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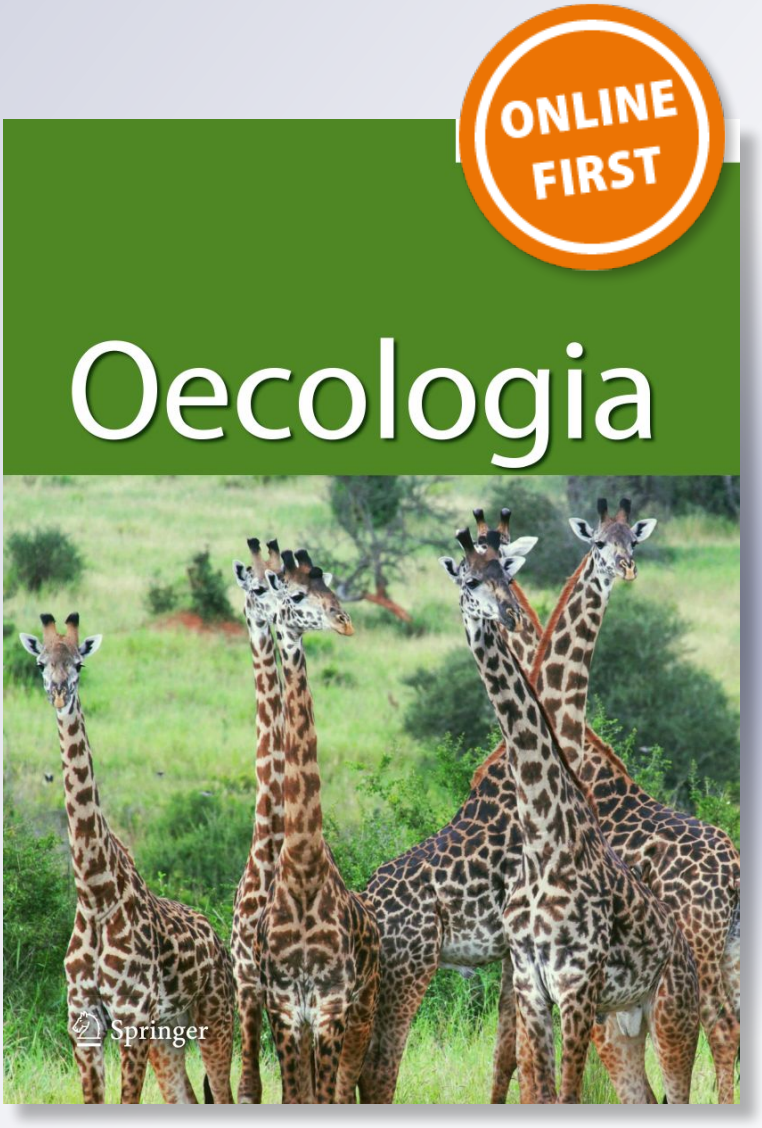

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PHYSIOLOGICAL ECOLOGY – ORIGINAL RESEARCH

Water stress tolerance tracks environmental exposure and exhibits a fluctuating sexual dimorphism in a tropical liverwort

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Abstract

Water shortage events negatively impact plant productivity, threaten ecosystem functioning, and are predicted to increase dramatically in the coming years. Consequently, building a detailed understanding of how plants respond to water stress is critical for improving predictions of ecological processes and species range shifts under climate change. Here, we characterized patterns of intraspecific variation in dehydration tolerance (DhT, also dehydration tolerant) across a variable landscape in the tropical plant, *Marchantia inflexa*. DhT enables tissues to survive substantial drying (below an absolute water content of − 10 MPa) and despite the ecological significance of DhT, many questions remain. We tested if DhT was correlated with an environmental exposure gradient, if male and female plants had contrasting DhT phenotypes, and if variation in DhT had a genetic component. To do so, we collected plants from five populations, spanning an environmental exposure gradient in the forests of northern Trinidad, Republic of Trinidad and Tobago. We measured DhT immediately after collection, and after growing plants for ~ 1 year in a common garden. We found that DhT varied significantly among populations and tracked the characterized exposure gradient. Additionally, we showed that phenotypic differences among populations in DhT were maintained in the common garden, suggesting that underlying genetic differences contribute to DhT variability. Finally, we detected a fluctuating sexual dimorphism where males had lower DhT than females in less exposed sites, but not in more exposed sites. Interestingly, this fluctuating sexual dimorphism in DhT was driven primarily by male variation (females exhibited similar DhT across sites).

Keywords Fluctuating sexual dimorphisms · Ecophysiology · Desiccation tolerance · Intraspecific variation · Diversity panel

Introduction

With climate change models predicting increased variation in global weather patterns, including frequent and intense droughts (Dai 2013), both ecological stability (Jentsch et al. 2007) and food security (Schlenker and Lobell 2010) are threatened. However, by drawing on natural variation in water stress tolerance, we can enhance our understanding of adaptive processes related to water scarcity and improve predictions of the ecological consequences of drought. Many mechanisms of water stress tolerance exist within land plants, spanning diverse life histories, morphologies, and physiologies, and this diversity represents a valuable repository of information that can be mined to gain insight into plant responses to water shortage.

Most plants are desiccation sensitive (DS) and die at water contents from − 5 to − 10 MPa (Proctor and Pence 2002; Proctor et al. 2007), but desiccation tolerant (DT, also desiccation tolerance) plants have a unique ability to survive extreme drying (to an absolute water content of < − 100 MPa) (Bewley 1979). Dehydration tolerance (DhT, also dehydration tolerant) is a less extreme version of DT (DhT plants can survive drying to < − 10 MPa, but not

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− 100 MPa) (Oliver et al. 2010; Marks et al. 2016). Building a comprehensive understanding of the ecology, physiology, and genomics of DT and DhT will inform ecological predictions, environmental management, and agricultural innovations related to water shortage. Extant DT and DhT plants are phylogenetically diverse, but tend to occupy similar ecological niches, suggesting that environmental selection has been a strong driving force in the retention and re-evolution of DT and DhT (Alpert 2005; Le and McQueen-Mason 2006). Plants spanning the entire spectrum from DS to DT have been identified, but the degree of intraspecific variation in DT and DhT is unclear, and whether this variation is driven by genetic differences among populations has rarely been tested. The limited studies on intraspecific variation in DT and DhT indicate that DT and DhT can vary seasonally (Farrant et al. 2009), among populations (Oliver et al. 1993; Farrant and Kruger 2001), under specific culturing methods (Stark et al. 2014; Brinda et al. 2016), among life stages (Stark et al. 2007), and among the sexes (Marks et al. 2016). Additional studies of intraspecific variation in DT and DhT will provide critical insight into possible species range shifts and population persistence.

In addition to abiotic stress, dioecious plants face the threat of distorted population sex ratios, which can lead to mate scarcity, and in extreme cases, a loss or reduction of sexual reproduction. Sex differences in stress tolerance may be an important driving force of population sex ratio distortions (Retuerto et al. 2018), and consequently, detecting sex-specific responses to stress will enhance our understanding of population dynamics in dioecious species. Broadly speaking, sexual dimorphisms in both plants and animals can arise due to distinct selective pressures on males and females (Shine 1989; Badyaev and Hill 2003). This often leads to unidirectional dimorphisms, but variable sexual dimorphisms have also been documented (Alexander et al. 1979; Berry and Shine 1980; Woolbright 1983; Badyaev et al. 2000). Although seemingly unlikely, complete reversals of sexual dimorphisms have also been observed (Kahlke et al. 2000). Taken together, this suggests that sexual dimorphisms are not fixed, and can in fact, fluctuate across space. Understanding the patterns of fluctuating sexual dimorphisms in DhT and other stress tolerance traits will improve predictions of local sex ratios, reproductive potential, and population persistence for dioecious plants.

