

## Sex differences and plasticity in dehydration tolerance: insight from a tropical liverwort

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- **Background and Aims** Adaptations allowing plants to cope with drying are particularly relevant in the light of predicted climate change. Dehydration tolerance (DhT, also dehydration-tolerant) is one such adaptation enabling tissue to survive substantial drying. A great deal of work has been conducted on highly DhT species. However, bryophytes showing less intense and variable DhT are understudied, despite the potential for these species to provide an informative link between highly tolerant and sensitive species. In this study, we tested the degree to which DhT varies across populations and the sexes of a species expected to exhibit a moderate DhT phenotype.
- **Methods** To test predicted patterns of tolerance we assessed DhT in males and females of *Marchantia inflexa* from two distinct habitat types that differ in water availability. Both common garden and field-collected tissue was subjected to drying assays at multiple intensities and recovery was monitored by chlorophyll fluorescence. Verification studies were conducted to confirm the level of dehydration, the rate of drying and the associated changes in photosynthetic physiology.
- **Key Results** We confirmed our expectation that *M. inflexa* is able to tolerate moderate dehydration. We also found that females exhibited higher DhT than males, but populations did not differ in DhT when cultured in a common garden. However, field-collected samples exhibited differences in DhT corresponding to environmental dryness, suggesting plasticity in DhT.
- **Conclusions** By studying a less extreme DhT phenotype we gained insight into how more sensitive (yet still tolerant) organisms cope with dehydration. Additionally, the identified sex-specific variation in DhT may explain ecological patterns such as female-biased sex ratios. Furthermore, plasticity in DhT has the potential to inform management practices aimed at increasing tolerance to drought conditions.

**Key words:** *Marchantia inflexa*, sex difference, acclimation, chlorophyll fluorescence, desiccation tolerance, dehydration, plasticity.

### INTRODUCTION

In a warming and increasingly variable climate, adaptations to cope with water scarcity are particularly relevant. Knowledge of such adaptive strategies can inform both natural and agricultural applications. For some plant species, unique adaptations provide a means for tissues to resurrect from near complete dehydration and apparent death. This capacity is known as desiccation tolerance, an extreme case of dehydration tolerance (DhT, also dehydration-tolerant). A variety of research has been conducted on the natural history (Alpert, 2000), physiology (Bewley, 1979), mechanisms (Le and McQueen-Mason, 2006; Gechev *et al.*, 2012) and prevalence (Wood, 2007) of DhT with particular focus on highly desiccation-tolerant species.

Dehydration tolerance is not common across the Tree of Life and most plant and animal tissue cannot withstand cellular water potentials below  $-10$  MPa (Gaff, 1997; Walters *et al.*, 2002). However, DhT tissues consistently recover from cellular water potentials below this level (Oliver *et al.*, 2010), and desiccation-tolerant tissues survive cellular water potentials below  $-100$  MPa (Gaff, 1971). Dehydration-tolerant organisms have a unique ability to leverage both protection and repair systems

to combat the myriad drying stresses, such as membrane rupture (Hoekstra and Golovina, 1999), protein aggregation and oxidative damage (Leprince *et al.*, 1990; Oliver *et al.*, 2005). To date, considerable effort has been devoted to understanding the mechanism of DhT. Soluble sugar accumulation, antioxidant production, cell wall flexibility and late embryogenesis abundant (LEA) protein accumulation have been associated with tolerance in multiple species (see Black and Pritchard, 2002; Lüttge *et al.*, 2011, and citations within). Much of this work has focused on driving positive economic impact in the biotech and agricultural industries (Alpert, 2005; Toldi *et al.*, 2009), and although progress has been made many unanswered questions remain.

Despite being uncommon, DhT is found across many life forms, from bacteria (Billi and Potts, 2002) to animals (Clegg, 2005; Gusev *et al.*, 2014) and plants. In plants DhT likely evolved as a necessary adaptation for the transition from water to land by early plants (Oliver *et al.*, 2000). Through evolutionary time most vascular plants lost DhT in their vegetative tissues, perhaps as a trade-off for more complex water storage and transport systems supporting larger growth forms (Alpert, 2006; Gaff and Oliver, 2013). However, species from multiple lineages of vascular plants re-evolved vegetative DhT

independently (Farrant and Moore, 2011). Interestingly, most dehydration-sensitive plants retain DhT in their pollen, seeds or spores, suggesting that a wider array of plants may have the genetic potential for DhT, but that it is constrained to specific tissue types and developmental stages as a product of taxon-specific life history (Walters *et al.*, 2002; Grene *et al.*, 2011). Although there are DhT representatives across taxonomic groups of land plants, DhT is most prominent among bryophytes, lichens and green algae (Proctor *et al.*, 2007; Wood, 2007).

