3596). Site is plotted on the x-axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between avg. gametophyte width cm (GW) and site. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

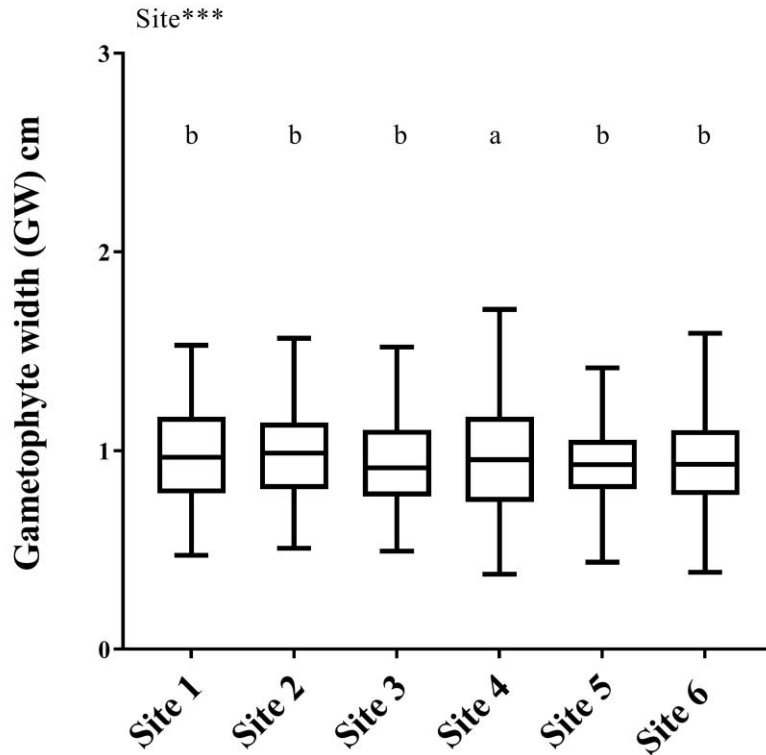


Figure 4.7 Gametophyte width (GW) cm versus sex and nitrogen treatment in *P. juniperinum* (n=3596). Nitrogen treatment is plotted on the x-axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between gametophyte width vs. nitrogen treatment and sex. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

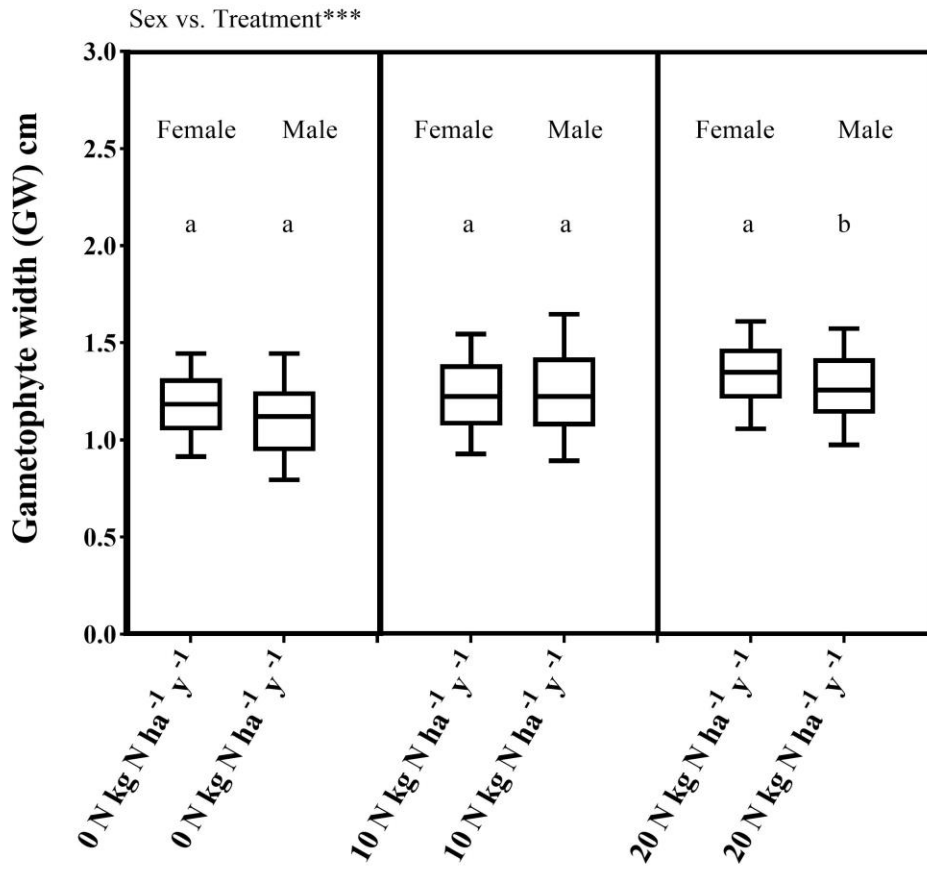


Figure 4.8 Reproductive effort versus sexual expression in *P. juniperinum* (n=1440). Sexual expression is plotted on the x-axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between total reproductive effort by sporophytes, splash cups, and non-sexual gametophytes. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