Here, we investigated intraspecific variation in DhT in the tropical liverwort, *Marchantia inflexa*, to gain insight into the evolutionary and ecological dynamics of DhT. Our primary aims were to characterize variability in DhT across environments, investigate if a previously identified sexual dimorphism in DhT fluctuated among populations, test for genetic differences in DhT among populations, and test if variability in DhT predicted population sex ratios. Building on a previous study that measured DhT of plants from a limited number of populations (Marks et al. 2016), we expanded the scope of our investigations to cover additional sites, intentionally targeting natural, relatively undisturbed streams. We collected plants from five populations, spanning an environmental exposure gradient in the tropical forests of northern Trinidad, Republic of Trinidad and Tobago. DhT was estimated by measuring plant recovery from dehydration events (either immediately after collection or after ~ 1 year of cultivation in a common garden). These data were analyzed to explicitly test the following four hypotheses: (1) plants collected from more exposed sites will recover from dehydration more consistently than plants collected from less exposed sites; (2) the degree and direction of sexual dimorphisms in DhT will fluctuate among populations, as a consequence of male variability; (3) measurable phenotypic variation in DhT will be retained under common conditions due to underlying genetic differences among populations; and (4) population sex ratios will be biased in favor of the sex that has higher DhT in that population.

Methods

Study organism and sample collection

Marchantia inflexa is a New World liverwort with unisexual individuals that is distributed from northern Venezuela to the southern United States (Bischler 1984). The dominant life stage of *M. inflexa* is the haploid gametophyte, which grows as a bifurcating thallus. Sex is chromosomally determined with one U/V sex chromosome and eight autosomes. Sexual reproduction produces spores, and asexual reproduction produces specialized asexual propagules (gemmae) or occurs via thallus fragmentation. *Marchantia inflexa* typically occupies low light, high humidity sites along tropical streams and rivers, but it can also colonize more exposed and disturbed sites, such as road cuts within the forest (Brzyski et al. 2014). *Marchantia inflexa* can recover from moderate dehydration, and prior studies found that females had higher DhT than males (Stieha et al. 2014; Marks et al. 2016). *Marchantia inflexa* exhibits numerous sexual dimorphisms, many of which vary among populations (Groen et al. 2010; Brzyski et al. 2014; Marks et al. 2016), and in some of the above examples, the fluctuation is driven by male variability.

Marchantia inflexa plants for the current study were collected from five sites on the island of Trinidad, Republic of Trinidad and Tobago in May 2016. All sites were located along separate streams (within 18 km of each other) in the moist lowland tropical forests of northern Trinidad (Table 1). Forty-eight samples (bifurcated thallus tips ~ 1 cm long) were collected from each site from May 20 to 27, 2016 (Fig. 1). Plants were sampled haphazardly along a roughly linear transect following the stream, with the restriction that

Table 1 Information on study sites in Trinidad, Republic of Trinidad and Tobago

Collection site	Coordinates	Elevation (m)	Canopy openness (%)	VPD (kPa)	
				2016	2019
N. Oropuche	10°40′09.4″N 61°08′14.9″W	71	9.4 ± 0.06a	0.111 ± 0.0075a	0.137 ± 0.0093b
Rio Seco	10°43′29.3″N 61°02′01.3″W	125	7.5 ± 0.17b	0.079 ± 0.0051ab	0.128 ± 0.0058b
Quare	10°40′29.7″N 61°11′47.5″W	145	6.9 ± 0.06bc	0.016 ± 0.0017bc	0.169 ± 0.0076a
West Turure	10°40′41.6″N 61°10′03.0″W	142	5.7 ± 0.74 cd	0.006 ± 0.0008c	0.067 ± 0.0020c
East Turure	10°41′22.7″N 61°09′37.6″W	140	4.5 ± 0.38d	0.001 ± 0.0003c	0.044 ± 0.0021d

Sites are ordered from most to least exposed. Coordinates and elevation were retrieved from a GPSMAP® 62sc (Garmin International, Inc, Olathe, Kansas USA). Canopy openness was determined via analyses of three hemispherical photographs taken at each site. Vapor pressure deficit (VPD) was quantified from local temperature and relative humidity data in both the rainy season (2016) and the dry season (2019). For canopy openness and VPD, mean and standard error around the mean are listed. Sites with the same letter are not significantly different from each other ($p > 0.05$)

no two samples were within a meter of each other. This sampling scheme was based on prior studies showing that the probability of collecting duplicate genotypes drops to zero at distances > 0.4 m (Brzyski et al. 2018). Upon collection, isolates were placed directly into 24-well plates, hydrated with stream water, and transported back to the William Beebe Tropical Research Station.

Field site characterization

Environmental differences among collection sites were characterized via mean canopy openness and mean vapor pressure deficit (VPD). Canopy openness, which is a measure of exposure that has been linked to plant responses in other studies (Fuselier and McLetchie 2004; Groen et al. 2010), was measured using hemispherical canopy photographs (taken with a Nikon CoolPix 4500 camera with a 180° lens attached). Three photographs were taken at each site ~ 0.5 meters above the plants at the beginning, middle, and end of the sampling area. Canopy photographs were analyzed with WinSCANOPY™ (Reagent Instruments, Québec, Canada), following the manufacturer instructions to quantify canopy openness (%).

VPD is a measure of the evaporative demand of the air and was calculated from temperature and relative humidity (RH) data monitored with sensors integrated in the WatchDog™ models 450 and 1000 data loggers (Spectrum® Technologies, Inc. Plainfield, IL, USA). Data were collected at the beginning of the wet season for 4–6 days in May 2016, and during the dry season for 5 overlapping days in mid-March 2019. Loggers, protected with radiation shields, were deployed at each site by placement in the center of the sampling area at ground level (~ 5 cm) to capture the local conditions of *M. inflexa*. To visually characterize these sites, we took aerial photographs in 2019 directly above each site at equivalent vertical distances, using a Mavic Pro drone (DJI, Shenzhen China) (Fig. 1).

Dehydration treatment

Changes in chlorophyll fluorescence were quantified to estimate the ability of plants to recover from dehydration and are expressed as F_v/F_m recovery (%) (the percentage of baseline F_v/F_m regained after dehydration). F_v/F_m is a measure of the maximum potential quantum efficiency of photosystem II (PSII), and F_v/F_m declines under stress, because PSII function is disrupted. F_v/F_m is frequently used in studies of bryophyte DT to estimate recovery after a drying event (Marschall and Proctor 1999; Proctor et al. 2007; Bader et al. 2013; Hájek and Vicherová 2014). Here, F_v/F_m of dark-adapted tissues (Krause and Weis 1984) was measured with an OS5-FL modulated chlorophyll fluorometer (Opti-Sciences, Tyngsboro, Massachusetts, USA). Measurement parameters were set to a saturation intensity of 100 (4000 µmol m^{-2} s^{-1}) and a pulse duration of 0.8 s. Gain and modulation were adjusted to achieve an adequate signal per manufacturer directions.