To date, a great deal of vegetative DhT research has focused on the extremely DhT (or desiccation-tolerant) species, which has contributed valuable insight into the process and mechanisms of DhT. However, such extreme phenotypes are not applicable to many plant forms and may obscure nuanced components of the process. There has been a relative paucity of research on bryophytes that show lower levels of tolerance, although some recent studies address this (Koster *et al.*, 2010; Cruz de Carvalho *et al.*, 2011, 2012, 2014, 2015; Pardow and Lakatos, 2013; Bader *et al.*, 2013; Stark *et al.*, 2013), and numerous studies have investigated moderate levels of dehydration in the drought response of vascular plants (Hsiao, 1973; Osakabe *et al.*, 2014). Bryophytes showing moderate DhT likely occupy a large ecological niche, and understanding their response to abiotic stress is important for predicting species range shifts due to environmental changes. Additionally, studying more moderate DhT in a relatively simple tissue could increase fundamental understanding of the spectrum of dehydration responses.

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

In this study we tested for intraspecific variation in DhT in the tropical dioecious liverwort *Marchantia inflexa*. We hypothesized that *M. inflexa* would show moderate DhT and also considerable intraspecific variability in DhT. For this study we used plants collected from populations located along a moisture availability gradient and subsequently grown in a common garden. Due to selection pressures, we predicted that *M. inflexa* would show lower DhT in plants originating from mesic habitats compared with less mesic habitats because plants originating from a moist environment would have little use for DhT. Based on previous studies (Stieha *et al.*, 2014), females were expected to be more DhT than males, offering a potential explanation for the female-biased sex ratios seen in this and many bryophyte populations. Additionally, we tested the predicted population difference in DhT in the field by using plants collected directly from field habitats.

## MATERIALS AND METHODS

### *Study organism, sampling and growth conditions*

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

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

Male and female plants were collected from randomly chosen locations (patches) along one stream site (East Turure River) and along one road site (Cumaca Road). Each patch was physically separated from the other to guarantee that individuals were genetically different, and uniqueness of each isolate was confirmed by PCR analysis (Brzyski *et al.*, 2014). Eighteen genotypes were selected for use in the current study: four males from the stream site, four males from the road site, five females from the stream site and five females from the road site.

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

### *Dehydration tolerance assay and recovery*

Dehydration conditions of differing intensity were generated using saturated salt solutions to modify the relative humidity (RH) in dehydration chambers. Experimental RHs, water vapour pressure deficits (VPDs) and corresponding treatment levels are shown in Table 1. Water vapour deficit was calculated to enable comparison of treatment intensity with our field assay (Anderson, 1936). Each experimental RH was calculated based on an equilibrium RH chart (Wexler and Hasegawa, 1954) and verified using a HOBO<sup>TM</sup> humidity sensor attached to a data logger (Onset Computer Corporation, Bourne, MA, USA).

Dehydration assays were conducted in dehydration chambers (airtight plastic boxes  $24 \times 10 \times 32$  cm) and were placed in a growth chamber with a 12-h light/12-h dark cycle period and a constant temperature of  $13^\circ\text{C}$ . Healthy vegetative thallus tips ( $\sim 7$  mm in length) from each of the 18 genotypes were collected from the greenhouse, saturated with distilled water and placed in the growth chamber for 24 h to ensure that each sample was fully hydrated before the assay began. After 24 h, each thallus tip was randomly assigned to one of four treatment groups and blotted to remove external water. Each thallus tip was then placed in a  $35 \times 10$  mm Falcon<sup>TM</sup> brand Petri dish along with a single filter paper disc and  $200\ \mu\text{L}$  of distilled water. These Petri dishes were placed on the internal perimeter of the dehydration chamber, and a bowl of saturated salt solution (differed between treatments) was placed in the centre of the box. A small battery-powered fan sat on a wire mesh stand above the salt solution (Fig. 1). Plants were dehydrated for 22 h, after which the fan was stopped and the plants were rehydrated with distilled water and maintained in the same growth chamber for 1 week. Treatments were replicated through time across all 18 genotypes: level 1 = three replications; level 2 = ten replications; level 3 = nine replications; level 4 = three replications.

Recovery of each individual thallus tip was analysed by chlorophyll fluorescence after 1 week of recovery to determine the

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

#### Relative water content

The relative water content (RWC) of tissue was measured to determine the percentage of water lost by samples in each treatment level. Vegetative tissue of *M. inflexa* was collected from the greenhouse and hydrated for 24 h in the growth chamber. Following hydration, the tissue was blotted dry to remove external water. Each plant was then weighed to the nearest nanogram using a Chan 29 electrobalance to determine the turgid mass ( $M_t$ ), randomly assigned to one of the four treatment groups, and subjected to the dehydration assay described above. However, following completion of the assay at 22 h, the tissue was not rehydrated. Instead, samples were weighed to determine sample fresh mass ( $M_f$ ), and the tissue was placed in a drying oven ( $80^\circ\text{C}$ ) for 3 d, after which its dry mass ( $M_d$ ) was taken. The RWC was calculated for each sample with the following formula:

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

TABLE 1. Treatment conditions for laboratory dehydration assays. Temperature was maintained in a growth chamber on a 12-h light/12-h dark cycle. Relative humidity was verified using a HOBO<sup>TM</sup> data logger with sensors attached. All assays were 22 h long. Vapour pressure deficit indicates the relative intensity of the combined temperature and relative humidity during the assay

Treatment level	Temperature ( $^\circ\text{C}$ )	Relative humidity (%)	Vapour pressure deficit (kPa)	Salt
Level 1	13	95.41	0.08	$\text{KNO}_3$
Level 2	13	85.92	0.21	KCl
Level 3	13	75.61	0.37	NaCl
Level 4	13	55.87	0.66	$\text{MgN}_2\text{O}_6$

#### Field study

A study was conducted to investigate corresponding patterns of DhT at the field sites in Trinidad during March 2015. Vegetative thallus tips ( $\sim 7$  mm in length) were collected from

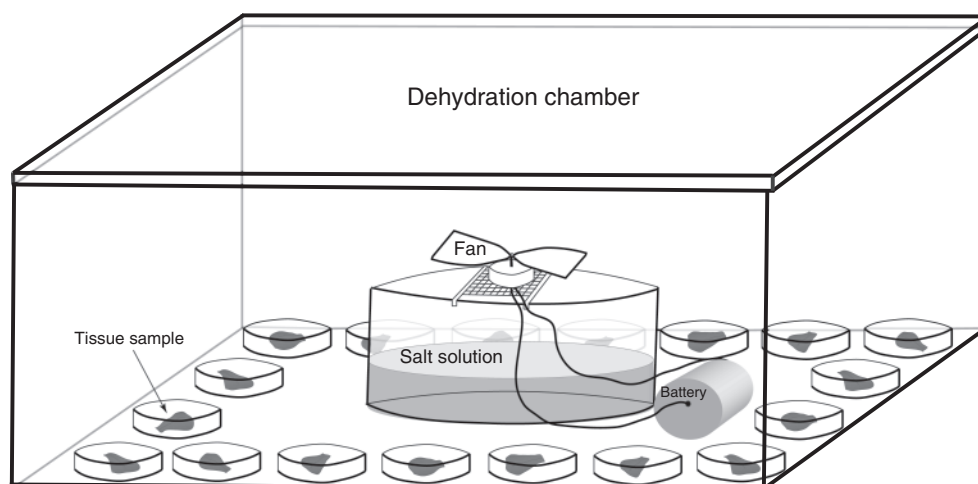


FIG. 1. Schematic of the dehydration assay set-up. Each box contained one tissue sample from each of the 18 genetic lines. Each sample was placed in a Petri dish along with a single filter paper disk and  $200\ \mu\text{L}$  of distilled water. The dehydration chamber was placed in a growth chamber with a constant temperature of  $13^\circ\text{C}$  and a 12-h light/12-h dark cycle.



plants growing in two habitat types (stream and road). The samples were collected from moist substrate and appeared hydrated when collected. Sampled thallus tips were saturated in stream water for 24 h, blotted dry, and then placed randomly into one of two dehydration treatment levels (Table 2). Three trails were conducted with ten thallus tips in each. Half of the thallus tips in each trial were from the stream habitat and half were from the road habitat. The assay was conducted following the same protocol as the laboratory dehydration assay (described above) with a few minor changes. First, because there was no access to a temperature-regulated growth chamber in Trinidad the dehydration assay was conducted under a raised building to avoid direct sunlight. Ambient temperatures ranged from 26 °C (night) to 30 °C (day). The corresponding VPDs and RHs for these treatments are listed in Table 2. The VPD was needed to compare the relative intensity of field and laboratory treatments because it accounts for the effect of temperature (Anderson, 1936). The thallus tips were allowed to dehydrate until they were noticeably dry, as indicated by visual changes in thallus curvature, after which they were rehydrated with stream water. Recovery was assessed by chlorophyll fluorescence 1 week after rehydration.

#### Verification of equilibration and rate of drying

To confirm that samples had equilibrated to the RH in the dehydration chambers after 22 h, vegetative thalli were collected from a single randomly selected genotype (1F) and subjected to the laboratory dehydration assay described above. Samples were weighed as in the RWC experiment and then randomly assigned to one of the four dehydration treatment levels. In order to minimize disturbance of the samples and potential changes to the treatment conditions, samples were not weighed for the first 16 h of the dehydration assay (except in treatment level 4 because these samples dried much faster than the others). Samples were then weighed at 16, 18, 20, 21 and 22 h using the Chan 29 electrobalance. When mass did not decline for three consecutive measurements, we considered tissues to be equilibrated to the RH in the chamber. If the samples were not equilibrated to experimental RH at 22 h the assay was extended for an additional hour. After completion, samples were oven-dried and weighed as described above. The RWC for each sample was calculated based on final  $M_d$ ,  $M_i$  and  $M_f$  at each time point using the equation for RWC.