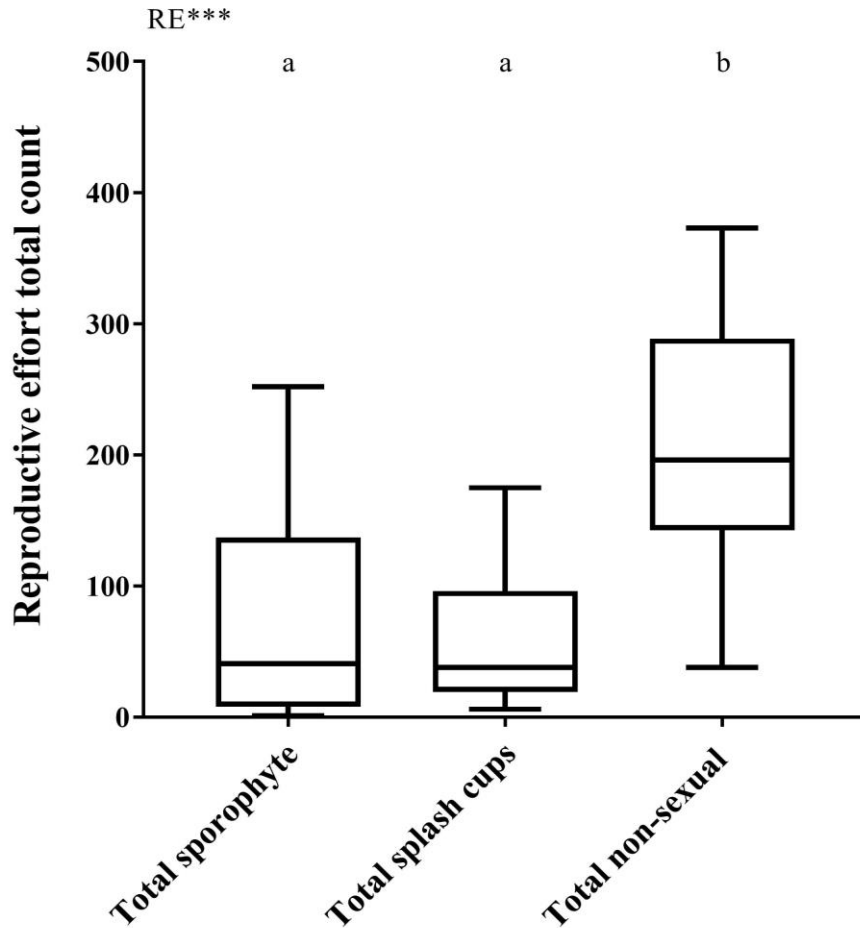


Figure 4.9 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) in the winter of 2015 versus site-specific differences in *P. juniperinum* gametophytes ($n=244$). Site is plotted on the x-axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between avg. isoprene emission and site. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

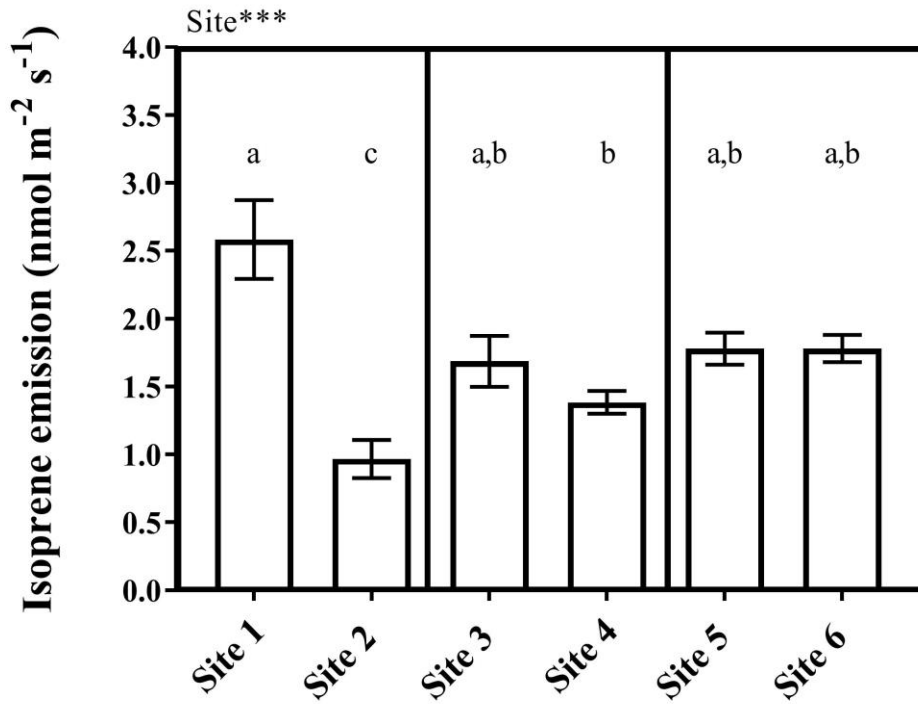


Figure 4.10 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) before N treatment versus within site-specific differences in *P. juniperinum* gametophytes (n=244). Site is plotted on the x-axis. Error bars represent $\pm 1\sigma$ (standard deviation). A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

