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RESEARCH ARTICLE

Interactions of plant growth responses to spring freezing and summer drought: a multispecies comparison

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PREMISE OF THE STUDY: Freezing and drought both result in cellular dehydration, and similar physiological responses to these stressors may result in cross acclimation, whereby prior freezing exposure increases subsequent drought tolerance. We examined how spring freezing influences summer drought tolerance for a range of herbaceous old field species: 6 graminoids (*Agrostis stolonifera*, *Arrhenatherum elatius*, *Bromus inermis*, *Festuca rubra*, *Lolium perenne*, *Poa compressa*) and 2 forbs (*Plantago lanceolata*, *Securigera varia*), with the goal of examining the generality of cross acclimation responses.

METHODS: We exposed the plants to –5°C in the spring and to a 3-week summer drought, and harvested the plants after a 3-week watering/recovery period. We also measured leaf soluble proteins and sugars to explore the potential mechanisms before and during drought stress.

KEY RESULTS: For *Agrostis stolonifera*, *Bromus inermis*, *Lolium perenne*, *Plantago lanceolata,* and *Poa compressa* there was evidence of cross acclimation based on aboveground or belowground biomass, with a reduction in the severity of the drought effect for the plants previously exposed to freezing. Freezing and drought effects were additive for *Arrhenatherum elatius*, and for the remaining two species the test of the freezing-drought interaction was inconclusive, because significant drought and freezing effects did not co-occur. When present, freezing-drought interactions were not correlated with changes in leaf soluble protein or sugars.

CONCLUSIONS: Our results reveal that the phenomenon of freezing-drought cross acclimation appears to be common in herbaceous species, and variation among species in cross acclimation indicates that multiple stresses could alter relative species abundances in plant communities.

KEY WORDS cross acclimation; dehydration; forb; frost; graminoid; herbaceous; legume; stress interactions; stress memory; water deficit.

Drought tolerance in plants can be positively correlated with freezing tolerance (Cloutier and Siminovitch, 1982; Blödner et al., 2005), and drought-freeze interactions (where prior drought exposure increases subsequent freezing tolerance) can significantly alter plant physiological responses (Hoffman et al., 2012) and growth (Kreyling et al., 2012a). Likewise, freeze-drought interactions (where prior freezing increases subsequent drought tolerance) also can impact plant physiology (Horváth et al., 2007; Grudkowska and Zagdanska, 2010; Hossain et al., 2013) and plant growth (Kong and Henry, 2016). Collectively, these responses have been described as cross acclimation or stress memory (Walter et al., 2013).

Drought and freezing stress likely interact because both stresses increase the concentration of osmolytes, antioxidants, and molecular chaperones within plant cells (Guy et al., 1992; Sasaki et al., 1998; Xiong et al., 2002). These compounds can help prevent cellular damage by eliminating reactive oxygen species and repairing damaged proteins formed during cellular dehydration (Mahajan and Tutega, 2005). When plants are exposed to freezing immediately after drought, they exhibit elevated concentrations of soluble sugars and antioxidants (Hoffman et al., 2012); this effect also has been observed when freezing is applied months after drought exposure (Kreyling et al., 2012b). When the order of the stressors is switched, and plants are exposed to drought immediately

after freezing, there also are higher concentrations of antioxidants when compared to drought-only plants (Horváth et al., 2007; Grudkowska and Zagdanska, 2010; Hossain et al., 2013). However, prolonged elevation of soluble sugars concentrations after drought was not responsible for increased freezing tolerance in *Poa pratensis* L. (Kong and Henry, 2018), nor did the retention of soluble sugars after freezing stress influence drought tolerance in this species (Kong and Henry, 2016).

Drought-freeze interactions, where prior exposure to drought increases freezing tolerance, have been documented for a wide range of species. For instance, this interaction was observed for over 18 species across 17 different studies, and it occurred in both herbaceous (Kreyling et al., 2012a) and woody species (Nelson et al., 1993; Anisko and Lindstrom, 1996; Melgar et al., 2009; O'Keefe et al., 2016). However, freeze-drought interactions only have been investigated in 3 species: *Brassica campestris* L., *Triticum aestivum* L., and *Poa pratensis* (Grudkowska and Zagdanska, 2010; Hossain et al., 2013; Kong and Henry, 2016). For the former two species, physiological responses were investigated with drought stress applied immediately after freezing. Although exposure to drought shortly after freezing can occur in select environments (e.g., high altitude or desert systems; Ewers et al., 2003; Lambrecht et al., 2007), for most species the time interval between freezing stress and drought stress is on the order of months. Freezing-drought interactions were examined on such a time scale by Kong and Henry (2016), who investigated the effects of spring freezing on the summer drought tolerance of *Poa pratensis*, and who observed that the plants exposed to both freezing and drought stress had higher survival and growth after drought compared to the drought-only plants. While these results confirmed that cross acclimation between freezing and drought can be detected when these stressors are separated by an interval of months, the generality of freeze-drought cross acclimation responses across herbaceous species remains unexplored.

In this study, we investigated the effects of prior freezing on drought tolerance for a range of herbaceous old field species (six grasses and two forbs). We used plant biomass as a response variable to assess freezing-drought interactions, and we also examined the changes in protective compounds (soluble proteins and sugars) in response to the treatments. We predicted that reductions in both shoot and root biomass caused by drought would be diminished by prior exposure to freezing (i.e., there would be increased drought tolerance), and that elevated leaf soluble sugar and protein concentrations following freezing would correspond with the increased drought tolerance. Variation among species in the strength of freezing-drought cross acclimation would imply that cross acclimation could alter the responses of plant community composition to multiple stresses.

