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PHYSIOLOGICAL ECOLOGY - ORIGINAL RESEARCH

Drought timing, not previous drought exposure, determines sensitivity of two shortgrass species to water stress

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Abstract

Climate change will alter global precipitation patterns, making it increasingly important that we understand how ecosystems will be impacted by more frequent and severe droughts. Yet most drought studies examine a single, within-season drought, and we know relatively little about the impacts of multiple droughts that occur within a single growing season. This distinction is important because many plant species are able to acclimate physiologically, such that the effects of multiple droughts on ecosystem function deviate significantly from the effects of cumulative, independent droughts. Unfortunately, we know relatively little about the ability of dominant species to acclimate to drought in drought-sensitive ecosystems like semi-arid grasslands. Here, we tested for physiological acclimation to multiple drought events in two dominant shortgrass steppe species: *Bouteloua gracilis* (C_4) and *Elymus elymoides* (C_3). Neither species exhibited physiological acclimation to drought; leaf water potential, stomatal conductance, and photosynthesis rates were all similarly affected by a single, late period drought and a second, late period drought. Biomass was lowest in plants exposed to two droughts, but this is likely due to the cumulative effects of both an early and late period drought. Our results suggest that late period droughts do exert weaker effects on biomass production of two dominant shortgrass species, but that the weaker effects are due to ontogenetic changes in plant physiology as opposed to physiological acclimation against multiple droughts. As a consequence, current ecosystem models that incorporate grass phenology and seasonal physiology should provide accurate predictions of primary production under future climates.

Keywords Leaf water potential · Photosynthesis · Stomatal conductance · Soil moisture · Plant phenology

Introduction

Climate change will drastically alter global precipitation patterns (IPCC 2014), making it increasingly important that we understand how ecosystems will be impacted by more frequent and severe droughts (Kayler et al. 2015). We know that intense rainfall shortages suppress primary production (Knapp et al. 2015a), alter community composition (Hoover et al. 2014), and modify regional-scale biogeochemical cycles (Knapp et al. 2002). Yet these effects are usually measured in response to a single, intense drought event, while climate change is expected to increase both the severity and frequency of droughts within a year (IPCC 2014). Indeed, severe droughts are becoming increasingly common in both North America and Europe (Vicente-Serrano et al. 2014; Diffenbaugh et al. 2015), but relatively few experiments have examined the consequences of recurring droughts on plant physiology and biomass production. Understanding the physiological and growth responses of dominant plant species to multiple droughts can refine our predictions of the consequences of climate change on ecosystem function in precipitation-limited systems.

The effects of repeated drought on plant production might deviate from the cumulative effects of multiple droughts because some plant species acclimate against repeated droughts. For example, drought can trigger the production

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and accumulation of numerous proteins, including heat shock proteins and transcription factors that enable a plant to quickly respond to subsequent stress events (Bruce et al. 2007). Drought stress can also induce epigenetic changes that allow plants to maintain constant stomatal conductance, transpiration, and photosynthetic rates in the face of subsequent drought events (Bruce et al. 2007; Neves et al. 2017). In this way, drought acclimation can buffer primary production against recurrent drought (Backhaus et al. 2014) and, in some cases, can even increase plant biomass in recurrent drought treatments (Walter et al. 2011). Not all species acclimate, however, making the effects of recurrent drought inconsistent among communities. In some cases, the initial drought exacerbates responses to later drought (Zavalloni et al. 2008; Dreesen et al. 2014), either increasing mortality or decreasing plant biomass for plants experiencing a second drought compared to plants exposed to a single drought (Wang et al. 2017). Whether or not initial droughts dampen or magnify the consequences of later droughts depends on the species present in a community and their specific physiological responses to drought (Backhaus et al. 2014); however, we currently lack a thorough understanding of which species acclimate effectively and which do not.