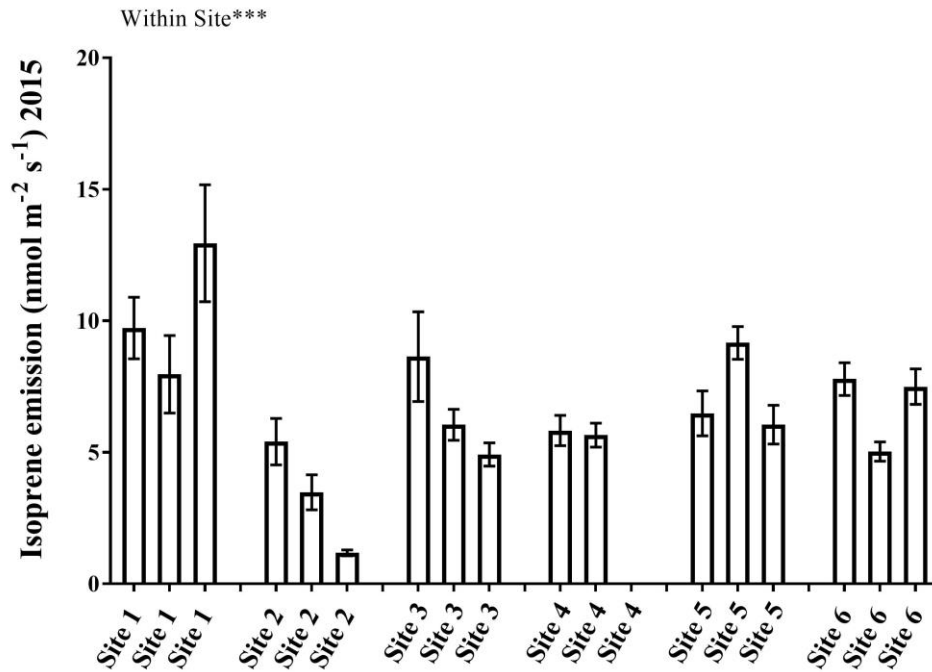


Figure 4.11 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) before N treatment versus sex in *P. juniperinum* gametophytes (n=244). Sex is plotted on the x-axis. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

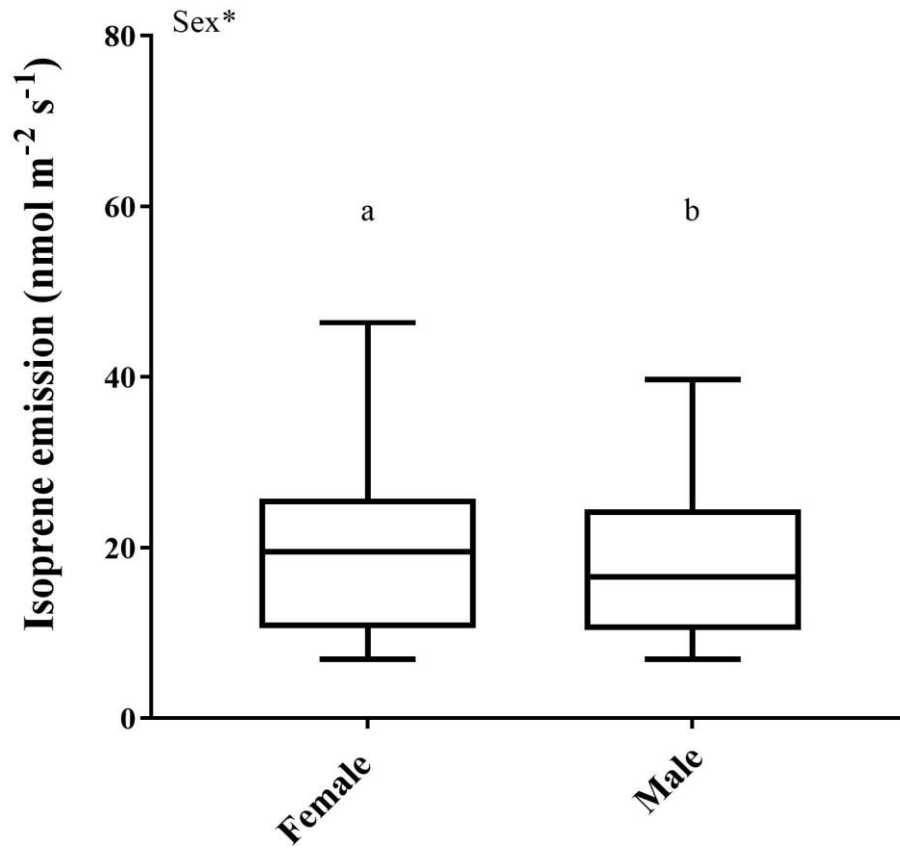


Figure 4.12 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) after N treatment versus site-specific differences in *P. juniperinum* gametophytes ($n=244$). Site is plotted on the x-axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between avg. isoprene emission and sites (1-6). Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

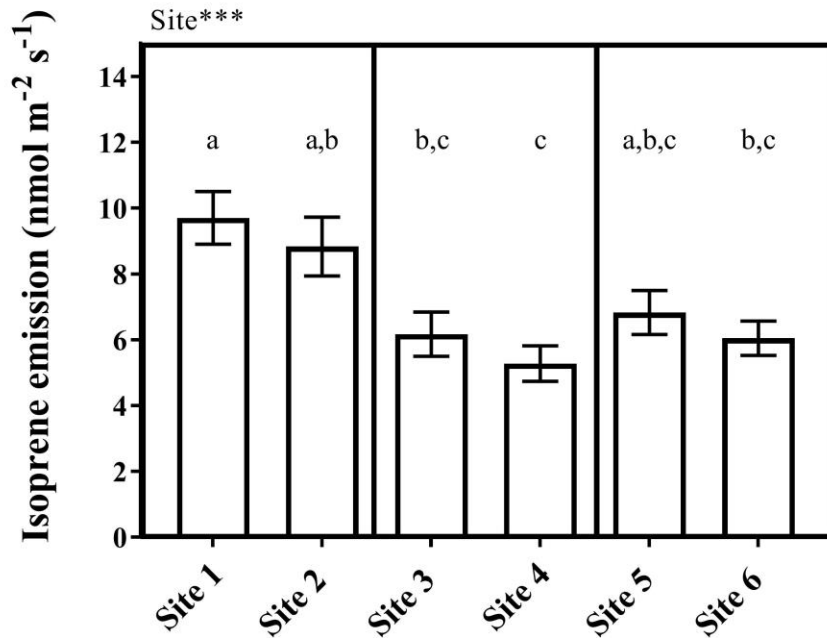


Figure 4.13 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) after N treatment versus within site-specific differences in *P. juniperinum* gametophytes (n=244). Site is plotted on the x-axis. Error bars represent $\pm 1\sigma$ (standard deviation). A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