To account for baseline variation among sites, initial F_v/F_m was measured in a random subset of plants from each collection site and used to estimate $\overline{\mathcal{X}}(F_v/F_m)_{\text{initial}}$ for each site. All the remaining isolates were subjected to dehydration treatment and $(F_v/F_m)_{\text{recovered}}$ was measured after re-wetting. These two measures $\overline{\mathcal{X}}(F_v/F_m)_{\text{initial}}$ and $(F_v/F_m)_{\text{recovered}}$ were used to compute F_v/F_m recovery (%) with the following equation:

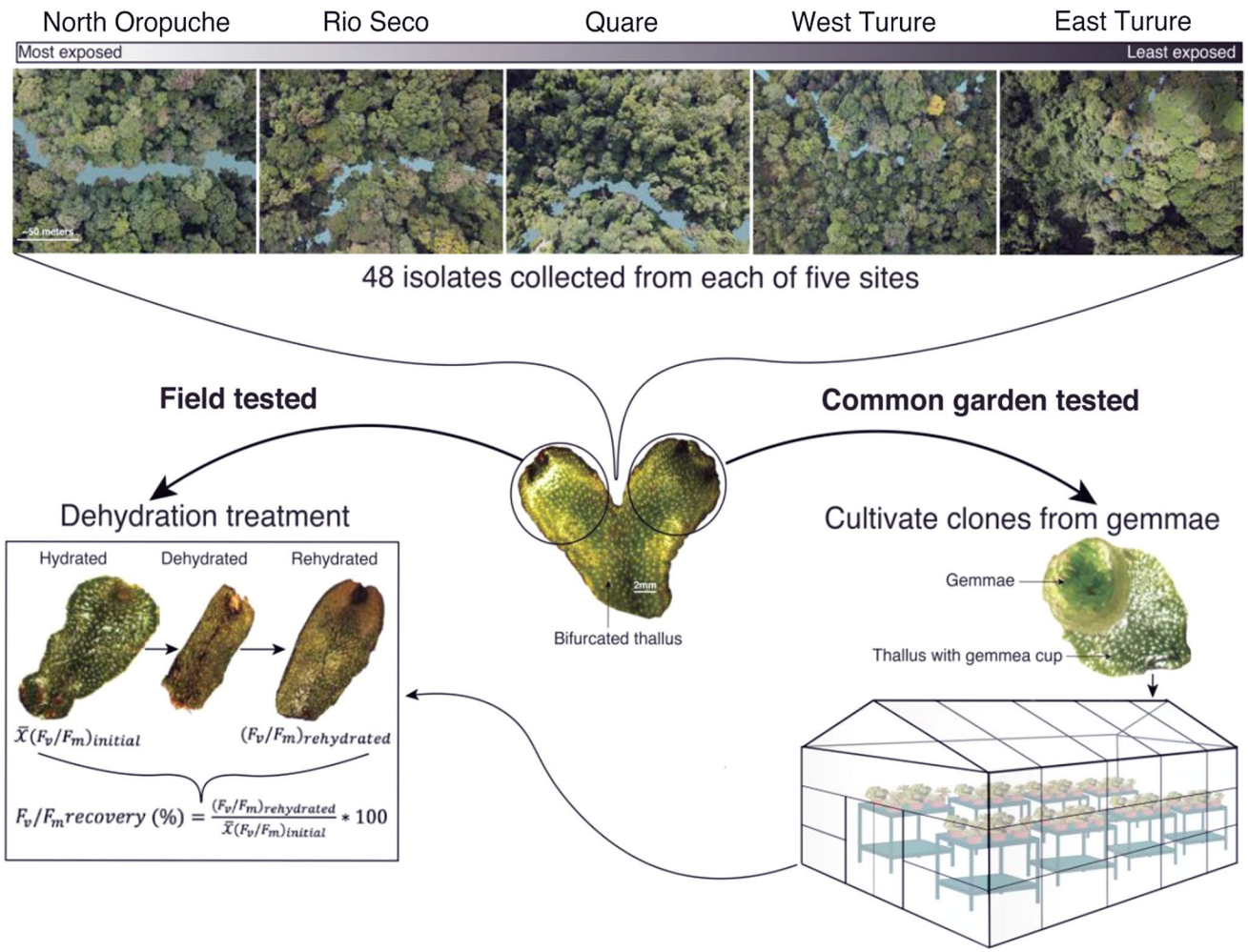

Fig. 1 This schematic outlines the experimental design of the current study. *Marchantia inflexa* plants were collected from five field sites that differed in environmental exposure in Trinidad, Republic of Trinidad and Tobago. Aerial photographs of the five sites (in order of most to least exposed) were taken with a Mavic Pro drone (DJI, Shenzhen China) directly above collection sites at equivalent vertical distances. Areas bordering the stream with an open canopy are shaded blue to increase contrast (original photographs are provided in supplementary information online). Plants were subjected to dehydration treatment 24 h after collection in the field. Subsequently, clones of each plant were propagated from gemmae in a common garden to remove field effects. After ~ 1 year of cultivation in a common garden, these clones were subjected to dehydration treatment again. The ability of plants to recover from dehydration was quantified by chlorophyll fluorescence to measure the maximum potential quantum efficiency of Photosystem II (F_v/F_m) before and after dehydration and is expressed as F_v/F_m recovery (%) (the percent of baseline F_v/F_m regained after dehydration treatment)

$$\left[F_v/F_m \text{recovered}(\%) = \frac{(F_v/F_m)_{\text{recovered}}}{(F_v/F_m)_{\text{initial}}} * 100\right]. \quad (1)$$

Field dehydration treatment

A total of 240 isolates ($n = 48$/site) were collected. Eight isolates were randomly selected from each site and used to estimate $\overline{\mathcal{X}}(F_v/F_m)_{\text{initial}}$. We did not take $\overline{\mathcal{X}}(F_v/F_m)_{\text{initial}}$ measures on experimental samples to minimize handling of tissues. The remaining 200 isolates ($n = 40$/site) were subjected to dehydration treatment, and $(F_v/F_m)_{\text{recovered}}$ was determined for 196 isolates (4 samples were lost). Dehydration treatments were carried out following the protocol outlined in Marks et al. (2016), with a few minor changes. Following collection, plants were held in fully hydrated, low light conditions (~ 10 µmol m^{-2} s^{-1}) at 25.8 ± 0.02 °C for 24 h before dehydration treatment. To initiate dehydration treatment, one side of the bifurcation (a thallus tip) was removed and placed in a dehydration chamber (Fig. 1). Dehydration chambers consisted of airtight plastic containers with tissue samples in individual Petri dishes placed around the interior perimeter of the chamber. Each chamber contained a total of 20 samples (10 plants each from 2 sites). The entire dehydration treatment was conducted under low light (~ 10 µmol m^{-2} s^{-1}) at 25.8 ± 0.02 °C. All samples were

dehydrated to equilibrium with 85% RH (maintained with a saturated KCl solution (Greenspan 1977)). Plants were allowed to dehydrate until visibly dry (~ 105 h), after which they were rehydrated and $(F_v/F_m)_{\text{recovered}}$ was measured 2 weeks after rehydration.

Confirmation that plants reached equilibrium with 85% RH after 105 h was determined by measuring the change in mass of an additional set of isolates from North Oropuche and Quare streams over the course of the dehydration treatment until no additional decrease in mass was observed. Mass measurements were also used to calculate the water content (WC) of dehydrated samples to estimate the intensity of the dehydration treatment (see Eq. 2). The precision of our scale (10 mg) at the field research station did not allow us to detect changes in the mass of a single sample, so measurements were made on four groups of ten tips. Sample fresh weight (FW) was measured at the most dehydrated condition (~ 105 h after assay initiation) and sample dry weight (DW) was measured after drying samples for 3 days in an 80 °C drying oven at the University of Kentucky, Lexington, KY, USA. The mean WC of samples after field dehydration treatment was 28.32 ± 2.23 g H_2O g^{-1} dry weight:

$$\text{WC} = 100 * \left(\frac{\text{FW} - \text{DW}}{\text{DW}}\right). \quad (2)$$

Common garden cultivation

Clones (the remaining side of the bifurcated thallus) of all 240 isolates were transported to the University of Kentucky, Lexington, KY, USA and placed in a growth chamber at 16 °C with a light intensity of 30 µmol m^{-2} s^{-1} and 12-h light/dark cycle to induce gemmae (asexual propagule) production. A single gemmea was used to generate a new clone of each isolate with the intention of removing field effects (Fig. 1). These clones were planted on steam sterilized local soil in 59 ml pots covered with neutral density acetate lids and grown in a climate-controlled greenhouse. Plants were watered daily and covered with a 15% shade cloth to mimic field light conditions.