Most drought acclimation studies examined either mesic herbaceous systems (Zavalloni et al. 2008; Backhaus et al. 2014; Dreesen et al. 2014) or trees (Yin and Bauerle 2017) and we know comparatively little about acclimation to repeated droughts by dominant species in arid- or semi-arid grasslands. This distinction is important because semi-arid grasslands are more sensitive to a single drought than mesic grasslands or forests (Knapp and Smith 2001; Huxman et al. 2004; Wilcox et al. 2017). The semi-arid shortgrass steppe of North America, for example, experiences significantly higher interannual variation in rainfall than mesic tallgrass prairies (Knapp et al. 2015b), and most precipitation is concentrated in early spring and fall months. Grasses of the shortgrass steppe must therefore undergo extreme intra- and interannual drought periods and, as a result, might possess considerably greater capacity for acclimation to a second drought than do mesic grasses. Unfortunately, we know of only one study examining drought acclimation in semi-arid grasses (Luo et al. 2011), highlighting the considerable knowledge gap in this area.

Here, we examined the potential for drought acclimation in two co-dominant grasses commonly found in semi-arid grasslands of North America (Hart 2001; Osterheld et al. 2001). We compared a C_3 and C_4 grass because C_4 grasses have higher water-use efficiencies (Pearcy and Ehleringer 1984; Taylor et al. 2010) and often tolerate drought better than C_3 grasses (Ward et al. 1999), although empirical data comparing the drought acclimation potential between photosynthetic pathways is lacking. Specifically, we tested the following hypotheses: (1) Grasses would reduce stomatal conductance and photosynthesis in response to early period drought, thereby experiencing severe reductions in biomass. (2) Both grass species would display acclimation to a second drought, maintaining higher photosynthesis rates under repeated drought and reducing declines in biomass relative to the first drought. To test these hypotheses, we employed a factorial design where we crossed early and late period droughts. This design allowed us to distinguish between the effects of a second drought versus the timing of drought, a common issue in studies of drought memory.

Methods

Study species

To evaluate the potential for acclimation against repeated droughts, we conducted a growth chamber experiment using two physiologically distinct grass species common to the shortgrass steppe of Colorado: *Bouteloua gracilis* (blue grama) and *Elymus elymoides* (squirreltail). *Bouteloua gracilis* is a C_4 grass that accounts for approximately 90% of plant cover and annual primary production in the shortgrass steppe (Osterheld et al. 2001). Thus, the tolerance or susceptibility of *B. gracilis* to repeated droughts likely determines the stability of both primary production and community composition of the shortgrass steppe. *Elymus elymoides* is the dominant cool season C_3 grass of the northern shortgrass steppe and is the main contributor to early season primary production in this region (Hart 2001). For each species, we sourced seeds from local Colorado populations (*B. gracilis* from Pawnee Butte Seeds, Greeley, Co; *E. elymoides* from Granite Seed Co., Denver, Co.).

Experimental conditions

We conducted our experiment in a controlled growth chamber environment (A1000 environmental chamber from Conviron, Winnipeg, MB) set to a 14/10 h photoperiod. Temperature and light were adjusted in a stepwise fashion over the course of four time periods: morning (6:00–10:00), midday (10:00–15:00), evening (15:00–20:00), and night (20:00–6:00). During the morning and evening periods, light and temperature were set at 250 μmol m^{-2} s^{-1} at 20 °C, respectively. At midday, light intensity increased to 1000 μmol m^{-2} s^{-1} and temperature increased to 30 °C. Night conditions were complete dark (0 μmol m^{-2} s^{-1}) at 15 °C. Relative humidity was held constant at 30% throughout the experiment. These light and temperature settings mimic natural summer conditions of the Colorado shortgrass steppe (Kemp and Williams III 1980).

Bouteloua gracilis and *E. elymoides* were germinated in pots (SC7 cone-tainers from Greenhouse Megastore, Danville, IL) filled with ≈ 93 cm^3 of greens grade topdressing ($n = 75$ cone-tainers per species, natural porous ceramic greens grade soil from Profile, Buffalo Grove, IL). These growth media are a blend of clay minerals converted under high temperatures into a permanent inorganic aggregate with high porosity and cation exchange capacity.[1] Since these soils lack nutrients, each pot received 260–270 mg of Osmocote Plus Indoor/Outdoor fertilizer (15% nitrogen), yielding approximately 0.66 g nitrogen (N) per g dry soil. This N density is similar to the bulk N density of 0.67 g N per g dry soil present in the Colorado shortgrass steppe (Burke et al. 1999). We planted 10–15 seeds of the appropriate species in each pot and provided 24 mL of water twice per week during the germination period. Once germinated, each pot was pruned to four individuals to standardize initial plant density across replicates.