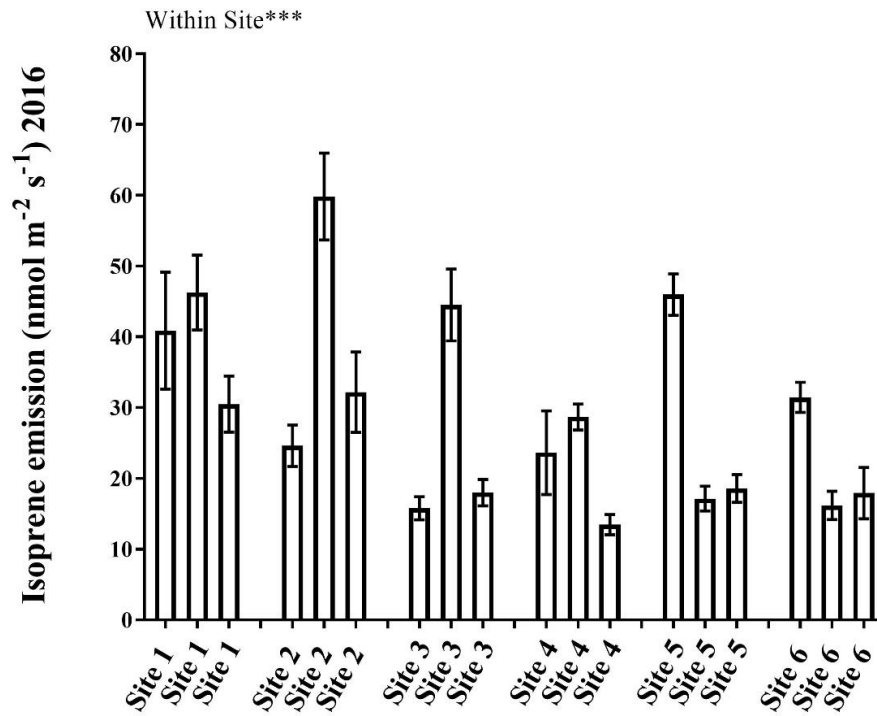


Figure 4.14 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) after N treatment versus sex in *P. juniperinum* gametophytes (n=244). Treatment is plotted on the x-axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between avg. isoprene emission vs. nitrogen treatment. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

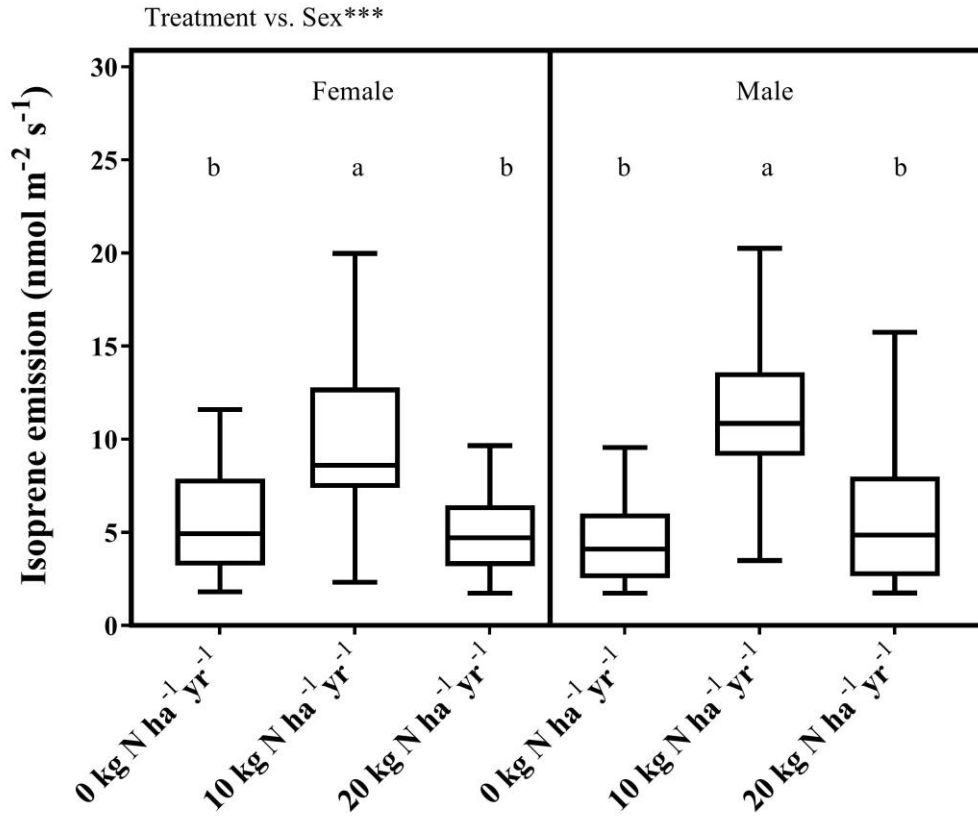


Figure 4.15 Isoprene emission ($\text{nmol m}^{-2} \text{s}^{-1}$) versus nitrogen treatment in *P. juniperinum* gametophytes (n=244). Nitrogen addition is plotted on the x-axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between avg. isoprene emission vs. nitrogen treatment. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