Common garden dehydration treatment

Plants were cultivated in a common garden for ~ 1 year to minimize the impact of any maternal effects. Subsequently, thallus samples from each isolate were subjected to dehydration treatment (Fig. 1). A randomly selected subset of isolates ($n = 8$) from each site were used to quantify $\overline{\mathcal{X}}(F_v/F_m)_{\text{initial}}$ to control for any baseline variation among sites that was retained in common garden conditions. Two hundred and one isolates were assayed for DhT after being cultured in a common garden. We targeted 40 samples per site, but due to mortality of some samples from Rio Seco and North Oropuche, we had less than 40 isolates from these sites, so we included additional isolates from the other sites if they were available. The dehydration treatment was designed to replicate field treatments as closely as possible, but a slight reduction in ambient temperature (from 25.8 ± 0.02 in the field to 20.5 ± 0.01 °C in the common garden) reduced the VPD of dehydration treatments in the common garden relative to the field (from 0.500 kPa in the field to 0.363 kPa in the common garden). Consequently, we made no direct comparisons between relative recovery of field and common garden plants. Paralleling field work, thallus tips were collected from isolates, hydrated in dH_2O for 24 h, placed into dehydration conditions, dehydrated until visibly dry (~ 120 h), rehydrated, and recovery was quantified 2 weeks after rehydration by measuring $(F_v/F_m)_{\text{recovered}}$. Because we had access to sufficient tissue to permit replication, isolates were sampled multiple (1–3) times each for a total of 412 samples (Fig. 1).

To confirm that plants reached equilibrium with 85% RH in the common garden dehydration treatments, we measured the WC of a subset of dehydrated samples. Mass measurements were made on a total of 57 samples at their most dehydrated condition and again after 3 days in an 80 °C drying oven using a Chan 29 electrobalance (0.1 µg). WC was calculated using Eq. 2. The mean WC of samples after laboratory dehydration treatment was 72.27 ± 7.23 g H_2O g^{-1} dry weight.

Sex expression

We identified the sex of all isolates that were included in this study though a combination of growth based and molecular methods. There were 12 isolates that died prior to being sexed, and these were removed from all analyses. Initially, we observed natural sex organ development in the common garden. This allowed us to determine the sex of the majority of our isolates. However, some plants did not produce sex organs under greenhouse conditions. For these specimens, we induced sex organ development by exposing plants to constant far-red light at 16 °C in a growth chamber. Plants, that still failed to produce sex organs ($n = 7$), were sexed using DNA sex markers for *M. inflexa* as described in Marks et al. (2019).

Statistical analyses

Statistical analyses were conducted in JMP12 (SAS Institute Inc. Cary, NC, USA). Canopy openness and VPD were analyzed separately to test for differences among sites using a mixed effects linear model. For VPD, the years were analyzed separately. In the statistical models, date and time were considered random effects and site was the fixed effect. To

test for a positive association between canopy openness and VPD in the 2016 data, we used Pearson *r* correlations.

To address the four central hypotheses of this study, the primary response variable analyzed was F_v/F_m recovery (%), which corresponds to the ability of a plant to recover from dehydration, and can be used to estimate DT and DhT (Hájek and Vicherová 2014). Initially, we analyzed the complete dataset to test for general patterns of DhT variability. We used a mixed effects linear model to test the fixed effects of sex, site, growth condition, and all second- and third-order interaction effects on F_v/F_m recovery (%). To account for different levels of replication in greenhouse and field studies, isolate ID (nested within site) was included in the model as a random effect. Dehydration chamber (nested within growth condition) was also included as a random effect and post hoc comparisons were made with Tukey's Honest Significant Difference (HSD) tests. To develop our general characterization of the relationship between DhT and local environmental conditions (hypothesis one), we considered the effect of site on F_v/F_m recovery (%), and we tested for correlations between site means of F_v/F_m recovery (%), VPD, and canopy openness.

Subsequently, to drill down into different aspects of *M. inflexa's* biology, we considered data from field and common garden plants separately. We used separate mixed effect linear models to test the fixed effects of sex, site, and their interaction on F_v/F_m recovery (%) in the field and common garden assays. Isolate ID and dehydration chamber were included as random effects and post hoc comparisons were made with Tukey's Honest Significant Difference (HSD) tests.

We analyzed data from field collected plants to address hypotheses one and two. To further characterize the relationship between DhT and local environmental conditions (hypothesis one), we considered the effects that include site on F_v/F_m recovery (%), and we tested for correlations between site means of F_v/F_m recovery (%) and VPD and canopy openness. To investigate sex-specific responses to the exposure gradient (hypothesis two), we tested for a sex effect on F_v/F_m recovery (%). Additionally, we computed site-specific F_v/F_m recovery (%) for each sex and tested for correlations between canopy openness (%), VPD, and F_v/F_m recovery (%) in males and females. Finally, we tested if the variance in DhT among sites was sex-specific using a two-sided *F* test.

We used the common garden assay to test for the maintenance of phenotypic variation in DhT under common conditions. If detected, this would indicate that genetic differences among populations contribute to DhT variability (assuming that common garden cultivation removed any baseline variation due to maternal effects of field plants) (hypothesis three). To do so, we tested for a significant site effect on F_v/F_m recovery (%) in common garden plants. We built upon this by specifically testing if patterns of sexual dimorphisms identified in field-assayed plants were maintained in the common garden.

To address hypothesis four, site-specific sex ratios (♂/(♂ + ♀) were calculated using the data from Table 2. We used the Goodness-of-fit test to examine sex ratios within each population (with the expected sex ratio of 0.5) and a heterogeneity test to identify significant differences among populations. Subsequently, the difference in DhT among the sexes ($\overline{\mathcal{X}}_{♂} F_v/F_m$ recovery (%)–$\overline{\mathcal{X}}_{♀} F_v/F_m$ recovery (%)) was computed for each site. We predicted that sites with significant sex differences in F_v/F_m recovery (%) would have sex ratios significantly different from 0.5 and in favor of the sex with higher DhT, and that sites with no significant sex difference in F_v/F_m recovery (%) would have sex ratios not significantly different from 0.5. We compared our observed data to these expectations and considered the proportion of cases in which our prediction was met.

Table 2 The difference in DhT between males and females was computed for each site ($\overline{\mathcal{X}}_{♂}$ (F_v/F_m recovery (%)–$\overline{\mathcal{X}}_{♀} F_v/F_m$ recovery (%))

Collection site	Sex difference in DhT (%)	Predicted sex ratio	Observed sex ratio	Number ♂	Number ♀
North Oropuche	9.15	> 0.5	0.43	20	26
Rio Seco	8.22	0.5	0.07*	3	39
Quare	− 1.76	0.5	0.18*	8	37
West Turure	− 7.24	0.5	0.68*	32	15
East Turure	− 20.63*	< 0.5	0.21*	10	38

Population sex ratios (♂/(♂ + ♀)) for each site were computed based on the number of male and female isolates collected at each site. Sites with significant sex differences in DhT or population sex ratios significantly different from 0.05 are indicated with an *. For predicted sex ratios, we expected that population sex ratios would be biased in favor of the sex with higher DhT. This prediction was met in only two out of the five cases (North Oropuche and East Turure), suggesting that DhT does not predict sex ratio

Results

Field site characterization

The environmental parameters of canopy openness and VPD varied significantly among sites. Canopy openness ranged from 4.5 ± 0.38% at East Turure to 9.4 ± 0.06% at North Oropuche, with significant differences among sites ($F_{4,8} = 27.93$, $P < 0.0001$, Table 1). In the rainy season (2016), VPD ranged from 0.001 ± 0.0003 kPa at East Turure to 0.111 ± 0.0075 kPa at North Oropuche ($F_{4,67.4} = 130.6$, $P < 0.0001$, Table 1). In the dry season (2019), VPD ranged from 0.044 ± 0.0021 kPa at East Turure to 0.169 ± 0.05076 kPa at Quare ($F_{4,7464} = 98.3$ $P < 0.0001$, Table 1). In 2016, VPD was significantly positively associated with canopy openness ($R^2 = 0.78$, $F_{1,3} = 15.19$, $P = 0.03$).