Drought treatments

In the fourth week, pots of *B. gracilis* and *E. elymoides* were randomly assigned to either control or early period drought treatments ($n = 30$ per species per treatment). Control replicates continued to receive 24 mL of water twice per week, while early period drought replicates received 24 mL of water once per week. After two additional weeks, the drought treatment was intensified by reducing the weekly watering to 15 mL. To assess the efficacy of our drought treatment, we gravimetrically measured soil volumetric water content (% soil moisture) every week on the driest day (control: 4 days without water, early period drought: 7 days without water). Because we measured soil moisture on the driest day, our estimates of gravimetric soil moisture do not capture periodic rewetting of control soils and likely underestimate the magnitude of difference between treatments. However, our measurements still serve as a useful indicator of treatment effectiveness: gravimetric soil moisture was approximately 40–50% in control treatments vs. 20% in drought treatments on the driest day (Fig. 1).

After 3 months, all plants entered a well-watered, recovery period and received 24 mL of water twice per week. This treatment successfully raised soil moisture of early period drought replicates to control levels, alleviating drought conditions (Fig. 1). After 4 weeks of well-watered conditions, we randomly assigned pots to one of four treatments: Control–Control plants received no drought at any point during the experiment, Control–Drought plants received a single late period drought, Drought–Control plants received a single early period drought, and Drought–Drought plants received a second, late period droughts to test for evidence of drought acclimation. The late-drought period was similar the first drought (15 ml of water added per week) and continued for 5 weeks until the end of the experiment (Fig. 1).

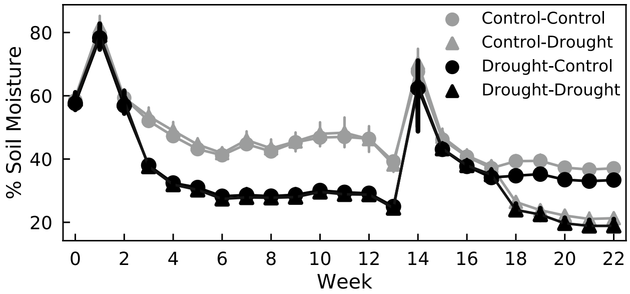

Fig. 1 Soil gravimetric water content (% soil moisture) in each of the four drought treatments over the course of the experiment. Weeks 0–2 are three pre-treatment weeks where all plants were well-watered. Weeks 3–13 are the early period drought, weeks 14–17 are the recovery period, and weeks 18–22 are the late period drought

Physiological measurements

We tested for evidence of drought acclimation by measuring several aspects of plant physiology throughout the experiment. To assess the extent of water stress experienced by each species, we measured midday leaf water potential at the end of the early period drought, recovery period, and late period drought. At the end of each period on the driest day (control: 4 days without water, early period drought: 7 days without water), we clipped a single leaf from a randomly chosen tiller within a pot ($n = 4$–7 per species per treatment) measured leaf water potential using a Scholander-style pressure chamber (PMS Instrument Company, Albany, OR). Our pressure chamber was unable to measure pressures below − 9 MPa, limiting our ability to detect severe water stress. However, most measurements of leaf water potential were above this lower limit except in a few cases. We chose to measure leaf water potential only on the driest day to capture maximum plant stress.

During the final week of each drought and the intervening recovery period, we also measured leaf gas exchange using an LI-6400XT gas exchange system (LI-COR Biosciences, Lincoln, NE). During each sampling event, we took measurements between 10AM and 2PM on both the day before (i.e, the driest day) and the day after watering to capture plant responses at the highest and lowest levels of stress. Because measurements from the driest day often fell below detection limits, here we report data from only the wet measurements. To estimate gas exchange, we randomly selected 5–7 individuals per species per treatment. For each individual, we enclosed the newest fully expanded leaf within the instrument leaf chamber. Leaf chamber conditions were set to mimic growth chamber conditions as closely

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[1] Chemical specifications available at: https://www.profileevs.com/resources/article/greens-grade-natural-standard-specifications.

as possible (Temperature: 25 °C, Humidity: ≈ 40%, Light: 1000 µmol m^{-2} s^{-1}). We recorded stomatal conductance and photosynthesis until measurements reached a steady state (5–10 min). Because individual leaves often did not fill the leaf chamber, we inserted multiple leaves into the chamber to fill the area; therefore, total leaf area was estimated from total leaf width and the length of the leaf chamber (3 cm^2) and gas exchange measurements were adjusted accordingly.