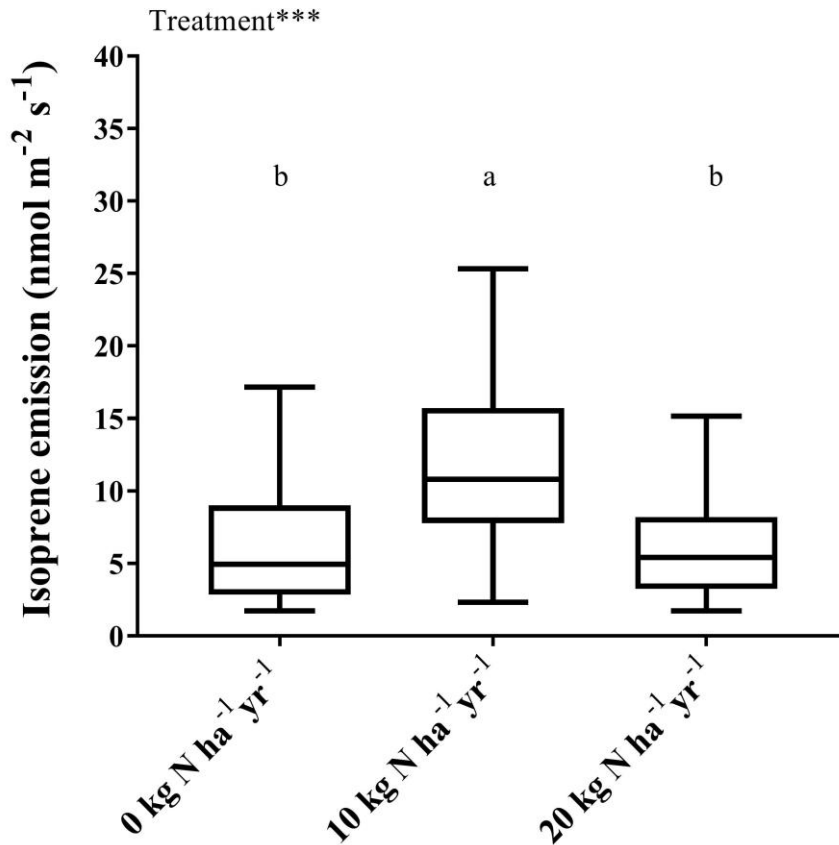


Figure 4.16 Chlorophyll Fluorescence Ratio (CFR) measures versus site in *P. juniperinum* gametophytes (n=244). Site is plotted on the x-axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between CFR vs. Site (1-6). Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.

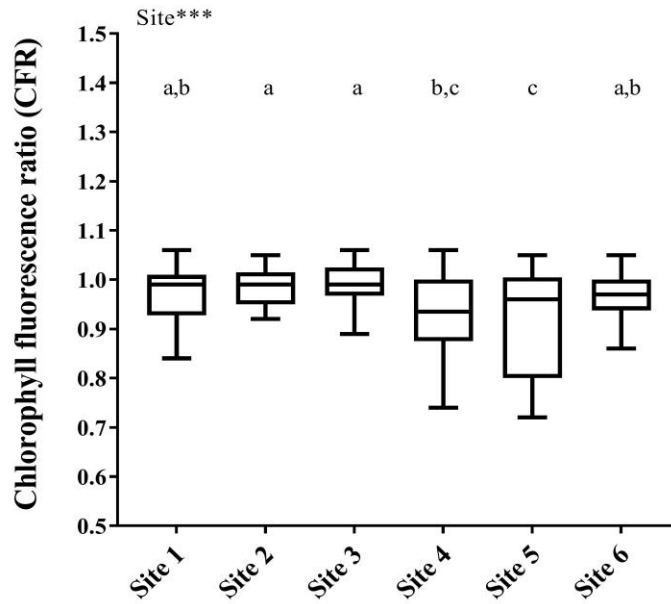
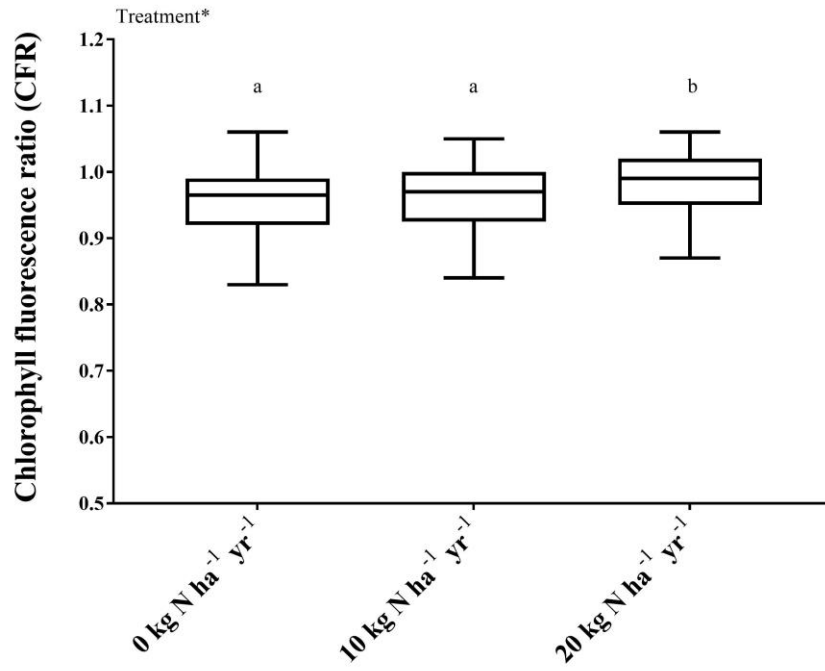