TABLE 2. Treatment conditions for dehydration assays conducted in the field. Temperature and humidity were confirmed using a HOBOTM data logger with sensors attached. The temperature varied with ambient air temperature in Trinidad and the assay was conducted in a location to avoid direct sunlight. Vapour pressure deficit indicates the relative intensity of the combined temperature and relative humidity during the assay

Treatment level	Temperature (°C)	Relative humidity (%)	Vapour pressure deficit (kPa)	Salt
Field level 1	26–30	83.63	0.50–0.64	KCl
Field level 2	26–30	75.09	0.80–1.07	NaCl

#### Physiological changes

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

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

Gas exchange was measured with an open-flow system (LI-6400, Li-Cor, Lincoln, NE, USA) equipped with a fluorescence attachment (6400-40, Li-Cor). Air temperature was allowed to vary with the ambient laboratory temperature (22–24 °C), and RH ranged from 72 to 80 %. Thus, the VPD varied from 0.53 to 0.84 MPa. Airflow rate was set at 100 µmol s<sup>-1</sup> and CO<sub>2</sub> was set at 450 µmol mol<sup>-1</sup>. Infrared gas analysers (sample and reference) were matched prior to the assays.

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

#### Statistical analyses

All statistical analyses were done in JMP®, Version 10 (SAS Institute Inc., Cary, NC). The complete data set for this project has been archived on Figshare.

**Dehydration tolerance assay** To account for genotypic variation we estimated initial  $F_v/F_m$  of healthy tissues of each genotype using five thallus tips per genotype. The mean  $F_v/F_m$  for each genotype was used as the initial  $F_v/F_m$  value and the recovery  $F_v/F_m$  value for each thallus tip was subtracted from the mean initial  $F_v/F_m$  value for that genotype. Thus, the measure of recovery used for analysis of dehydration response was (initial  $F_v/F_m$  – recovery  $F_v/F_m = \Delta F_v/F_m$ ). Larger values indicate less recovery. The difference in recovery across treatment level was analysed using a mixed linear model with  $\Delta F_v/F_m$  as the dependent variable. The fixed effects tested were treatment level, sex, habitat and the sex  $\times$  habitat interaction. Genotype (nested within sex) and trial (replications) were random effects. *Post hoc* comparisons were made with a Tukey honest significant difference (HSD) test.

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

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

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

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

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

The response variable of photosynthetic rate was examined using a mixed linear model in which photosynthetic rate was

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

## RESULTS

### Dehydration tolerance assay and recovery

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

**Sex and habitat patterns** Analysis of recovery from treatment level 3 revealed a sex effect ( $F_{1,13.37} = 7.60$ ,  $P = 0.016$ ) and a sex  $\times$  habitat interaction ( $F_{1,13.37} = 4.94$ ,  $P = 0.044$ ). Females were less damaged by dehydration than males (Fig. 3A). More specifically, females were less damaged than males from the stream populations, but this sex difference was not evident in plants from road populations (Fig. 4). No overall population difference was found between individuals from stream or road habitats (Fig. 3B).

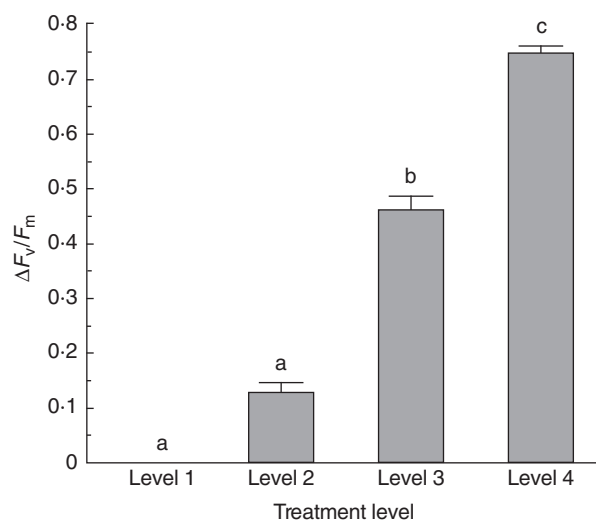


FIG. 2.  $\Delta F_v/F_m$  of samples for each of the four levels of dehydration treatment. Recovery was measured after 1 week. Sample sizes used to calculate survival were: level 1 = three; level 2 = ten; level 3 = nine; level 4 = three. Higher  $\Delta F_v/F_m$  indicates more damage. Bars with different lowercase letters are significantly different at  $P < 0.05$ .