MATERIALS AND METHODS

Plant selection and establishment

We selected 8 common old field species (2 forbs and 6 grasses) to examine the interaction between spring freezing and summer drought. *Plantago lanceolata* L. is a weedy forb from the family Plantaginaceae that is well-adapted to drought conditions (Cavers et al., 1980), and *Securigera varia* L. is a legume in the family Fabaceae that is often used for erosion control (Symstad, 2004). The remaining species belong in the family Poaceae and included *Poa compressa* L., *Agrostis stolonifera* L., and *Festuca rubra* L., which are commonly used as turfgrasses (Carroll, 1943), and *Bromus inermis* Leyss, which is considered to be a rapidly spreading invasive species in North America (Otfinowski et al., 2007). We also selected *Arrhenatherum elatius* (L.) P.Beauv. ex J.Presl & C.Presl, an invasive grass in North America (Wilson and Clark, 2001) and *Lolium perenne* L., because these species have been used in previous drought-freeze studies (Hoffman et al., 2012; Kreyling et al., 2012a).

We grew *Arrhenatherum elatius* 'Ruffner', *Agrostis stolonifera*, *Bromus inermis*, *Lolium perenne* 'Amazing GS', *Festuca rubra*, *Poa compressa*, *Securigera varia* 'Penngift' (Ernest Seeds, Meadville, Pennsylvania, USA), and *Plantago lanceolata* seeds (Stokes Seeds, Thorold, Ontario, Canada) in trays filled with Pro-Mix BX Mycorrhizae soil medium (Premier Horticulture Inc., Quakertown, Pennsylvania, USA). Individual seedlings were transplanted into 9 cm wide by 13 cm deep square pots in late summer (August-September 2016), depending on the timing of seed germination. These pots were filled with soil (Bryanston silt loam, pH = 7.4; Hagerty and Kingston, 1992; Zhou et al., 2017) that was collected from the Environmental Sciences Western field station near London, Ontario, Canada. This soil was air-dried, sieved and mixed with perlite in a 1:3 ratio in Ziploc® bags and shaken thoroughly. The pots were weighed to determine the amount of dry soil they contained prior to seedling transplantation, and weeds that germinated throughout the experiment were removed.

We maintained the plants in a greenhouse (20°C day/15°C night) under a natural photoperiod, and they were watered daily until 29 November 2016, when they were placed outside in sand beds. To mimic field conditions, the pots were placed level with the surrounding soil and dried hay was placed on top of the plants to minimize excessive freezing exposure. To monitor soil temperatures during the winter, temperature loggers (LogTag Trix-8 Recorders, Auckland, New Zealand) were placed 1 cm below the surface (Fig. 1).

Freezing treatments

Based on preliminary freezing trials (Appendix S1), we selected a duration of 3 d and a target temperature of –5°C for all species, with the exception of *Securigera varia*, which was frozen at –2.5°C, to impose sufficient freezing stress while minimizing mortality. Freezing treatments were initiated on 31 March 2017 and the chambers were set at 15°C and ramped down at a rate of 1.67°C /h (12 h). The plants were held at a –5°C (48 h), and then warmed up at a rate of 1.67°C /h (12 h) to 15°C. For control plants, the chambers were set initially at 15°C and cooled down at 1.67°C /h (6 h) to 5°C (54 h) and warmed up at 1.67°C /h (6 h) back to 15°C (6 h). To ensure that sufficient freezing stress occurred, *Arrhenatherum elatius*, *Agrostis stolonifera*, *Bromus inermis*, *Lolium perenne*, *Festuca rubra*, and *Plantago lanceolata* were frozen for a second time between 10 and 13 April 2017. These chambers were cooled down from 5°C at 2°C /h (5 h) to –5°C (62 h) and warmed up to 5°C at 2°C/h (5 h); control plants were kept at a constant 5°C. To assess freezing damage, we determined the number of green leaves for both frozen (n = 6) and non-frozen (n = 6) plants immediately before the drought.

Drought treatments

Based on preliminary drought trials (Appendix S2), which showed that sufficient drought stress can occur within 3 weeks, we withheld