Figure 4.17 Chlorophyll Fluorescence Ratio (CFR) measures versus N treatment in *P. juniperinum* gametophytes (n=244). Nitrogen treatment is plotted on the x-axis. Tukey-Kramer HSD Multiple Comparisons Test shows areas of significant difference between CFR vs. nitrogen treatment. Error bars represent $\pm 1\sigma$ (standard deviation). Letters show statistical significance between means. Means with the same letter are not significantly different. A * indicates significant difference, ** indicates a highly significant difference, *** indicates high statistical significance, NS indicates no significance.



Chapter 5

Conclusion

We still do not understand the functional role of isoprene production and emission from vascular plants (Sharkey et al. 2017) or non-vascular plants. The ability to emit isoprene has been gained and lost multiple times during plant evolution (Sharkey et al. 2007, 2013; Fineschi et al. 2013; Monson et al. 2013; Dani et al. 2014; Loreto et al. 2015). Isoprene is the second most abundant BVOC in the atmosphere, and it represents one of the most important interactions between plants and the atmosphere (Monson et al. 1995; Sharkey et al. 1999; Niinemets et al. 2004; Peñuelas et al. 2010; Loreto et al. 2010; Guidolotti et al. 2019; Nagori et al. 2019; Li et al. 2019). Isoprene is the most abundant non-methane hydrocarbon in air near forests and has the greatest potential for transport from forest to urban areas (Khalil et al. 1992). In many ecosystems and even some urban areas, isoprene may dominate photochemical reactions in the atmospheric boundary layer (Chameides et al. 1988; Goldstein et al. 1998; Arneth et al. 2007; Park et al. 2011; Chang et al. 2014; Kota et al. 2015).

BVOC emission data are quite limited for non-vascular plants which do not possess structures such as stomatal pores, complex leaves, stems, or roots regulating water loss (Duckett et al. 2009; Ruzsala et al. 2011; Chater et al. 2016). Since the signaling pathways for these emissions are present in both vascular plants and mosses (Cove et al. 2009), mosses make ideal systems to study the origin, diversification, and early function of isoprene emission. These often-overlooked early land plants might

provide the crucial link to finally understand why plants in general produce and emit isoprene, partially because emission is more common in mosses than any other major land plant group (Hanson et al. 1999).

Isoprene emission from *Polytrichum juniperinum* protonema

In Chapter 2, we examined at what life stage mosses emit isoprene and at what cost. We have discovered that after spore germination, *P. juniperinum* is capable of isoprene emission in the protonema phase of its life cycle. We hypothesize that mosses are releasing isoprene as it is produced, and thus in their earliest life stages, as they do not possess guard cells or stomata in their gametophytic leaves (Woodward 1998; Raven 2002; Chater et al. 2011; Merced et al. 2013). Given mosses are rarely limited by nutrients (Bergamini et al. 2002), they must use direct uptake of dissolved nutrients from the atmosphere over their entire gametophytic leaf surface. Mosses gaseous exchange can be impacted by the slightest change in atmospheric humidity (Miyata et al. 1961). We suggest that mosses may always be in an opportunistic state, where emission and growth are tightly bound, and carbon gained from photosynthetic process is allocated to either growth or isoprene emission. *Polytrichum* species could lose water as reserves are deposited primarily within storage tissues in rhizoids, displacing water from the cells. With isoprene production, *Polytrichum* mosses could retain more of that water if through bioengineering the nearby atmospheric hydrological microenvironment.

We hypothesized that isoprene emission is fundamental to the emergence of life on land. The feedback mechanism that supplies BVOCs aerosols for cloud formation and

precipitation helps to sustain the hydrological cycle in an ecosystem. Isoprene emission encourages cloud nuclei formation via isoprene/OH reaction to secondary organic aerosols (SOA), which in turn affects cloud droplet number (Claeys et al. 2004; Engelhart et al. 2011; Kerminen et al. 2012). Model simulations support this hypothesis, indicating that 100% of the observed changes in cloud properties can be attributed to isoprene secondary organic aerosols (SOA) (Pöschl et al. 2010). It is our suggestion that moss may employ a similar strategy throughout its lifecycle with an additional more direct water retention strategy when it reaches its adult gametophyte stage. Isoprene emission in both mature and protonemal moss is sensitive to both increased insolation and thermal stress. Both of these stress mechanisms have a direct effect on a plant's ability to maintain water levels, and both thermal and insolation changes can in theory be mitigated through isoprene emission leading to SOA water droplet nucleation particles.

The examination of isoprene (BVOCs) in the context of water strategies may shed significant light on the evolution of the earliest plants to colonize areal environments, with possible implications for our understanding of colonization strategies for all of Earth's plant life. Future work should address cost benefit analyses in isoprene emission and mosses as they are novel model system to test fundamental theories of ecological and ecosystem evolution. A better characterization of isoprene emissions from non-vascular plant species will be required, not just among the basal group of mosses, but also in later divergent species of mosses.