Biomass and shoot height measurements

To quantify the consequences of drought, and the resultant physiological stress, on primary production, we measured both the length of the tallest living shoot (cm) via nondestructive sampling and aboveground biomass production (g) via destructive harvest of all aboveground tissue. At the end of the early period drought, we recorded tallest living shoot height of every replicate ($n = 30$ per species per treatment) and randomly selected six replicates per species per treatment for aboveground biomass harvest. At the end of the recovery period, we again measured shoot height of every replicate to test the capacity for recovery following an early period drought ($n = 24$ per species per treatment), but we did not conduct destructive biomass harvests to maximize replication for the late period drought. At the end of the late period drought, we measured shoot height and collected aboveground biomass from all remaining individuals ($n = 12$ per species per treatment). Collected biomass was dried at 60 °C for 48 h prior to weighing.

Statistical analyses

To test whether drought timing (early vs. late period) affected plant responses to drought, we compared single-drought treatments using a two-way ANOVA with drought treatment (control vs. drought) crossed with time period (early vs. late). A significant interaction between treatment and timing would indicate that the effect of drought varies with time period. We conducted this analysis for all physiological measures as well as aboveground biomass for both *B. gracilis* and *E. elymoides*. Leaf senescence at the end of the experiment reduced the height of the tallest living shoot uniformly across treatments, and so we were only able to compare shoot height between early period control vs. drought treatments via a one-way ANOVA. Additionally, leaf water potential and stomatal conductance of *E. elymoides* during late period drought declined below detectable levels, and we were only able to statistically compare control vs. drought treatments during early period droughts using a one-way ANOVA for these two response variables.

We assessed recovery of physiological function and plant growth using a one-way ANOVA comparing physiological and shoot height measurements taken at the end of the recovery period between control and early drought treatments. This analysis was performed separately for *B. gracilis* and *E. elymoides*.

We tested for acclimation against repeated drought using a one-way ANOVA to compare end-of-experiment physiology, shoot height, and aboveground biomass between single, late period drought (Control–Drought) and multiple drought (Drought–Drought) treatments (Walter et al. 2011). This comparison enabled us to determine whether an early period drought modified plant response to a late period drought. A lack of significant difference in this test suggests that repeated drought is identical in effect to a single, late period drought and that plants exhibit no drought acclimation.

All analyses were conducted within a hierarchical Bayesian framework in order to place weakly informative priors on hyperparameters that constrain estimates of effect size in the presence of small sample sizes (Lemoine et al. 2016). Briefly, each analysis was a linear model of the form:

$$\mathbf{y} \sim N\left(\hat{\mathbf{y}}, \sigma^2\right)$$

$$\hat{\mathbf{y}} = \mathbf{XB}$$

where $\mathbf{X}$ is the design matrix for each analysis and $\mathbf{B}$ is the vector of coefficients. Coefficients were modeled hierarchically, to allow for partial pooling and to constrain effect sizes (Gelman et al. 2012):

$$\mathbf{B} \sim N\left(0,\ \sigma_{\mathrm{B}}^2\right)$$

The parameter σ_{B}^2 was given a weakly informative *Cauchy* (0, 2.5) prior, which states that coefficients should be small unless strongly supported by the data. To assess the influence of prior choice on results, we repeated all analyses with the uninformative prior $\mathbf{B} \sim N(0, 10000)$. Results using uninformative priors were qualitatively similar, but also less conservative (i.e., stronger effect sizes, Appendix A in Supplementary material). However, we choose to favor conservative estimates of effect sizes to help prevent Type M errors (see Lemoine et al. 2016).

For each analysis, we checked the assumptions of normality and homogenous variances using posterior Pearson residual plots. Severe heteroscedasticity was remedied by fitting a model that allowed for unique variances within each treatment; posterior Pearson residual plots confirmed that this was an effective technique in all cases. Although posterior probabilities are best judged as providing a continuous estimate of evidence for an effect, we assessed significance of the results by calculating the probability that a parameter was either positive or negative (Pr), wherein $\Pr > 0.90$ indicates a moderately significant effect, and $\Pr > 0.95$ indicates a statistically significant effect (Lemoine and Shantz 2016; Lemoine et al. 2017; Rode et al. 2017). All models were fit via the STAN programming language (v2.17) accessed from Python v3.6.