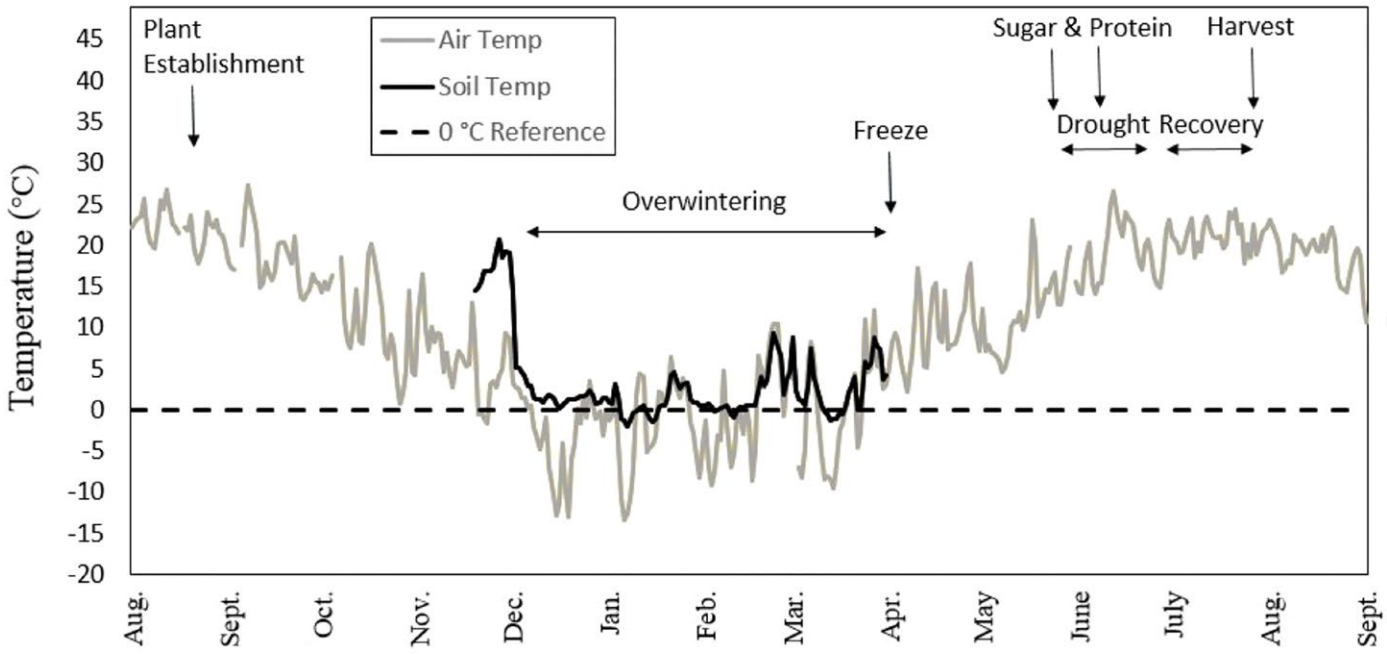

FIGURE 1. Average air temperatures (grey solid line) in London, Ontario, Canada (Environment Canada, National Climate Data and Information Archive: Canadian Climate Normals 1981–2010) and average soil temperatures at the 1 cm depth (solid black line), with reference to 0°C (dashed black line). Important dates during the experiment from August 2016 to September 2017 have been indicated with vertical and horizontal arrows.

water from the drought treatment plants for the latter amount of time (control plants were supplied daily with 200 mL of water to saturate the soil). The drought treatments were initiated at the beginning of June 2017. Pots for each species were weighed every other day to determine soil water content, and we assessed drought stress by examining turgor loss, discoloration, and leaf wilting. After the drought, the plants were well-watered every day until harvest.

Soluble protein and sugar assays

A set of plants was grown for each species to examine both soluble sugar (glucose, sucrose, and fructose) and soluble protein concentrations in the leaves. These plants were established in the fall of 2016 (see above at Plant selection and establishment) and they experienced the same treatments as the plants used for the biomass analysis. The plants were harvested either immediately before the drought or at the onset of drought stress (i.e., when there were visual signs of turgor loss and wilting). Drought symptoms appeared on day 3 for *Securigera varia*, day 9 for *Arrhenatherum elatius*, *Agrostis stolonifera*, *Bromus inermis*, *Festuca rubra*, *Poa compressa*, and *Plantago lanceolata*, and on day 14 for *Lolium perenne*; these dates corresponded to the days that the plants used for the protein and sugar assays were harvested.

Overwinter survival was low for *Poa compressa* at 57%, and the freezing treatments had a significant impact on *Lolium perenne* shoot biomass. Therefore, we did not conduct sugar and protein analyses on these two species, with the concern that mortality would be too high during the drought (i.e., we needed to ensure that we had a sufficient sample size for the growth analysis).

We measured soluble proteins in leaves using a modified Bradford assay (Hoffman et al., 2012). Green leaf-tissue was randomly selected and frozen using liquid nitrogen, and the plant material was subsequently wrapped in aluminum foil and stored at –80°C until further analysis. Leaf tissue was ground using liquid nitrogen in a 15 mL test tube, and 8 mL of 150 mM ice-cold phosphate buffer (1 M potassium phosphate; pH modified using 1 M NaOH) was added and the plant material was homogenized using a vortex. The test tubes were centrifuged at 15,000 g at 4°C for 20 min using a Beckman Avanti J-30I Centrifuge and JA.25.15 rotor (Beckman Coulter, Brea, California, USA). In a disposable cuvette, 0.1 mL of the sample was mixed with 1.5 mL of the Bradford reagent, and absorbency was determined after a 20 min color development period at a wavelength of 595 nm using a Cary 50 Bio UV-Visible Spectrophotometer (Varian, Palo Alto, California, USA). For each sample run, a standard curve was generated using bovine serum albumin (Sigma-Aldrich, St. Louis, Missouri, USA).

We determined combined soluble sugars (glucose, fructose, and sucrose) in leaves using a modified phenol-sulfuric acid method (Buysse and Merckx, 1993; Wang et al., 2003; Kong and Henry, 2016). Briefly, green plant leaves were randomly selected and dried at 60°C for a minimum of 3 d. The plant tissue was ground into a fine powder using a 2000 Geno/Grinder ball mill (SPEX CertiPrep, Metuchen, New Jersey, USA), and 50 mg was weighed out and filtered with 30 mL of 80% ethanol through Whatman™ grade 1 filter paper, and then 20 mL of deionized water was added. Subsequently, 0.5 mL of this solution was mixed with 0.5 mL of 18% (w/v) phenol and 2.5 mL of concentrated sulphuric acid. Absorbance at 490 nm was determined using a UV-VIS Recording Spectrophotometer UV-160 (Shimadzu, Kyoto, Japan), and a standard curve for sucrose was used to determine soluble sugar concentrations.

Non-destructive biomass estimation

To assess the freezing effect on biomass prior to the drought treatment, we grew individual plants for each species in 10 cm diameter by 9 cm deep round pots with Pro-Mix BX Mycorrhizae soil medium

and watered them daily. The plants were harvested at various times to obtain a range of plant sizes, the number of green leaves in a single plant was quantified, and the leaves were dried at 60°C for a minimum of 3 d. Allometric equations between leaf count and total dry leaf biomass were created for each species, where the best fit was selected through AIC. These equations were used to convert leaf counts to dry leaf biomass estimates for aboveground tissue (refer to equations shown in Appendix S3).