General patterns of DhT across field and common garden assays

Our analyses revealed that F_v/F_m recovery (%) varied significantly among sites ($F_{4,115} = 3.05$ $P = 0.02$) (Fig. 2). Plants from drier, more exposed sites had higher F_v/F_m recovery (%) than plants from moist, less exposed sites. Differences among sites in F_v/F_m recovery (%) were tightly correlated with environmental exposure. Mean F_v/F_m recovery (%) was positively correlated with both canopy openness ($R^2 = 0.83$, $F_{1,3} = 14.2$, $P = 0.033$) and 2016 VPD ($R^2 = 0.94$, $F_{1,3} = 55.6$, $P < 0.005$). There was a significant interaction between site and sex on F_v/F_m recovery (%) ($F_{4,265} = 3.43$ $P = 0.009$), but the effects of sex, growth condition, and all other second- and third-order interaction effects were not significant (data not shown).

Targeted characterization of site and sex effects on DhT

In field-assayed plants, there was no significant effect of site or sex on F_v/F_m recovery (%) ($F_{4,27} = 1.52$ $P = 0.226$; $F_{4,178} = 1.8$ $P = 0.174$, respectively), but there was a significant interaction between site and sex on F_v/F_m recovery (%) ($F_{4,178} = 3.52$ $P = 0.009$) (Fig. 3). Specifically, females had higher F_v/F_m recovery (%) than males at East Turure, but the sexes did not differ in DhT at the remaining sites. Site-specific F_v/F_m recovery (%) was not significantly correlated with canopy openness or VPD ($R^2 = 0.64$, $F_{1,3} = 5.4$, $P = 0.103$; $R^2 = 0.65$, $F_{1,3} = 5.5$, $P = 0.101$, respectively), but sex-specific patterns were identified. F_v/F_m recovery (%) in males was positively associated with canopy openness ($R^2 = 0.82$, $F_{1,3} = 20.02$, $P = 0.02$), but not in females ($R^2 = 0.003$, $F_{1,3} = 1.01$, $P = 0.388$). There was a similar pattern for VPD in 2016, where F_v/F_m recovery (%) was positively associated with VPD in males ($R^2 = 0.93$, $F_{1,3} = 53.75$, $P = 0.0052$), but not in females ($R^2 = 0.52$, $F_{1,3} = 5.41$, $P = 0.102$). Additionally, males had significantly higher variance across sites in mean F_v/F_m recovery (%) than females ($F_{4,4} = 12.7$ $P = 0.03$). F_v/F_m recovery (%) ranged from 46.7 to 86.2% in males and 67.3 to 77.9% in females (Fig. 3).

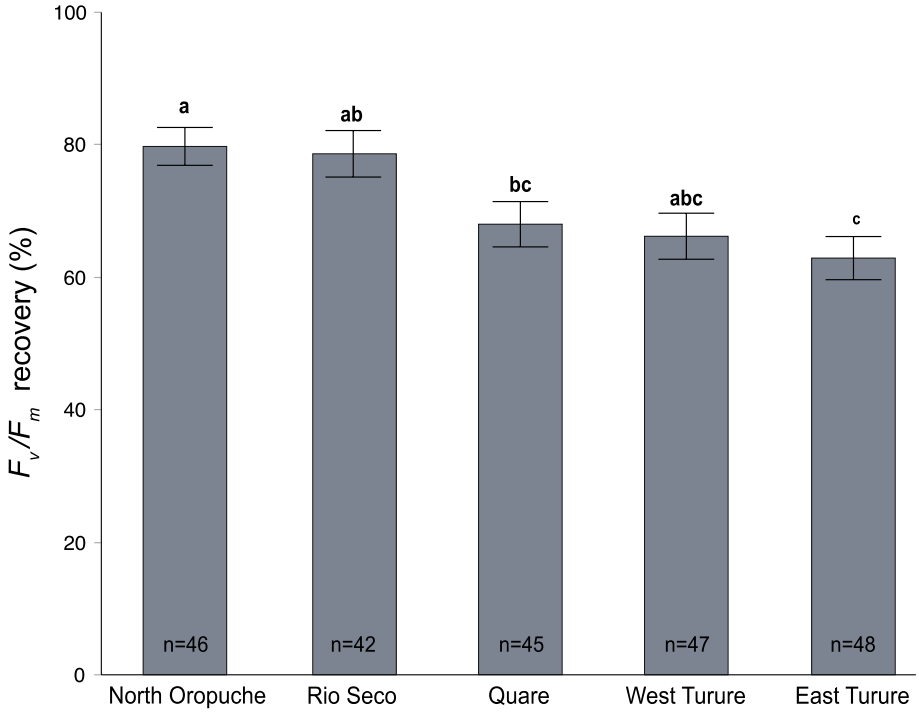

Fig. 2 *Marchantia inflexa* plants collected from five streams in Trinidad, Republic of Trinidad and Tobago (ordered from most to least exposed based on canopy openness) differed in DhT. There was a significant effect of site on F_v/F_m recovery (%). Specifically, plants from more exposed sites had higher DhT than plants from less exposed sites. Both field collected and common garden specimens are included in these analyses and the reported sample sizes refer to the number of unique isolates included in analyses. There are 12 isolates that are not represented, because they died prior to sex determination. Differences in DhT were tightly correlated with canopy openness and vapor pressure deficit. Bars are standard error about the mean

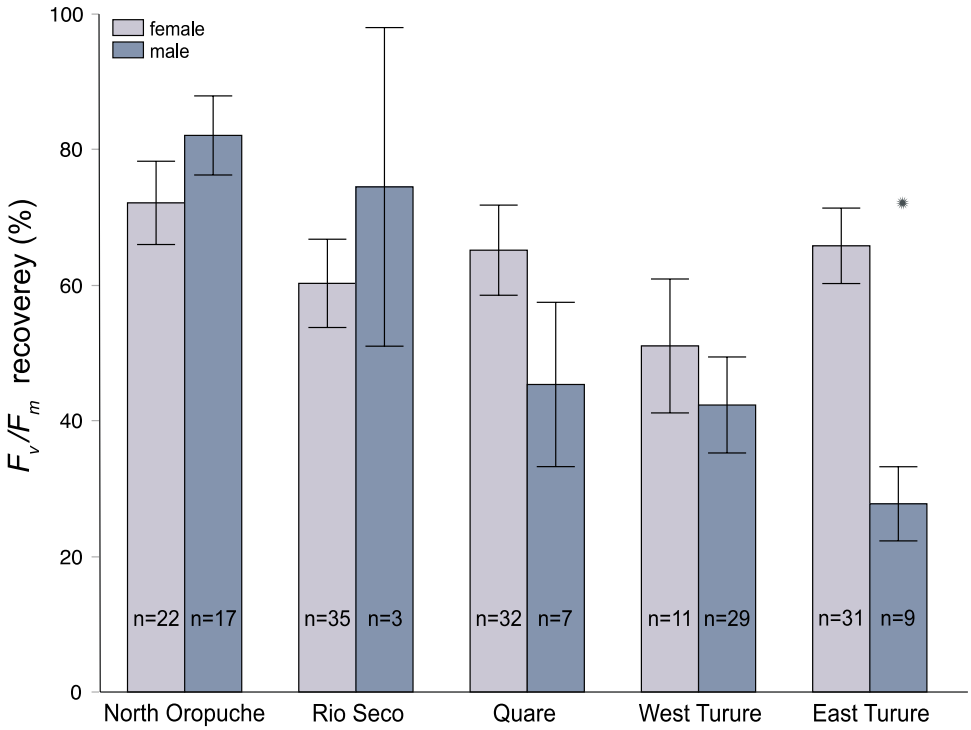

Fig. 3 Relative DhT of *Marchantia inflexa* males and females fluctuated among the sites. Only field plants are included in this analysis. Sites are ordered from most to least exposed. Females were significantly more DhT than males at East Turure, but the sexes did not differ at other sites. This fluctuating sexual dimorphism was driven primarily by male variability. The number of isolates is reported, and bars are standard error about the mean