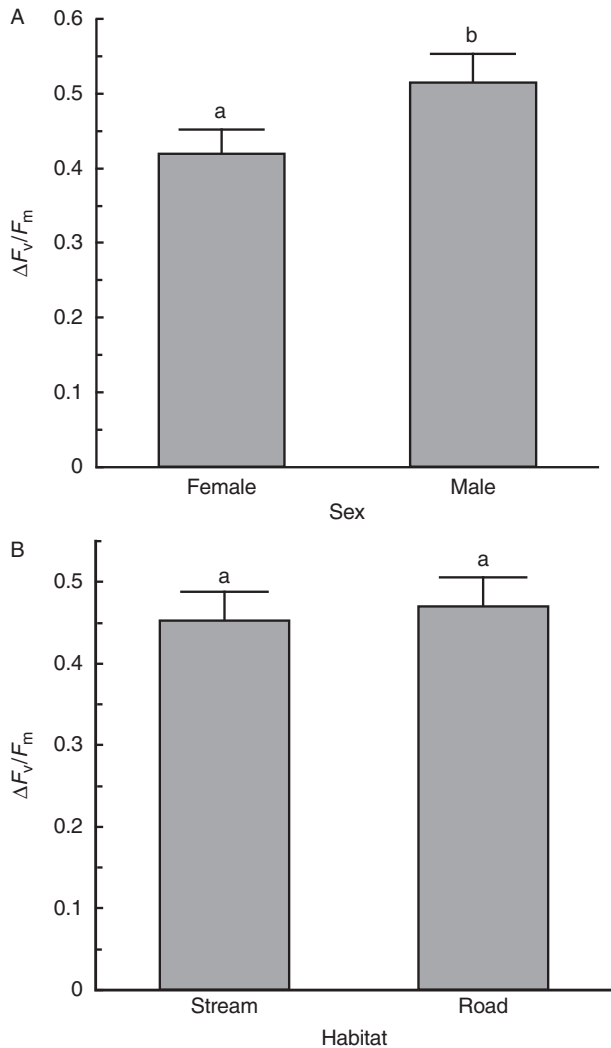


FIG. 3. (A) Difference in damage incurred from dehydration at level 3 between the sexes was significant, with females being less damaged than males ( $P = 0.016$ ). (B) Difference in damage incurred for plants from wet versus dry source habitats at level 3 is not significant. Recovery was measured after 1 week. There were nine trials. Bars with different lowercase letters are significantly different at  $P < 0.05$ .

#### Relative water content

Differences in mean RWC across treatment level were significant ( $F_{3,195} = 278.25$ ,  $P < 0.001$ ; Fig. 5). Tissue in the level 1 treatment maintained full saturation. Tissue in the level 2 treatment lost some cellular water (RWC =  $79.9 \pm 4.4$  %) and that in the level 3 treatment lost a substantial amount of cellular water (RWC =  $19.7 \pm 2.9$  %). Tissue in the level 4 treatment lost almost all cellular water.

#### Field study

Plants sampled from the dry road habitat recovered more completely from dehydration treatment than those collected from the moist stream habitat ( $F_{1,26} = 4.49$ ,  $P = 0.044$ , Fig. 6).

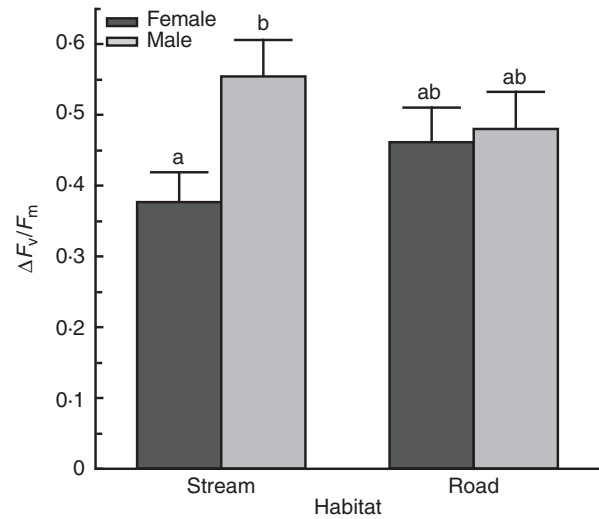


FIG. 4. Females from the stream were less damaged than males from the stream, whereas females and males from the road did not differ from one another. This interaction was statistically significant at treatment level 3 ( $P = 0.044$ ). Recovery was measured after 1 week. There were nine trials. Bars with different lowercase letters are significantly different at  $P < 0.05$ .

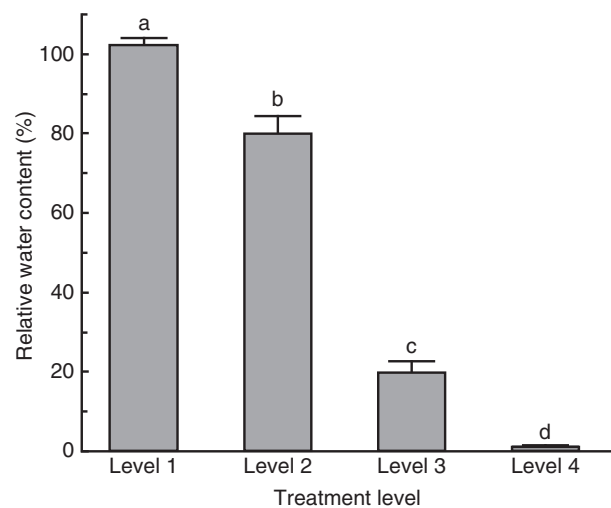


FIG. 5. Mean RWC of *M. inflexa* tissue after treatment at each of the four dehydration intensities (Table 1). Sample size for each treatment group was three replicated dehydration chambers each containing 18 individuals. Bars with different lowercase letters are significantly different at  $P < 0.05$ .