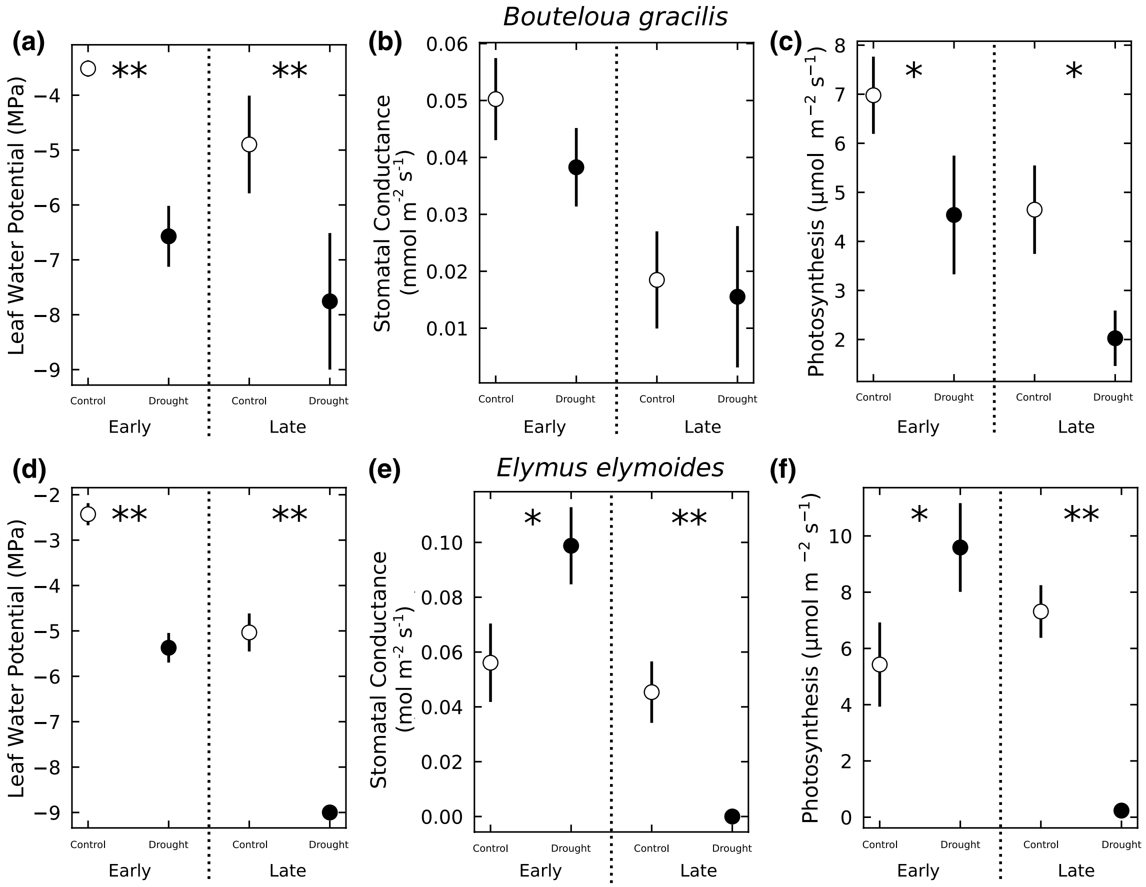

Fig. 2 Leaf water potential (**a**, **d**), stomatal conductance (**b**, **e**), and photosynthesis (**c**, **f**) under single-drought treatments for *Bouteloua gracilis* and *Elymus elymoides.* Data are shown separately for the two droughts imposed (left = early period drought, right = late period drought). Points and bars denote means ± 1 SE. The effect of drought is denoted as either moderately (asterisk) or significantly (double asterisk) different