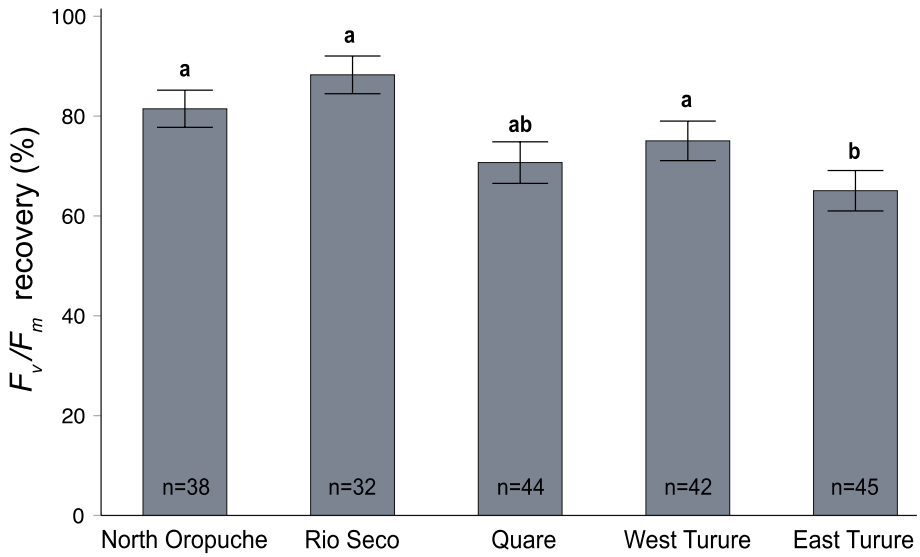

Fig. 4 DhT of *Marchantia inflexa* plants cultivated in a common garden varied significantly among sites. Collection sites are ordered from most to least exposed. Plants from more exposed sites had higher DhT than plants from the less exposed sites even under common growth conditions. These findings were driven primarily by plants from the least exposed site (East Turure) having significantly reduced DhT relative to other sites. The number of isolates from each site is reported. Bars are standard error about the mean

Genetic differences in DhT

To test if phenotypic variation in DhT was maintained under common conditions, we analyzed F_v/F_m recovery (%) of common garden plants. There was a significant effect of site on F_v/F_m recovery (%) ($F_{4,143}=3.8$ $P=0.006$), which was driven primarily by plants from the least exposed site (East Turure) having reduced DhT relative to plants from other sites (Fig. 4). F_v/F_m recovery (%) ranged from $65.1 \pm 4.1\%$ for plants from East Turure to $88.3 \pm 3.8\%$ for plants from Rio Seco. The effects of sex, and the site and sex interaction were not significant ($F_{1,133}=0.3$ $P=0.582$; $F_{4,143}=1.2$ $P=0.300$, respectively). However, focusing on males that were found to be variable in the field assay, males F_v/F_m recovery (%) tended to differ by site, ranging from $54.5 \pm 9.9\%$ in East Turure to $92.1 \pm 13.6\%$ Rio Seco.

Population sex ratios

Overall population sex ratios were heterogeneous, ranging from 0.058 to 0.64 ($G_4=45.43$, $P<0.0001$). The sex ratio at North Oropuche did not differ from 0.5, Rio Seco, Quare, and

East Turure were significantly female biased ($P < 0.0001$), and West Turure was male biased ($P = 0.012$) (Table 2). Despite the fluctuating sexual dimorphism in DhT, our prediction that sex ratios would be biased in favor of the sex with higher DhT was met in only two out of the five populations (Table 2).

Discussion

Our findings indicate that *M. inflexa* harbors substantial intraspecific variation in the ability to recover from dehydration. We found that plants collected from the drier of five natural sites recovered from dehydration more consistently than plants collected from the moister sites. This pattern was driven, at least in part, by underlying genetic differences among populations, as indicated by retention of population-specific differences in DhT in the common garden. Interestingly, we found that population differences in the ability to recover from dehydration were sex-specific. In less exposed sites, females recovered more consistently than males, but in more exposed sites, there were no differences in DhT among the sexes. This fluctuating sexual dimorphism was most evident in field collected plants and appeared to be driven by male variability, suggesting that *M. inflexa* males may be more responsive to environmental variation than females. The fluctuating sexual dimorphism in DhT did not scale up to population sex ratios.

Our data suggest that intraspecific variation in DhT is linked to environmental exposure and that it has a genetic component in *M. inflexa*. We characterized an environmental exposure gradient of canopy openness and VPD (also evident in aerial photographs of field sites taken in 2019) (Fig. 1). In general, we found that differences in the ability to recover from dehydration were correlated with environmental exposure. Furthermore, differences among sites in DhT were retained under common conditions (plants from the least exposed site (East Turure) had lower recovery than plants from more exposed sites), suggesting that variation in DhT has a genetic component in *M. inflexa*. Studies using other species have shown that variation in DT corresponds with environmental differences (Beckett and Hoddinott 1997; Dilks and Proctor 1976; Farrant et al. 2009; Stark et al. 2007), but these studies did not explicitly test for genetic differences among plants. Our study adds to this body of work by utilizing a common garden design to identify genetic differences among populations in DhT. Previous studies of *M. inflexa* have suggested that plasticity may also play a role in DhT variation (Marks et al. 2016). However, data from the current study were not used to explicitly test for plasticity. Our analyses indicated that site differences in DhT in *M. inflexa* were impacted by genetic variation among populations and correlated with environmental exposure, suggesting that spatially variable selection may drive local adaptation in DhT.

We found that DhT was sexually dimorphic, but that sex differences in DhT fluctuated among populations. Specifically, females exhibited higher recovery than males in less exposed sites, but the sexes did not differ in DhT at more exposed sites. Prior work in *M. inflexa* showed that females had higher DhT than males at the least exposed site (East Turure) (Marks et al. 2016) and that finding was confirmed in the current study. However, here, we found that females were not more DhT than males at other sites, indicating that sex differences in DhT are more complex than previously appreciated. DhT is not the first trait to display a fluctuating sexual dimorphism in *M. inflexa* (Groen et al. 2010; Brzyski et al. 2014), and we speculate that contrasting selective pressures on males and females could drive the fluctuating sexual dimorphisms observed in *M. inflexa*. Differences in reproductive function between females and males may lead to sex-specific tradeoffs between reproduction and other plant functions (Dawson and Geber 1999; Dudley and Galen 2007; Retuerto et al. 2018), which could be accentuated under stressful conditions and exacerbated by intra-sexual competition (Eppley 2006). In the context of the current study, we speculate that differences in the duration of reproductive processes in males and females may impact relative exposure to DhT selection. Females require more time in hydrated conditions to complete their reproductive cycle (gamete development through sporophyte maturation) and may, therefore, be exposed to selection for DhT even at the least exposed sites. In contrast, males require less time to complete their reproductive cycle (gamete development) and may experience little-to-no selection for DhT in the least exposed sites because their reproductive processes are completed well before the onset of drying events. However, in the more exposed sites, selection for DhT may be extended to males, which could lead to a local increase in male DhT. Over time, this may even result in a reduction of females in stressful sites, as has been suggested in other taxa (Freeman et al. 1976; Dawson and Ehleringer 1993; Eppley 2001).