#### Verification of equilibration and rate of drying

There was a significant effect of time in all treatment levels ( $F_{5,3} = 529.77$ ,  $P < 0.001$ ) as well as a significant time  $\times$  treatment interaction (Wilks'  $\lambda$ ,  $F_{15,8.68} = 17.69$ ,  $P < 0.001$ ), indicating that the rate of drying differed across treatments (Fig. 7). Samples in both treatment levels 3 and 4 equilibrated to the treatment RH well within the timeframe of our assay, as evidenced by a lack of change in RWC during the last 4 h of the assay for treatment level 3 and the last 12 h for level 4. Samples in treatment levels 1 and 2 took the entire 22 h to equilibrate. During the final 3 h of the assay there was no significant time effect on RWC at any treatment level (level 1:  $F_{2,1} = 96.53$ ,

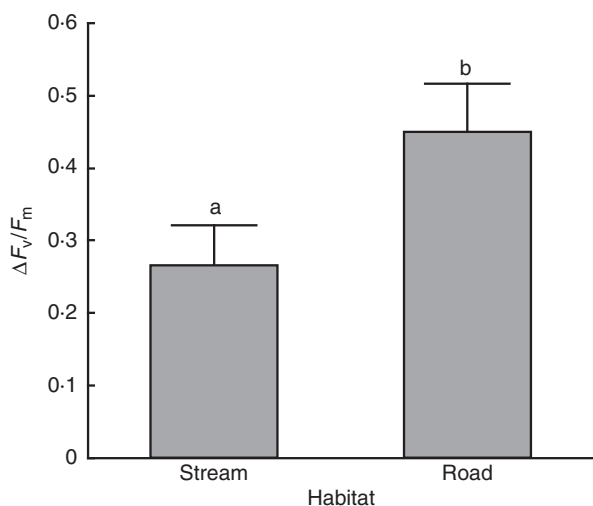


FIG. 6. The difference in recovery  $F_v/F_m$  in plants collected directly from the field at stream or road sites and subjected to dehydration conditions was significant ( $P=0.0438$ ). Recovery was measured after 1 week. There were three trials with ten thallus tips in each. Bars with different lowercase letters are significantly different at  $P < 0.05$ .

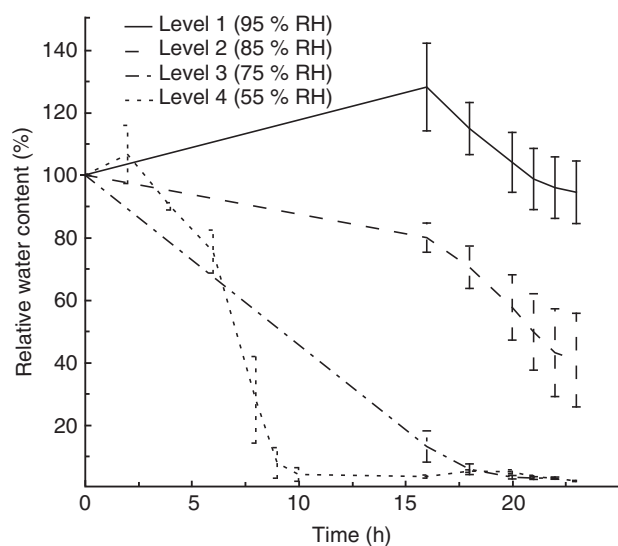


FIG. 7. Drying rate as change in RWC over time for the four treatment levels. Three samples of a single genotype (1F) were used in each treatment level. RWC was calculated using the fully hydrated sample mass, the mass measured over the course of the assay and the final oven-dry mass. The slight initial increase in RWC seen in level 1 and level 4 treatments is a result of the samples picking up external water prior to drying. The equilibration RWC here is not consistent with that seen in Fig. 5 because in Fig. 5 there was a single level 3 trail that did not equilibrate and level 2 trails contained substantial variation.

$P = 0.072$ ; level 2:  $F_{2,1} = 3.36$ ,  $P = 0.36$ ; level 3:  $F_{2,1} = 0.38$ ,  $P = 0.75$ ; level 4:  $F_{2,1} = 0.55$ ,  $P = 0.4$ , demonstrating that tissue was equilibrated to treatment conditions during this final time period.

#### Physiological changes

Tissue samples in the light equilibrated to  $\sim 75\%$  RH after  $\sim 3$  h, as indicated by the lack of difference in

mmol  $H_2O\ mol^{-1}$  of input and output air (Fig. 8A). Samples stopped assimilating  $CO_2$  after  $\sim 3$  h (Fig. 8B).