Results

Single drought

The single-drought treatment induced significant water stress in *B. gracilis*, which manifested as a 73% reduction in leaf water potential during both early and late period droughts [Pr(Drought) = 0.998, Fig. 2a]. Time period affected neither baseline leaf water potential [Pr(Time) = 0.876] nor the reduction in leaf water potential caused by drought [Pr(Interaction) = 0.555], suggesting that *B. gracilis* experienced similar water stress during both early and late period droughts (Fig. 2a). Drought did not affect stomatal conductance of *B. gracilis* during either time period [Pr(Drought) = 0.718], although late period plants had moderately lower stomatal conductance rates than early period plants [Pr(Time) = 0.939, Fig. 2b]. Despite maintaining constant stomatal conductance under water stress, drought caused a moderate decline in photosynthesis rates of *B. gracilis* [Pr(Drought) = 0.908], and this effect was consistent across time periods [Pr(Interaction) = 0.721, Fig. 2c]. Reduced photosynthesis rates led to shorter shoots and less aboveground biomass in response to drought, but the effect size varied with time period (Pr(Interaction) = 0.954). Under early season drought, *B. gracilis* suffered a 57% reduction in aboveground biomass [Pr(Drought) = 0.997, Fig. 3a] and a 43% reduction in shoot height [Pr(Drought) = 1.000, Fig. 3b]. In contrast, *B. gracilis* exposed to late period drought experienced only a 19% reduction in aboveground biomass [Pr(Drought) = 0.995, Fig. 3a]. These results suggest that *B. gracilis* does not alter its water-use strategy under either early or late period drought, and that the consequences of drought on *B. gracilis* biomass weaken as plants age.

Compared to *B. gracilis*, *E. elymoides* demonstrated greater physiological drought responses. Water stress caused a 121% decline in leaf water potential under early period drought [Pr(Drought) = 1.000, Fig. 2d], while late period drought caused such hydraulic stress that all measurements of leaf water potential exceeded the detection threshold

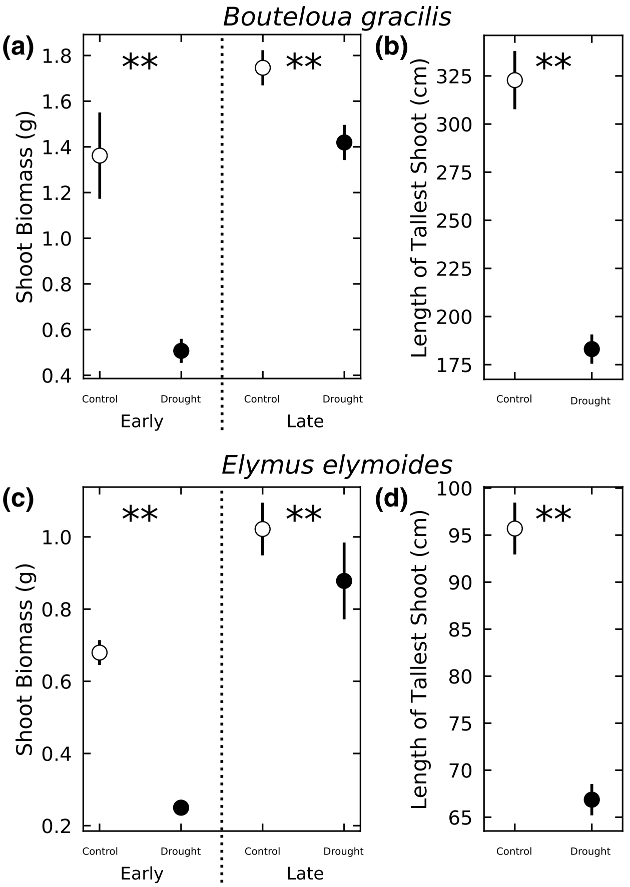

Fig. 3 Aboveground biomass (**a**, **c**) and length of the tallest living shoot (**b**, **d**) under single-drought treatments for *Bouteloua gracilis* and *Elymus elymoides.* Data are shown separately for the two droughts imposed (left = early period drought, right = late period drought). Due to senescence, we only recorded tallest living shoot from the early period drought and do not show data for the late period drought. Points and bars denote means ± 1 SE. The effect of drought is denoted as either moderately (asterisk) or significantly (double asterisk) different