Final harvest

Aboveground biomass was harvested destructively from 13 to 14 July 2017, and the pots were stored in the dark at 5°C and the roots were harvested during the following weeks. Shoot and root tissue were dried at 60°C for a minimum for 3 d, and subsequently weighed. Aboveground tissue was separated into living green tissue and senesced material.

Statistical analyses

To compare estimated aboveground biomass prior to the drought treatment between the frozen and non-frozen plants we used two-tailed *t*-tests. For the final harvest data (post-drought recovery), three-way ANOVAs were run to assess the effects of freezing, drought, species and their interactions on shoot and root biomass; because of a significant interaction between freezing, drought and species for this global analysis, we subsequently performed two-way ANOVAs to assess the interaction between freezing and drought for each species independently.

A two-way ANOVA was used to assess the effect of freezing and species on leaf soluble sugars and protein prior to the onset of drought; two-tailed *t*-tests were then run to assess the effect of freezing for each species. Three-way ANOVAs were used to assess the effects of freezing, drought, species, and their interactions on soluble sugars and protein at the onset of drought symptoms; for each species, a single two-way ANOVA was used to assess freezing and drought effects. Statistics were conducted using JMP version 14.1 (SAS Institute, Cary, North Carolina, USA), and the effective sample sizes for each set of measurements are provided in Appendix S4.

RESULTS

Prior to drought, the freezing treatment suppressed aboveground biomass (estimated non-destructively) for *Plantago lanceolata* (frozen: 305 ± 20 mg; non-frozen: 648 ± 113 mg; $t = 3.0$, $df = 11$, $P = 0.01$), *Agrostis stolonifera* (frozen: 261 ± 63 mg; non-frozen: 531 ± 25 mg; $t = 4.0$, $df = 11$, $P < 0.01$), and *Lolium perenne* (frozen: 97 ± 42 mg; non-frozen: 354 ± 22 mg, $t = 5.4$, $df = 11$, $P < 0.01$). By the final biomass harvest (i.e., post-drought recovery), significant drought and freezing effects did not co-occur for *Securigera varia* and *Festuca rubra*; therefore, further testing for cross acclimation in these two species would have been inconclusive. However, for the combined analysis of the remaining six species, there was a significant interaction between freezing and drought for shoots (Table 1) and for roots (Table 2), with a reduction in the severity of the drought effect for plants previously exposed to freezing (Figs. 2 and 3).

For the individual species responses, there was an interaction between freezing and drought for *Agrostis stolonifera* shoot biomass (Appendix S5), where previously frozen plants experienced a 19% decline in response to drought, whereas non-frozen plants experienced a 37% decline (Fig. 2A). There also was an interaction for *Bromus inermis* shoot biomass (Appendix S5), where previously frozen plants experienced a 1% decline in response to drought, while non-frozen plants experienced a 38% decline (Fig. 2C), and for *Plantago lanceolata* shoot biomass (Appendix S5), where frozen plants experienced a 10% decline in shoot biomass and non-frozen plants experienced a 40% decline in biomass after the drought (Fig. 2F).

For *Agrostis stolonifera* root biomass there also was an interaction between freezing and drought (Appendix S6), where previously frozen plants experienced a 16% increase in response to drought, but non-frozen plants experienced a 26% decrease (Fig. 3A). Similarly, *Lolium perenne* root biomass increased by 25% response to drought for previously frozen plants, whereas it decreased by 5% for non-frozen plants (Appendix S6; Fig. 3E). For *Poa compressa*, the root biomass of previously frozen plants increased by 23% in response to drought, while it declined by 32% for non-frozen plants (Appendix S6; Fig. 3G). Only in *Arrhenatherum elatius* were there significant freezing and drought effects, but no evidence for cross acclimation (i.e., the effects of freezing and drought were additive) for both root and shoot biomass.

For total soluble protein (Table 3) and sugars (Table 4) there were no interactive effects between freezing and drought. There was, however, a marginally significant freezing effect for *Festuca rubra* before the start of the drought ($t = 1.9$, $df = 22$, $P = 0.07$), and at the onset of drought symptoms soluble protein concentrations increased

TABLE 1. Summary table for three-way ANOVA examining the effects of freezing, drought, and species (*Arrhenatherum elatius, Agrostis stolonifera, Bromus inermis, Lolium perenne, Poa compressa, Plantago lanceolata*) on green shoot biomass. Individual plants were exposed to spring freezing and summer drought. The degrees of freedom for the *F* values are represented by df_1 (numerator) and df_2 (denominator).

Source	*F*	df_1	df_2	*P* value
Freeze	50.1	1	314	<0.01
Drought	56.6	1	314	<0.01
Species	103.6	5	314	<0.01
Freeze × Species	0.7	5	314	0.60
Drought × Species	2.9	5	314	0.02
Freeze × Drought	19.9	1	314	<0.01
Freeze × Drought × Species	2.8	5	314	0.02

TABLE 2. Summary table for three-way ANOVA examining the effects of freezing, drought, and species (*Arrhenatherum elatius, Agrostis stolonifera, Bromus inermis, Lolium perenne, Poa compressa, Plantago lanceolata*) on root biomass. Individual plants were exposed to spring freezing and summer drought. The degrees of freedom for the *F* values are represented by df_1 (numerator) and df_2 (denominator).