Neither initial ( $F_{1,4} = 0.85$ ,  $P = 0.408$ ) nor final ( $F_{1,4} = 3.37$ ,  $P = 0.14$ ) photosynthetic rates were significantly different between genotypes, but there was a significant change in photosynthetic rate during the process of equilibration to  $\sim 75\%$  RH (Fig. 8B). The mean initial photosynthetic rate was  $5.48\ \mu\text{mol}\ CO_2\ m^{-2}\ s^{-1}$  (95% CI 5.10 to  $5.86\ \mu\text{mol}\ CO_2\ m^{-2}\ s^{-1}$ ). The mean final photosynthetic rate was  $-0.02\ \mu\text{mol}\ CO_2\ m^{-2}\ s^{-1}$  (95% CI  $-0.19$  to  $0.16\ \mu\text{mol}\ CO_2\ m^{-2}\ s^{-1}$ ). Because the confidence intervals do not overlap, we conclude that the initial and final photosynthetic rates are significantly different from one another. Furthermore, the 95% CI for the final photosynthetic rate includes 0, suggesting that photosynthesis was likely shutdown at this point. We found that initial respiration levels were too low to detect a change over time.

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

## DISCUSSION

Our results indicate that *M. inflexa* displays a moderate degree of DhT, which confirms our expectation and extends existing knowledge of the diversity of DhT phenotypes within bryophytes. *Marchantia inflexa* also exhibits a sex difference in DhT, with females showing a higher degree of DhT than males. This sex difference may be caused by differences in male and female reproductive strategies, which in turn may explain biased population sex ratios. The sex difference is particularly intriguing because it was only observed for plants originating from the moist stream habitat. We did not find evidence of genetic adaptation to dry habitats, but we did detect plasticity in DhT as plants showed acclimation to different environments.

#### Recovery

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

*Marchantia inflexa* females exhibited a higher degree of DhT than males (Fig. 3A), which suggests contrasting male and female reproductive strategies or functions. This contradicts the typical expectation that females of dioecious plants should be less stress-tolerant due to higher resource investment in reproduction (Juvany and Munné-Bosch, 2015). However, there is mixed evidence on the direction of sex-specific stress tolerance



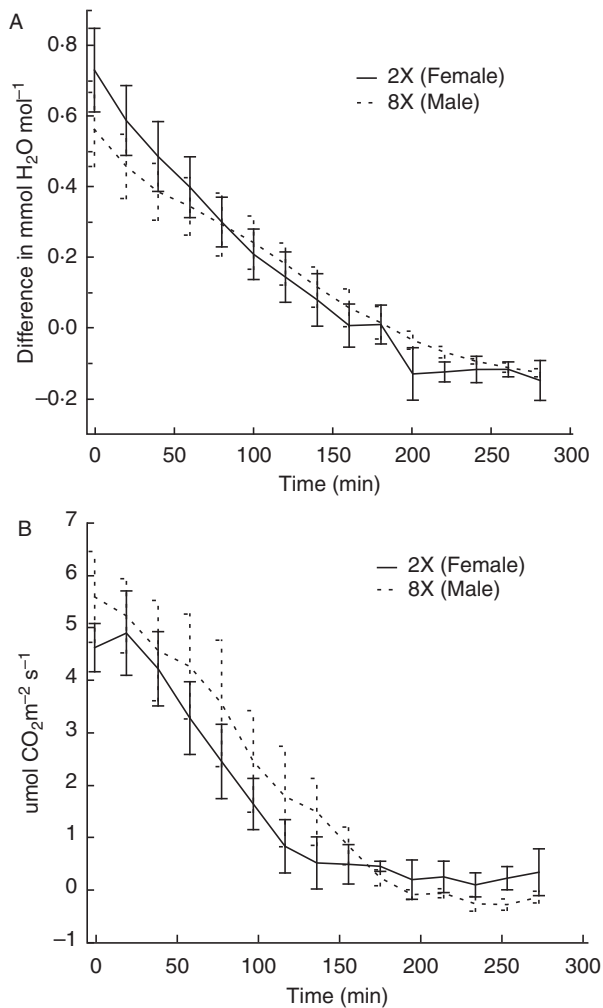


FIG. 8. (A) The dehydration state of samples over time was measured as difference in  $\text{mmol H}_2\text{O mol}^{-1}$  input to  $\text{mmol H}_2\text{O mol}^{-1}$  output. (B) The change in photosynthetic rate was measured over the same time period. Photosynthetic rates were measured as  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  and decreased to 0 between 2 and 3 h of drying at  $\sim 75\%$  RH. There was no difference between genotypes in their dehydration state or photosynthetic rate ( $n=6$ ).