of −9 MPa (Fig. 2d). Stomatal conductance, however, moderately increased by 64% under early period drought [Pr(Drought) = 0.900], while late period drought caused stomatal conductance to cease (Fig. 2e). Photosynthetic rates of *E. elymoides* followed a similar pattern, whereby the drought response was influenced by timing [Pr(Interaction) = 0.986]. Early period drought caused a 42% increase in photosynthesis rates, likely due to increased chlorophyll concentrations (Lemoine, Griffin-Nolan, and Lock *pers. obs.*), whereas late period drought led to a 90% reduction in photosynthetic rates (Fig. 2f). Despite the relatively greater physiological response to drought of *E. elymoides*, the effects of water stress on shoot height and aboveground biomass were qualitatively similar to those of *B. gracilis*. Early period drought reduced shoot height by 30% [Pr(Drought) = 1.000, Fig. 3d] and aboveground biomass by 62% [Pr(Drought) = 0.999, Fig. 3c]. Late period drought, in contrast, had minimal effects on *E. elymoides* biomass [Pr(Drought) = 0.880, Fig. 3c], suggesting that the impact of drought on *E. elymoides* biomass weakens as plants age despite strong physiological adjustment.

Recovery period

During the regrowth phase, leaf water potential of previously droughted *B. gracilis* returned to, and even moderately exceeded, leaf water potential of non-droughted plants [Pr(Drought) = 0.902, Fig. 4a]. Similarly, stomatal conductance [Pr(Drought) = 0.879] and photosynthesis [Pr(Drought) = 0.844] of prior-drought *B. gracilis* both returned to pre-drought levels (Fig. 4b, c). Yet despite the return of photosynthesis rates to non-stressed levels, *B. gracilis* shoot height could not match undroughted levels, as shoot height was still 42% lower in prior-drought plants than in non-droughted plants [Pr(Drought) = 1.000, Fig. 4d].

Elymus elymoides also experienced recovery in physiological function following the cessation of drought. Leaf water potential [Pr(Drought) = 0.654], stomatal conductance [Pr(Drought) = 0.710], and photosynthesis rates [Pr(Drought) = 0.634] were all indistinguishable between prior-drought and non-droughted plants by the end of the regrowth period (Fig. 4e–g). The recovery of physiological function enabled prior-drought *E. elymoides* to outgrow non-droughted plants, achieving an 11% increase in shoot height by the end of the regrowth period [Pr(Drought) = 0.999, Fig. 4h].

Second drought

The response of *B. gracilis* to a second drought was similar to the response of *B. gracilis* to a single, late period drought. Compared to the single, late drought, a second drought did not differ in its effect on leaf water potential [Pr(Drought) = 0.672], stomatal conductance [Pr(Drought) = 0.580], or photosynthesis [Pr(Drought) = 0.580] (Figs. 5a–c). Biomass, however, was significantly lower in *B. gracilis* exposed to two droughts compared to a single, late period drought [Pr(Drought) = 1.000, Fig. 5d]. This is likely because early period drought reduced *B. gracilis* biomass at the onset of a second drought. For example, a single, late period drought reduced *B. gracilis* shoot biomass by 17% compared to well-watered controls. A second, late period drought reduced *B. gracilis* biomass by 25% compared to plants recovering from an early period drought. Thus, it appears that the impact of a second drought on *B. gracilis* is similar in magnitude to the effect of single drought and that the reduction in biomass is simply cumulative throughout the year.