Source	*F*	df_1	df_2	*P* value
Freeze	209.9	1	314	<0.01
Drought	9.4	1	314	<0.01
Species	29.8	5	314	<0.01
Freeze × Species	8.0	5	314	<0.01
Drought × Species	1.7	5	314	0.14
Freeze × Drought	18.1	1	314	<0.01
Freeze × Drought × Species	1.5	5	314	0.21

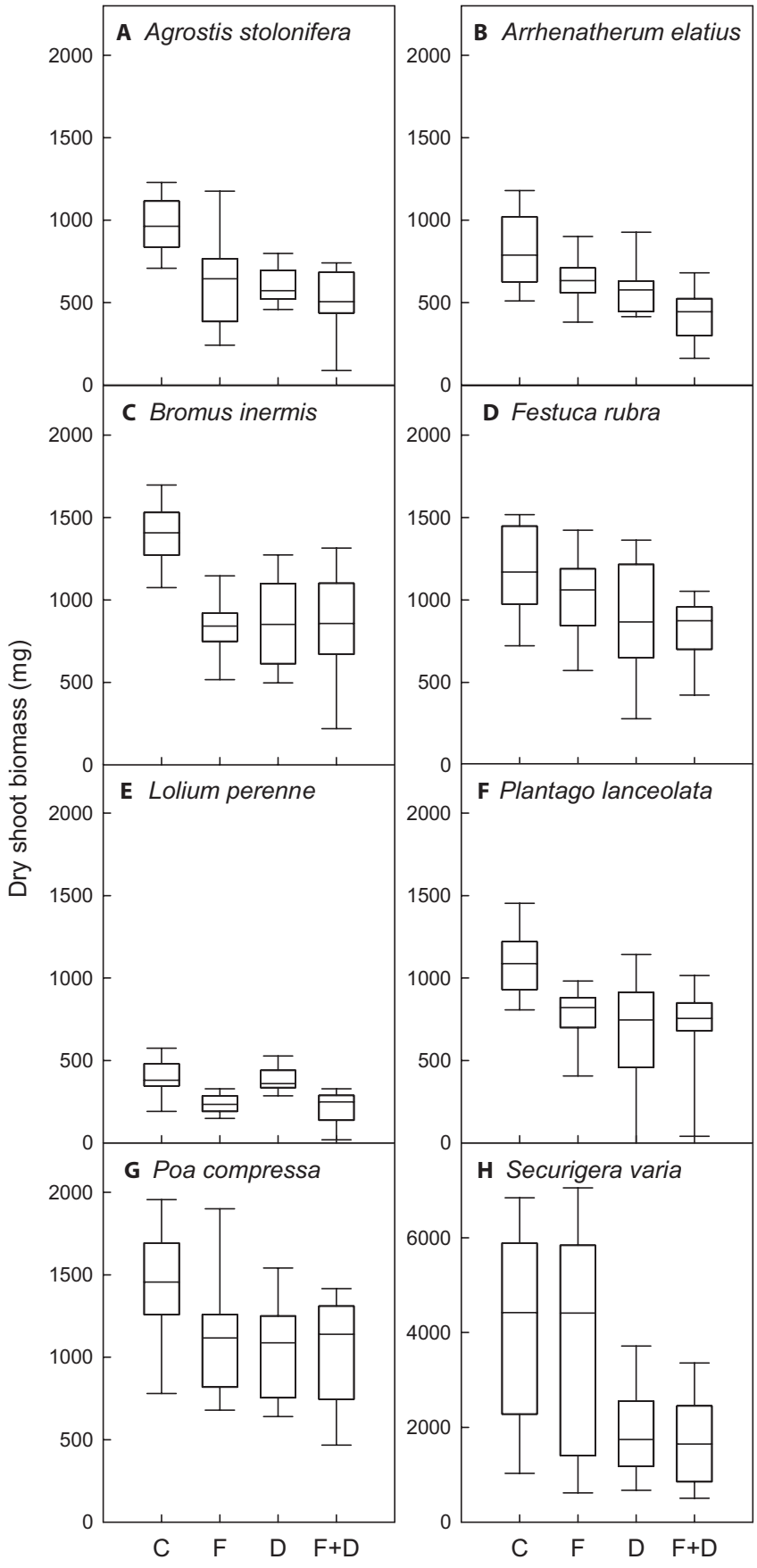

FIGURE 2. Dry green shoot biomass for: Panel A, *Agrostis stolonifera*; Panel B, *Arrhenatherum elatius*; Panel C, *Bromus inermis*; Panel D, *Festuca rubra*; Panel E, *Lolium perenne*; Panel F, *Plantago lanceolata*; Panel G, *Poa compressa*; and Panel H, *Securigera varia*. The plants were frozen for a total of 6 d (3 d for *Poa compressa*) in the spring at –5°C, and control plants were kept at 5°C. The plants then experienced drought stress, where water was withheld, and control plants were watered daily. The plants were then harvested and dried after a 3-week recovery period, and box plots were created for the control (C), freeze-only (F), drought-only (D), and freeze-drought (F+D) treatments. The lower boundary of the box plot represents the 25th percentile and the upper boundary of the box represents the 75th percentile with the horizontal line representing the median green shoot biomass; the top and bottom whiskers indicate the 90th and 10th percentiles, respectively. Note: The vertical scale for Panel H, *Securigera varia,* is different from all other species.

in the plants that experienced drought ($F_{1, 19}$ = 7.5, P = 0.01; Table 5). At the onset of drought symptoms, soluble protein concentrations also increased for the drought plants in *Agrostis stolonifera* ($F_{1, 19}$ = 9.8, P < 0.01; Table 5). Sugar concentrations were significantly lower for frozen plants than for non-frozen plants before the drought in *Bromus inermis* (t = 4.2, df = 20, P < 0.01) and *Plantago lanceolata* (t = 2.3, df = 22, P = 0.03; Table 6). However, drought stress increased sugar concentrations in *Festuca rubra* ($F_{1, 19}$ = 12.1, P < 0.01) and *Agrostis stolonifera* ($F_{1, 17}$ = 24.1; P < 0.01; Table 6).