differences in dioecious plants. While most studies indicate that males have higher water stress tolerance than females (Juvany and Munné-Bosch, 2015), multiple examples of higher tolerance in females have been observed as well (Ward et al., 2002; Sánchez-Vilas and Retuerto, 2009; Melanie et al., 2013). Further complicating matters, some studies have found that sex-linked differences in stress tolerance depend on life stage and environmental conditions (Rakocevic et al., 2009; Juvany and Munné-Bosch, 2015). Notably, higher DhT has been observed for females versus males in some bryophyte species (Newton, 1972; Stieha et al., 2014). In *M. inflexa*, like other bryophytes (McLetchie, 1992; Stark et al., 2000), males likely allocate more resources to pre-fertilization sexual investment than females, possibly at the expense of traits related to survival. Conversely, females may invest more in self-preservation than males as they must persist for longer during offspring maturation. Although plants were in a non-reproductive stage in our study, the underlying differences in male and female

reproductive strategies could be important. For instance, *M. inflexa* males consistently reach sexual maturity more readily than females (Brzyski et al., 2014), and this precocious trait might trade off with DhT. This could explain the higher degree of DhT in females compared with males, but resolving this will require more systematic investigation of sex-specific trade-offs in DhT and sexual reproduction.

We predicted that plants collected from dry habitats (roadsides) would show higher recovery from dehydration than those collected from moist habitats (streams). However, we found no difference in the DhT of these two groups when plants were grown in a common garden. This suggests that there are no adaptive differences in DhT between plants from these divergent habitat types (Fig. 3B), but the road habitats are novel and thus it is not particularly surprising that we did not detect evidence of DhT adaptation to this environment. However, when plants were collected directly from these two habitat types (field study) and subjected to a dehydration treatment, samples from dry habitats had higher DhT than plants from moist habitats (Fig. 6). This pattern fits our initial prediction but contradicts our laboratory study, and suggests a strong potential for acclimation. In other words, it implies that DhT in *M. inflexa* is a plastic trait. The ability of *M. inflexa* to acclimate to dry conditions corroborates previous work on protoplasts showing that slight decreases in water potential can lead to increased DhT and multiple studies demonstrating hardening in vegetative tissue (Schonbeck and Bewley, 1981; Beckett, 1999; Walters et al., 2002; Beckett et al., 2005; Cruz de Carvalho et al., 2011). We conclude that genetic adaptations are not responsible for the habitat difference in DhT, but rather that acclimation to dry conditions can increase DhT in *M. inflexa*.

The sex  $\times$  habitat interaction detected in the common garden study was driven by plants derived from the stream habitat (Fig. 4). Females from the stream showed higher DhT than males, whereas plants from roadsides had similar responses across the sexes. While this pattern is complex, it is consistent with previous studies on *M. inflexa* showing habitat  $\times$  sex interactions for life history traits. For example, the pattern of habitat-specific sexually dimorphic DhT is similar to what Brzyski et al. (2014) found for growth rate. In that study there was no overall habitat difference for growth rate, but stream-collected plants had a sexually dimorphic growth rate and road-collected plants did not have a dimorphic growth rate. One possible explanation for this pattern is that because the stream populations are native and well established, there has been more time for selection to generate the dimorphic patterns observed. Conversely, the roads are novel habitats and there has been less time for selection to act on secondary sexual dimorphism. In addition, the road populations engage in sexual reproduction much more frequently than those along the streams (Brzyski et al., 2014), which could lead to more mixing of male and female traits. Coupled with reduced evolutionary time, this mixing may explain the more homogeneous DhT of the sexes in road versus stream populations.

#### Verification

In bryophyte dehydration studies, equilibration to different RHs is often used as a measure of dehydration stress (Proctor



et al., 2007; Wood, 2007). Thus, it was necessary for us to demonstrate that the length of our assay was sufficient for tissue to equilibrate to each treatment RH. In doing so we showed that samples achieved equilibration during our assay, and also that the rate of drying varied across treatments (Fig. 7). Samples dehydrated at the lower RHs not only dried more completely, but also dried faster, which very likely influenced their survival. A great deal of work has shown that drying rate strongly affects survival and recovery from dehydration, with faster drying typically being more damaging for intact vegetative tissues (Farrant, 1999; Cruz de Carvalho et al., 2012, 2015), although in many seeds slow drying is more damaging (Pammenter et al., 1998).

Most of the work on bryophyte DhT has been done using equilibration to lower RHs than were used in our study (Wood, 2007). The standard RH used for dehydration treatment is ~50 %, which has been very informative in studying highly desiccation-tolerant species, but for understanding moderate levels of DhT we find it useful to use less intense dehydration treatments. Although other studies have used similarly less intense treatments (Pardow and Lakatos, 2013), there may be some concern as to the physiological effect of our treatments. To address this concern, we demonstrated that photosynthetic cessation is synchronized with equilibration to ~75 % RH in *M. inflexa* (Fig. 8A, B). Although the cessation of photosynthesis does not imply that all metabolic activity has ceased, it is a central plant process, and its shutdown indicates that substantial physiological changes are associated with this level of dehydration (Dilks and Proctor, 1979; Deltoro et al., 1998).

### Conclusions

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

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