Additional work should distinguish genetic vs. environmental factors at work in the control of isoprene emission in mosses, and future work should focus on molecular

work. A number of isoprene synthase enzymes have been characterized in vascular plants (Kuzma et al. 1993; Silver et al. 1991, 1995; Sharkey et al. 2005; Schnitzler et al. 2005). As of now, isoprene synthase enzyme has been characterized in only the heath star moss *Campylopus introflexus* (Lantz et al. 2015). This could pave the way for using mosses as a model system for understanding the evolution of isoprene in land plants that emit isoprene. It could also shed light of why not all plants emit isoprene and why there is such high variation in emission capability even within families that emit isoprene (Harley et al. 1999).

To further understand isoprene emission in protonema, the next step would be to suppress isoprene and assess the overall effect of mosses response to the loss of the ability to produce and emit isoprene. We have seen that protonema growth exhibited a negative correlation between isoprene emission and growth. It would be necessary to successfully suppress isoprene emission and evaluate growth. A study comparing isoprene emission and protonema mortality under various stress regimes would also be useful in elucidating the role of isoprene. A further step will be to utilize new methodology to suppress isoprene emission that will differ from vascular plant methods. No known literature exists on this subject, and it could provide useful and invaluable information to what is known in isoprene emission suppression in vascular plants (Niinemets et al. 1999; Sharkey et al. 2001; Grote et al, 2013).

The role of isoprene emission in Polytrichaceae sexual systems

In Chapter 3, we wanted to address the hypothesis that isoprene emission is related to the evolution of sexual systems in the mosses. Previous research in our lab has indicated that BVOCs can influence sexual strategies in mosses (Rosenstiel et al. 2012). Of particular interest to us was to understand how sex-specific isoprene emissions (both quantity and composition) change throughout the moss life cycle, and particularly during gametangia formation. We found that the highest isoprene emission was actually seen from non-sexually expressing gametophytes. Isoprene emission may confer benefits to species to negate the effects of selection pressure from loss of carbon and energy (Velikova et al. 2012).

These results suggest that the benefits associated with isoprene production may be too costly to maintain during periods of investment in reproductive structures. This would indicate that bulk isoprene emission does not play a role in sexual reproduction strategies other than to be shed and free up resources to invest in sexual organs. Bulk emission would seem to be involved in some non-sexual survival strategy. In *Polytrichum* mosses, male and female sexual organ formation does not occur synchronously (Watson 1974), and sexual reproduction is resource limited.

While sex has been investigated in these species independently from isoprene emission (Lloyd et al. 1977; Brown et al. 1990; Korpelainen et al. 1992, 1998; Rydgren et al. 2003; Stark et al. 2000, 2005, 2010), no one has invested in exploring isoprene emission and sexual expression in mosses. BVOCs taken together with sexual expression might explain some of the differences in sex and allocation of resources seen in *Polytrichum* species. Isoprene emission might suppress other chemical compounds that

the moss emits, and since we categorized only one compound, it is necessary to run emission profiles for species within *Polytrichaceae* using analyses by gas chromatography–mass spectrometry (GC-MS). This could elucidate if a host of chemical compounds are suppressed while isoprene emission dominates. From non-isoprene emitting *Ceratodon purpureus*, we know that emission of over ~281 chemical compounds are present, some that are analogous to floral volatiles (Rosenstiel et al. 2012). There is an interest in understanding if mosses that emit isoprene would have dissimilar chemical profiles and the evolutionary significance is between mosses that emit and mosses that do not emit isoprene. It is also evident, that large population of mosses contain non-sexually expressing gametophytes, of which there are no known chemical profiles. It seems that sex is costly in mosses (Stark et al. 2000), but not for isoprene emission in *Polytrichaceae* species.

The effect of nitrogen addition on moss morphology, sexual reproduction, and isoprene production

In Chapter 4, we tested the effects of nitrogen addition on moss morphology and physiology. In our experiment, we saw isoprene emission varied with season and site, and increased significantly with moderate increases in nitrogen. Also, treatment-specific differences were evident in morphology measurements. *Polytrichum* mosses are pioneer species and are highly adaptable across environments. In poor nitrogen conditions, low resource availability will limit growth of plants, and the allocated carbon could be used for secondary compounds instead of growth (Bryant et al. 1983). There is a complicated

and poorly understood interaction between isoprene emission and biomass accumulation in vascular plants (Hong et al. 2012), where trade-offs exist between carbon partitioning and biomass accumulation. Understanding the reasons for the increased isoprene emission we saw after nitrogen addition and the observed resource allocation will require a better understanding of the benefits of isoprene production versus growth. Studies are needed to examine the ontogenic patterns associated with isoprene emission in mosses. In vascular plants, the rates of emission are dependent on a host of environmental factors, and biotic interactions such as life stage, age, genotype, day length, temperature regimes, and water status (Harley et al. 1997; Hakola et al. 2003; Owen et al. 2002; Pio et al. 2005; Grabmer et al. 2006; Steinbrecher et al. 2009; Holopainen et al. 2010).