DISCUSSION

Our results demonstrate that freezing-drought cross acclimation effects on growth, previously only investigated and demonstrated in *Poa pratensis* (Kong and Henry, 2016), are present for a range of herbaceous species (both grasses and forbs). In all cases, the decline in biomass for plants exposed to drought was minimized for plants previously exposed to freezing. Interestingly, although drought-freezing cross acclimation (the opposite of the interaction explored in the current study) has been demonstrated previously in *Arrhenatherum elatius* under field conditions and over an ecologically-relevant time interval (Kreyling et al., 2012a), this was the one species for which freezing followed by drought did not interact. In both

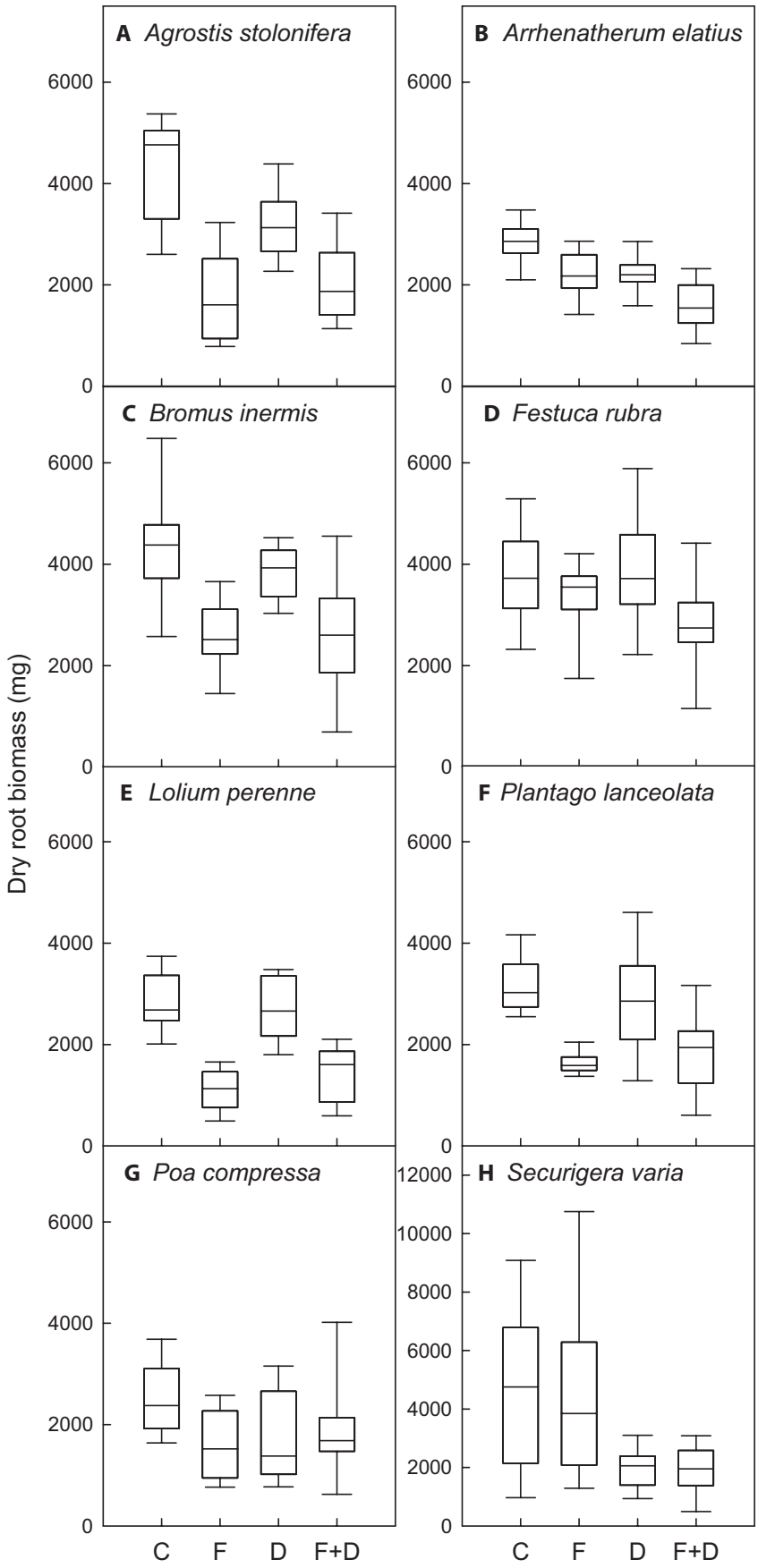

FIGURE 3. Dry root biomass for: Panel A, *Agrostis stolonifera*; Panel B, *Arrhenatherum elatius*; Panel C, *Bromus inermis*; Panel D, *Festuca rubra*; Panel E, *Lolium perenne*; Panel F, *Plantago lanceolata*; Panel G, *Poa compressa*; and Panel H, *Securigera varia*. The plants were frozen for a total of 6 d (3 d for *Poa compressa*) in the spring at –5°C, and control plants were kept at 5°C. The plants then experienced drought stress, where water was withheld, and control plants were watered daily. The plants were then harvested and dried after a 3-week recovery period, and box plots were created for the control (C), freeze-only (F), drought-only (D), and freeze-drought (F+D) treatments. The lower boundary of the box plot represents the 25th percentile and the upper boundary of the box represents the 75th percentile with the horizontal line representing the median root biomass; the top and bottom whiskers indicate the 90th and 10th percentiles, respectively. Note: The vertical scale for Panel H, *Securigera varia,* is different from all other species.

studies, the plants were exposed to –5°C and relatively similar drought durations, but the acclimation state of plants may have differed. In the study conducted by Kreyling et al. (2012a), plant tissue subjected to drought may have senesced during the winter, and the new growth may have differed in stress tolerance, which may have attributed to the differences between studies. In contrast, for *Lolium perenne*, both drought-freezing cross acclimation (Hoffman et al., 2012) and freeze-drought cross acclimation have been demonstrated.