Interestingly, we found that the fluctuating sexual dimorphism in DhT was driven by male variability. Mean male DhT was highly variable across sites and tightly correlated with environmental exposure, whereas female DhT was similar across all sites and not correlated with environmental exposure. We speculate that females are relatively constrained by the reproductive biology associated with offspring maturation, and therefore, exhibit low variability in DhT. In contrast, the more permissive male reproductive biology may facilitate rapid adaptation or acclimation to local conditions. There are many examples of animal taxa where multiple male "types" have been identified (Liley 1966; Hayashi 1985; Sinervo and Lively 1996; Taborsky 1998; Zamudio and Sinervo 2000), but we

are unaware of studies describing multiple male types in dioecious plants (but see Moore et al. 2016). Our recent genomic work in *M. inflexa* also points to high male variability, showing that genes on the male-specific V chromosome are diversifying (relative to *M. polymorpha*) faster than genes on the female-specific U chromosome (Marks et al. 2019). Diversification of male-specific genes and high male variability in multiple phenotypic traits suggests that *M. inflexa* males may be undergoing rapid adaptation. We speculate that greater plasticity in males relative to females may also contribute to the high male variability observed, but we did not explicitly test for this here. We detected the fluctuating sexual dimorphism in field plants but not the common garden, which could be due to sex-specific plasticity or the lower dehydration intensity in the common garden (VPD ~ 0.363 kPa) relative to the field (VPD ~ 0.500 kPa). Because we cannot distinguish among these alternative explanations currently, we do not make any conclusions on the mechanism (genetic vs. plastic) of male variability.

It has been suggested that differences in male and female responses to environmental conditions can favor spatial segregation of the sexes (Bierzychudek and Eckhart 1988), but we found that sex differences in DhT did not scale up to population sex ratios. We found no relationship between sex differences in DhT and population sex ratios, suggesting that sex-specific DhT does not predict population sex ratio. *Marchantia inflexa* population sex ratios can be highly variable (they range from 0 to 1 in Quare stream (Brzyski et al. 2018)) and are likely impacted by numerous ecological and stochastic factors, such as the size of the substrate (Brzyski et al. 2018), sex-specific life-history traits (García-Ramos et al. 2007), and spatial distribution of populations (Stieha et al. 2017). Sexual dimorphisms in colonization, establishment, growth, and asexual reproduction also likely impact population sex ratio, so the finding that sex differences in DhT did not predict population sex ratios was not surprising.

In conclusion, this study builds on prior studies of DhT in *M. inflexa* and demonstrates that patterns of DhT are more complex than previously described. We showed that DhT varied along an environmental gradient and that genetic differences among populations contributed to differences in DhT. Evidence of genetic variation among *M. inflexa* populations in DhT suggests that local adaptation to an environmental gradient may be occurring in this system. This adaptive response could enhance population persistence and contribute to ecological stability. Prior studies of *M. inflexa* found that females were more DhT than males (Stieha et al. 2014; Marks et al. 2016), but these conclusions were based primarily on a single population (Marks et al. 2016), or specialized tissues (Stieha et al. 2014). Here, we found that sex differences in DhT varied among populations, with females exhibiting higher DhT than males in some, but not all populations. This finding highlights the importance of population-specific assessments of stress tolerance and demonstrates that sexual dimorphisms can fluctuate in response to environmental conditions.

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Author contribution statement RAM and DNM conceived of the project and designed the methodology. RAM, BDP, and DNM collected data. RAM, BDP, and DNM contributed to data analyses. RAM led the writing of the manuscript. All authors read and gave final approval of the manuscript for publication.

Data accessibility All data associated with this study are deposited on Figshare (https://doi.org/10.6084/m9.figshare.6736934) and will be made publicly available upon publication.

References

Alexander RD, Hoogland JL, Howard RD et al (1979) Sexual dimorphisms and breeding systems in pinnipeds, ungulates, primates, and humans. In: Chagnon NA, Irons W (eds) Evolutionary biology and human social behavior: an anthropological perspective. Duxbury Press, North Scituate, pp 402–435

Alpert P (2005) The limits and frontiers of desiccation-tolerant life. Integr Comp Biol 45:685–695. https://doi.org/10.1093/icb/45.5.685

Bader MY, Reich T, Wagner S et al (2013) Differences in desiccation tolerance do not explain altitudinal distribution patterns of tropical bryophytes. J Bryol 35:47–56. https://doi.org/10.1179/1743282012Y.0000000033

Badyaev AV, Hill GE (2003) Avian sexual dichromatism in relation to phylogeny and ecology. Annu Rev Ecol Evol Syst 34:27–49. https://doi.org/10.1146/annurev.ecolsys.34.011802.132441

Badyaev AV, Hill GE, Stoehr AM et al (2000) The evolution of sexual size dimorphism in the house finch. II. Population divergence in relation to local selection. Evolution (N Y) 54:2134–2144

Beckett R, Hoddinott N (1997) Seasonal variations in tolerance to ion leakage following desiccation in the moss Atrichum androgynum from a KwaZulu-Natal afromontane forest. S Afr J Bot 63:276–279

Berry JF, Shine R (1980) Sexual size dimorphism and sexual selection in turtles (order testudines). Oecologia 44:185–191. https://doi.org/10.1007/BF00572678

Bewley JD (1979) Physiological aspects of desiccation tolerance. Annu Rev Plant Physiol 30:195–238. https://doi.org/10.1146/annurev.pp.30.060179.001211

Bierzychudek P, Eckhart V (1988) Spatial segregation of the sexes of dioecious plants. Am Nat 132:34–43

Bischler H (1984) *Marchantia* L: the new world species (Bryophytorum Bibliotheca, Band 26). Lubrecht & Cramer Ltd, Port Jervis

Brinda JC, Stark LR, Clark TA, Greenwood JL (2016) Embryos of a moss can be hardened to desiccation tolerance: effects of rate of drying on the timeline of recovery and dehardening in *Aloina ambigua* (Pottiaceae). Ann Bot 117:153–163. https://doi.org/10.1093/aob/mcv136

Brzyski JR, Taylor W, McLetchie DN (2014) Reproductive allocation between the sexes, across natural and novel habitats, and its impact on genetic diversity. Evol Ecol 28:247–261. https://doi.org/10.1007/s10682-013-9672-9

Brzyski JR, Stieha CR, McLetchie DN (2018) The impact of asexual and sexual reproduction in spatial genetic structure within and between populations of the dioecious plant *Marchantia inflexa* (Marchantiaceae). Ann Bot. https://doi.org/10.1093/aob/mcy106

Dai A (2013) Increasing drought under global warming in observations and models. Nat Clim Chang 3:52–58

Dawson T, Ehleringer J (1993) Gender-specific physiology, carbon isotope discrimination, and habitat distribution in boxelder, Acer negundo. Ecology 74:798–815

Dawson T, Geber M (1999) Sexual dimorphism in physiology and morphology. In: Geber M, Dawson T, Delph L (eds) Gender and sexual dimorphism in flowering plants. Springer, New York, pp 175–215