Future work in understanding the effect of nitrogen on isoprene emission in mosses should focus on allocation trade-offs as well as on biotic interactions. Further work could deconstruct the belowground morphological traits, such as rhizoidal biomass, nitrogen in soil and nitrogen within moss tissue both above and belowground to determine their effects on isoprene emissions. It has been shown that trait variation and inter-population differences must have a genetic basis and are associated with metabolic tradeoffs (Hedderson et al. 2009). Additionally, research has found that bacterial and fungal taxa are involved in degrading isoprene in vascular plant systems. Vascular plants emit isoprene that is deposited on their surfaces and in surrounding soil; microbial communities subsequently degrade isoprene on vascular plant leaves and in nearby soils (Rasmussen 1970; Cleveland et al. 1998; Fall et al. 2000; Gray et al 2005; Holopainen et al. 2013, 2017; El Khawand et al. 2016; Johnston et al. 2017; Crombie et al. 2019).

Further work is required to assess if this holds true for non-vascular plants. Assessment must be made to estimate the number of bacterial and fungal taxa that can degrade isoprene in the soil or on the leaves of mosses. These interactions could regulate soil communities and affect chemical ecology both above and belowground. Isoprene emission and surface adsorption may play an additional role in water regulation by making the surface of the plants emitting isoprene and the surrounding soil much more hydrophilic than it otherwise would be. This would aid in amelioration of both water and heat stress through reducing the internal/external moisture gradient to zero while providing water for evaporation buffering against rapid thermal changes.

Another biotic interaction that might influence isoprene emission in mosses is herbivory. Isoprene has been shown to mediate plant-herbivore interaction in vascular plants (Laothawornkitkul et al. 2008). Whether isoprene emission deters or attracts herbivores in mosses is yet to be seen, as it has not been investigated before. In *P. juniperinum*, we have documented for the first time the destructive eating habits of the invasive generalist herbivore *Noctua pronuba*. Although mosses appear to have an extensive arsenal of chemical defenses, bryophyte herbivory by invertebrates is remarkably poorly characterized, creating a fundamental gap in our basic understanding of plant-animal interactions and the ecology of Earth's earliest terrestrial plants. In our preliminary data collection, we have found *N. pronuba* larvae fed on both grass and moss tissue, however survival rates were highest for *N. pronuba* larvae feeding on the grass *Poa annua*. Relative growth rate of the larvae was significantly influenced by food choice, and its attendant C: N ratio. Despite our relatively poor understanding of the role

of herbivory in bryophyte ecology, we found that mosses may be an important alternative food source for *Noctua pronuba* larvae, although strong moss species-specific effects may influence this plant-animal interaction. Allocation to resources such as isoprene may come at the expense of reproduction affecting optimal growth, fitness and chemical composition of other BVOCs within *Polytrichum* species. Future studies should focus on allocation of resources in ontogeny between different life stages and tissue type, above or belowground sources and sinks and nutrient demands between seasons and climates. In order to address isoprene emission and understand its multiple ecological functions and evolutionary implications, mosses provide the ideal study system and should be assessed as a model that may eventually be applied to both vascular and non-vascular plants.

Our research testing the effects of nitrogen addition on mosses is timely and imperative as we measured isoprene emission increases in moss plants with nitrogen addition. Further, we found that isoprene emissions increased strongly with temperature, and we saw species level variation, which could feed into functional type modeling. Isoprene emission is expected to increase at a global level because of increased climate warming (Feng et al. 2019; Guidolotti et al. 2019). Mosses must be accounted for in climate modeling, as they take up 350 million hectares on land in 175 countries (Harenda et al. 2018). One of the primary mosses to occupy these areas is *Polytrichum* species, a high isoprene emitter among mosses, and the focus of this research. We must consider what we know about general model predictions of global average temperatures increase by 2-9°C by the end of the 21st century, and an increase in nitrogen from anthropogenic sources, which now surpass a 4-fold increase of nitrogen available to organisms

(Christensen et al. 2007; Erwin 2009). Future work should focus on understanding how isoprene emission from mosses influences climate change.

Both temperature and nitrogen deposition will influence the carbon/nitrogen ratio of leaf tissue in mosses, and we must consider the species level response to these changes. As nitrogen deposition increases and global temperatures rise, an increase in higher percentage of photosynthetically fixed carbon may be utilized as isoprene from *Polytrichum* species altering the total ecosystem budget of carbon and nitrogen, which up to now has been largely overlooked. We do not understand how these changes will affect increased isoprene emission from mosses.

Carbon allocation could be used to supply primary processes such as growth either below or aboveground, translocation to growth of fungal or microbial populations, reproduction or as storage in the rhizoidal mat. If mosses are such great ecosystem engineers and they are translocating nutrients, recycling resources out of the atmosphere to include water and regulating flow of carbon and nitrogen, these mosses clonal integration provide several potential benefits to all ecosystems and as such this places them at the forefront of understanding why some plants emit isoprene and others do not.

Future work should focus on analyzing leaf tissue nitrogen, rhizoidal mat nitrogen content and soil nitrogen content as mosses can retain nitrogen anywhere from three to ten years in their biomass (Eckstein 2000; Du et al. 2014). This will be greatly affected by an increase in temperature and increase in nitrogen deposition at the species level, and a more thorough evaluation needs to be carried out. This would be especially important to assess as mosses are important elements in forest carbon and nitrogen cycling because

they can utilize and access nitrogen from the atmosphere, their rhizoidal mat and soil. We conclude that some mosses will be at higher risk from global warming and nitrogen deposition at the species level. Mosses that emit isoprene might have an advantage and must be further investigated as the wider consequences of isoprene emission from mosses remain to be understood. This work highlights the importance of understanding how mosses shape local and global climate at a species level under enhanced temperature and nitrogen we can expect isoprene emission to increase from non-vascular plants.

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