Contrary to our prediction, the cross acclimation responses we observed were not correlated with changes in leaf soluble proteins. The lack of interactive effects on soluble proteins during the onset of drought symptoms would suggest that prior freezing did not elicit a stronger soluble protein response upon exposure to drought. In addition, there was no significant freezing effect on leaf soluble proteins immediately before the start of the drought, which indicated that the retention of soluble proteins after freezing could not explain any of the observed cross acclimation. However, it is important to note that the enzyme Rubisco accounts for >20% of total soluble protein in plant leaves, and changes in other proteins may be challenging to detect against the high background concentrations (Chapin et al., 1987; Panković et al., 1999).

Similarly, elevated concentrations of soluble sugars did not appear to correlate with cross acclimation. Surprisingly, we found that

non-frozen *Bromus inermis* and *Plantago lanceolata* had higher soluble sugar concentrations compared to frozen plants immediately prior to the drought, but this effect was not apparent during the drought period. In this case, frozen plants may have allocated sugars to cellular maintenance. Based on our results, interactions may be linked to genes responsible for protein folding, rather than genes related to osmotic adjustment (Xiong et al., 2002; Budak et al., 2013), and further investigations on the responses of plant chaperones in the context of cross acclimation therefore are warranted. Furthermore, epigenetic modifications (e.g., DNA methylation and histone acetylation; Bossdorf et al., 2008; Crisp et al., 2016) that occur during freezing should be examined as potential mechanisms for enhanced drought tolerance in plants.

Legacy effects of freezing on plant size could influence the observation of cross acclimation. Freeze-damaged plants may be smaller in size at the onset of the drought, and therefore experience drought stress later during the dry-down cycle than non-frozen plants, because of their reduced water demand (i.e., smaller plants use less water). In this case, the plants subjected to freezing would have enhanced drought avoidance, opposed to higher drought tolerance. Based on our non-destructive leaf measurements, this confounding size effect could potentially explain the interaction for *Plantago lanceolata* and *Agrostis stolonifera*. However, this could not explain the interaction observed for *Bromus inermis* and *Poa compressa*, because the frozen plants had similar aboveground biomass as the non-frozen plants.

Variation in cross acclimation can potentially alter plant community composition, and species that have high drought tolerance and exhibit cross acclimation may outcompete less competitive species. These stress interactions can therefore have consequences for plant community biodiversity. In addition, cross acclimation may impact aboveground biomass differently from belowground biomass (e.g., *Plantago lanceolata* and *Lolium perenne*), which suggests there are different strategies in response to drought stress after freezing. For *Holcus lanatus* L. and *Alopecurus pratensis* L. (Gargallo-Garriga et al., 2015), shoot tissue metabolites differed from the metabolites found in the root tissue of plants exposed to drought, and it would not be surprising if this had consequences on the drought tolerances. Thus, exposure to freezing may increase the competitive ability of certain species during and after drought, and increase their uptake of below- and/or aboveground nutrients.

In summary, cross acclimation between freezing and drought occurs in a variety of herbaceous species and appears to be relatively common under our experimental conditions. For species that do not show cross acclimation, increased stress intensity may reveal that this interaction can exist. The importance of cross acclimation may become more apparent as freezing and drought stress become more prevalent for herbaceous plants in northern temperate regions. Higher average air temperatures may reduce snow cover and expose

TABLE 3. Summary tables for statistical analyses examining the treatment effects on leaf soluble proteins in *Arrhenatherum elatius, Agrostis stolonifera, Bromus inermis*, *Festuca rubra, Plantago lanceolata*, and *Securigera varia*. A two-way ANOVA was run using freezing and species for the plants harvested before the drought and a three-way ANOVA was run using freezing, drought and species as factors for the plants harvested during the drought. The degrees of freedom for the *F* values are represented by df_1 (numerator) and df_2 (denominator).

	Source	*F*	df_1	df_2	*P* value
Before drought					
	Freeze	1.4	1	114	0.24
	Species	70.0	5	114	<0.01
	Freeze × Species	0.4	5	114	0.87
During drought					
	Freeze	0.3	1	114	0.60
	Drought	13.3	1	114	<0.01
	Species	141.8	5	114	<0.01
	Freeze × Species	0.3	5	114	0.91
	Drought × Species	7.8	5	114	<0.01
	Freeze × Drought	0.3	1	114	0.59
	Freeze × Drought × Species	0.3	5	114	0.92

TABLE 4. Summary tables for statistical analyses examining the treatment effects on leaf soluble sugars in *Arrhenatherum elatius, Agrostis stolonifera, Bromus inermis*, *Festuca rubra, Plantago lanceolata*, and *Securigera varia*. A two-way ANOVA was run using freezing and species for the plants harvested before the drought and a three-way ANOVA was run using freezing, drought, and species as factors for the plants harvested during the drought. The degrees of freedom for the *F* values are represented by df_1 (numerator) and df_2 (denominator).

	Source	*F*	df_1	df_2	*P* value
Before drought					
	Freeze	3.7	1	114	0.06
	Species	8.3	5	114	<0.01
	Freeze × Species	4.9	5	114	<0.01
During drought					
	Freeze	1.3	1	114	0.26
	Drought	10.7	1	114	<0.01
	Species	23.1	5	114	<0.01
	Freeze × Species	0.1	5	114	0.99
	Drought × Species	2.2	5	114	0.06
	Freeze × Drought	< 0.1	1	114	0.95
	Freeze × Drought × Species	0.6	5	114	0.71

TABLE 5. Average ± SE soluble protein content in plant leaves immediately before the drought and at the onset of drought symptoms, for previously frozen or non-frozen plants. We withheld water from the plants during the summer for 3 wks or watered the soil to field capacity. Protein concentrations are expressed in mg/mL/dry leaf (g).