Dilks TJK, Proctor MCF (1976) Seasonal variation in desiccation tolerance in some British bryophytes. J Bryol 9:239–247

Dudley LS, Galen C (2007) Stage-dependent patterns of drought tolerance and gas exchange vary between sexes in the alpine willow, *Salix glauca*. Oecologia 153:1–9. https://doi.org/10.1007/s00442-007-0712-4

Eppley S (2001) Gender-specific selection during early life-history stages in the dioecious grass *Distichlis spicata*. Ecology 82:2022–2031

Eppley S (2006) Females make tough neighbors: sex-specific competitive effects in seedlings of a dioecious grass. Oecologia 146:549–554

Farrant JM, Kruger LA (2001) Longevity of dry *Myrothamnus flabellifolius* in simulated field conditions. Plant Growth Regul 35:109–120. https://doi.org/10.1023/A:1014473503075

Farrant JM, Lehner A, Cooper K, Wiswedel S (2009) Desiccation tolerance in the vegetative tissues of the fern *Mohria caffrorum* is seasonally regulated. Plant J 57:65–79

Freeman D, Klikoff L, Harper K (1976) Differential resource utilization by the sexes of dioecious plants. Science 193:597–599

Fuselier L, McLetchie DN (2004) Microhabitat and sex distribution in *Marchantia inflexa*, a dioicous liverwort. Bryologist 107:345–356. https://doi.org/10.1639/0007-2745(2004)107%5b0345:masdim%5d2.0.co;2

García-Ramos G, Stieha CR, McLetchie DN et al (2007) Persistence of the sexes in metapopulations under intense asymmetric competition. J Ecol 95:937–950. https://doi.org/10.1111/j.1365-2745.2007.01264.x

Greenspan L (1977) Humidity fixed points of binary saturated aqueous solutions. J Res Natl Bur Stand A Phys Chem 81:89–96

Groen KE, Stieha CR, Crowley PH, McLetchie DN (2010) Sex-specific plant responses to light intensity and canopy openness: implications for spatial segregation of the sexes. Oecologia 162:561–570. https://doi.org/10.1007/s00442-009-1473-z

Hájek T, Vicherová E (2014) Desiccation tolerance of Sphagnum revisited: a puzzle resolved. Plant Biol 16:765–773. https://doi.org/10.1111/plb.12126

Hayashi K (1985) Alternative mating strategies in the water strider *Gerris elongatus* (Heteroptera, Gerridae). Behav Ecol Sociobiol 16:301–306. https://doi.org/10.1007/BF00295542

Jentsch A, Kreyling J, Beierkuhnlein C (2007) A new generation of climate-change experiments: events, not trends. Front Ecol Environ 5:365–374. https://doi.org/10.1890/1540-9295(2007)5%5b365:angoce%5d2.0.co;2

Kahlke V, Angele MK, Schwacha MG et al (2000) Reversal of sexual dimorphism in splenic T lymphocyte responses after trauma-hemorrhage with aging. Am J Physiol Cell Physiol 278:C509–C516. https://doi.org/10.1152/ajpcell.2000.278.3.C509

Krause GH, Weis E (1984) Chlorophyll fluorescence as a tool in plant physiology: II. Interpretation of fluorescence signals. Photosynth Res 5:139–157. https://doi.org/10.1007/BF00028527

Le TN, McQueen-Mason SJ (2006) Desiccation-tolerant plants in dry environments. Rev Environ Sci Biotechnol 5:269–279

Liley NR (1966) Ethological isolating mechanisms in four sympatric species of poeciliid fishes. Behav, Suppl

Marks RA, Burton JF, McLetchie DN (2016) Sex differences and plasticity in dehydration tolerance: insight from a tropical liverwort. Ann Bot 118:347–356. https://doi.org/10.1093/aob/mcw102

Marks RA, Smith JJ, Cronk Q et al (2019) Genome of the tropical plant *Marchantia inflexa*: implications for sex chromosome evolution and dehydration tolerance. Sci Rep 9:8722. https://doi.org/10.1038/s41598-019-45039-9

Marschall M, Proctor MCF (1999) Desiccation tolerance and recovery of the leafy liverwort *Porella platyphylla* (L.)Pfeiff.: chlorophyll-fluorescence measurements. J Bryol 21:257–262. https://doi.org/10.1179/jbr.1999.21.4.257

Moore JD, Kollar LM, McLetchie DN (2016) Does selection for gamete dispersal and capture lead to a sex difference in clump water-holding capacity? Am J Bot 103:1449–1457. https://doi.org/10.3732/ajb.1600096

Oliver MJ, Mishler BD, Quisenberry JE (1993) Comparative measures of desiccation-tolerance in the *Tortula ruralis* complex. I. variation in damge control and repair. Am J Bot 80:127–136. https://doi.org/10.1002/j.1537-2197.1993.tb13779.x

Oliver MJ, Cushman JC, Koster KL (2010) Dehydration tolerance in plants. In: Sunkar R (ed) Plant stress tolerance. Methods in molecular biology (methods and protocols). Humana Press, Totowa, New Jersey, pp 3–24

Proctor MCF, Pence VC (2002) Vegetative tissues: bryophytes, vascular resurrection plants and vegetative propagules. Desiccation and survival in plants: drying without dying. CABI, Wallingford, pp 207–237

Proctor MCF, Oliver MJ, Wood AJ et al (2007) Desiccation-tolerance in bryophytes: a review desiccation-tolerance in bryophytes: a review. Bryologist 110:595–621

Retuerto R, Sánchez-Vilas J, Varga S (2018) Sexual dimorphism in response to stress. Environ Exp Bot 146:1–4

SAS Institute Inc. JMP®, Version 10

Schlenker W, Lobell DB (2010) Robust negative impacts of climate change on African agriculture. Environ Res Lett 5:014010. https://doi.org/10.1088/1748-9326/5/1/014010

Shine R (1989) Ecological causes for the evolution of sexual dimorphism: a review of the evidence. Q Rev Biol 64:419–461. https://doi.org/10.1086/416458

Sinervo B, Lively CM (1996) The rock–paper–scissors game and the evolution of alternative male strategies. Nature 380:240–243. https://doi.org/10.1038/380240a0

Stark LR, Oliver MJ, Mishler BD, McLetchie DN (2007) Generational differences in response to desiccation stress in the desert moss Tortula inermis. Ann Bot 99:53–60. https://doi.org/10.1093/aob/mcl238

Stark LR, Greenwood JL, Brinda JC, Oliver MJ (2014) Physiological history may mask the inherent inducible desiccation tolerance strategy of the desert moss *Crossidium crassinerve*. Plant Biol 16:935–946. https://doi.org/10.1111/plb.12140

Stieha CR, Middleton AR, Stieha JK et al (2014) The dispersal process of asexual propagules and the contribution to population persistence in *Marchantia* (Marchantiaceae). Am J Bot 101:348–356. https://doi.org/10.3732/ajb.1300339

Stieha CR, García-Ramos G, McLetchie DN, Crowley P (2017) Maintenance of the sexes and persistence of a clonal organism in spatially complex metapopulations. Evol Ecol 31:363–386. https://doi.org/10.1007/s10682-016-9841-8

Taborsky M (1998) Sperm competition in fish: 'bourgeois' males and parasitic spawning. Trends Ecol Evol 13:222–227. https://doi.org/10.1016/S0169-5347(97)01318-9

Woolbright LL (1983) Sexual selection and size dimorphism in Anuran amphibia. Am Nat 121:110–119. https://doi.org/10.1086/284042

Zamudio KR, Sinervo B (2000) Polygyny, mate-guarding, and posthumous fertilization as alternative male mating strategies. Proc Natl Acad Sci USA 97:14427–14432. https://doi.org/10.1073/pnas.011544998