	Treatment	*A. elatius*	*A. stolonifera*	*B. inermis*	*F. rubra*	*P. lanceolata*	*S. varia*[1]	*S. varia*[2]
Before drought								
	Freeze	4.96 + 0.34	7.76 ± 0.65	0.77 ± 0.06	0.58 ± 0.05	1.40 ± 0.21	29.89 + 4.7[3]	N/A
	No freeze	4.46 + 0.31	7.14 ± 0.52	0.77 ± 0.06	0.44 ± 0.05	1.40 ± 0.15	25.80 + 2.6[3]	N/A
During drought								
	Freeze/Drought	5.11 ± 0.37	40.53 ± 5.05	1.37 + 0.54	0.69 ± 0.03	3.04 + 1.67	1.95 + 0.35	1.56 + 1.00
	Freeze/No drought	6.09 ± 0.57	28.09 ± 3.58	0.91 + 0.06	0.61 ± 0.07	0.76 + 0.09	0.57 + 0.17	0.35 + 0.15
	No freeze/Drought	5.14 ± 0.29	40.38 ± 6.59	0.92 + 0.13	0.79 ± 0.09	1.28 + 0.26	2.89 + 1.09	2.90 + 2.34
	No freeze/No drought	5.23 ± 0.72	22.61 ± 1.92	1.05 + 0.15	0.52 ± 0.05	0.82 + 0.16	1.21 + 0.72	0.19 + 0.07

Notes: [1]Compound leaflets; [2]Secondary leaflets; [3]Different methodology used, where tissue was ground with buffer using a mortar and pestle. All other sample runs were ground using liquid nitrogen as described in the Materials and Methods section.

TABLE 6. Average ± SE soluble sugar content (glucose, sucrose, fructose) in plant leaves immediately before the drought and at the onset of drought symptoms, for previously frozen or non-frozen plants. We withheld water from the plants during the summer for 3 wks or watered the soil to field capacity. Sugar concentrations are expressed in µmol/mL/dry leaf (g).

	Treatment	*A. elatius*	*A. stolonifera*	*B. inermis*	*F. rubra*	*P. lanceolata*	*S. varia*[1]
Before drought							
	Freeze	455 ± 41	102 + 23	137 ± 37	485 ± 60	287 ± 58	468 ± 59
	No freeze	523 ± 62	70 + 42	447 ± 66	368 ± 40	520 ± 79	405 ± 34
During drought							
	Freeze/Drought	286 ± 119	273 ± 25	253 ± 53	763 ± 89	469 ± 119	329 ± 50
	Freeze/No drought	287 ± 58	158 ± 11	197 ± 26	525 ± 54	402 ± 129	205 ± 26
	No freeze/Drought	334 ± 90	345 ± 52	211 ± 56	860 ± 36	512 ± 86	303 ± 65
	No freeze/No drought	281 ± 63	126 ± 19	350 ± 106	572 ± 97	409 ± 48	252 ± 72

Notes: [1]Secondary leaflets.

overwintering plant structures to cold stress (Groffman et al., 2001) and declining precipitation may increase the occurrence of drought in many regions (Mishra and Singh, 2010; PaiMazumder et al., 2013). Therefore, understanding how cross acclimation will impact plant productivity and the mechanisms behind this phenomenon may refine predictions of plant responses to climate change.

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The authors declare that there are no conflicts of interest. RSK and HALH designed this study and were responsible for drafting this manuscript. RSK was responsible for setting up the experiments and data collection.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. The number of plants that survived relative to the total of number of plants exposed to a freezing treatment. These plants were established from seed May 2016 and were cold acclimated between 27 June 2016 and 11 July 2016. To cold acclimate seedlings, we placed them into controlled environmental growth chambers that were cooled down from 20°C to 5°C at 2°C/h, and these plants received 9.5 h of light each day. Individual plants were frozen at either –10, –7.5, –5, –2.5, or 0°C for 3 d between 8 and 14 July 2016. During freezing, the chamber temperature was ramped down in the dark to the target temperature at 2°C/h, and ramped up at the same rate on the third day of freezing.

APPENDIX S2. Number of plants surviving relative to the total of number of plants exposed to a severe drought. The plants were established from seed May 2016 and watered daily. From 7 to 28 July 2016, water was withheld from plants until the onset of drought symptoms. Thereafter, plants were watered with a constant amount of water (10 mL), and survival was assessed on 18 Aug 2016.

APPENDIX S3. Allometric equations associating the number of green leaves with total dry leaf biomass for various herbaceous species. The plants were grown under various conditions, and the coefficient of determination (R^2) and sample size (n) for each equation is presented. For each equation, X = number of green leaves and Y = the estimated dry green aboveground biomass in grams.

APPENDIX S4. Effective sample size (n) for each treatment and species combination for the biomass and physiological measurements. Total soluble proteins and sugars are separated by sampling date (immediately before the drought occurred and at the onset of drought symptoms). No physiological measurements were made for *Lolium perenne* and *Poa compressa*.

APPENDIX S5. Summary tables for two-way ANOVAs examining the effect of freezing and drought on green shoot biomass for each species. The degrees of freedom for the *F* values are represented by df_1 (numerator) and df_2 (denominator).

APPENDIX S6. Summary tables for two-way ANOVAs examining the effect of freezing and drought on root biomass for each species. The degrees of freedom for the *F* values are represented by df_1 (numerator) and df_2 (denominator).

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