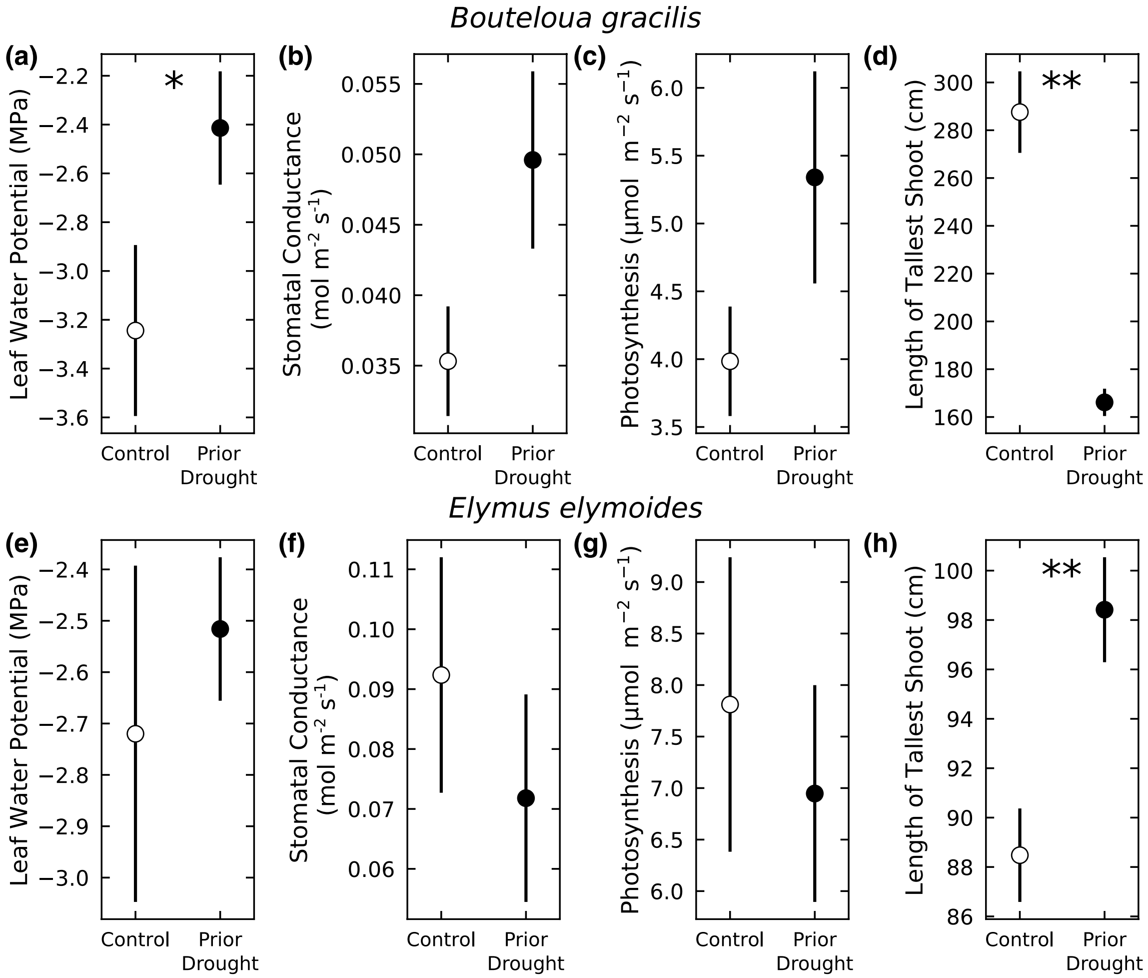

Fig. 4 Leaf water potential (**a**, **e**), stomatal conductance (**b**, **f**), photosynthesis (**c**, **g**), and height of the tallest living shoot (**d**, **h**) during the regrowth period for *Bouteloua gracilis* and *Elymus elymoides*. Control plants are those that received adequate water during the early period drought, while Prior Drought plants are those that were water-stressed during the early period drought. Points and bars denote means ± 1 SE. The effect of drought is denoted as either moderately (asterisk) or significantly (double asterisk) different

As with *B. gracilis*, the effects of a second, repeated drought on *E. elymoides* physiology paralleled the effects of a single, late period drought. Late period drought reduced leaf water potential, stomatal conductance, and photosynthesis rates below detectable levels across both single and multiple drought treatments. Multiple droughts reduced aboveground biomass by 43% compared to a single, late period drought [Pr(Drought) = 0.996, Fig. 5e], but as with *B. gracilis*, the reduction in biomass caused by multiple droughts is the cumulative impact of drought throughout the year. A single, late period drought reduced biomass by 10% compared to well-watered controls, while a repeated drought reduced biomass by 16% compared to plants recovering from an early period drought.

Discussion

The consequences of recurrent drought events on ecosystem function often diverge from the cumulative impacts of independent drought because many plant species undergo acclimation that can buffer them against repeated stress events (Bruce et al. 2007; Walter et al. 2011; Backhaus et al. 2014; Neves et al. 2017). Yet evidence for drought acclimation is variable among plant species (Dreesen et al. 2014), and studies from drought-sensitive, arid ecosystems are remarkably rare. Our results support our first hypothesis that early period drought will impair gas exchange and photosynthesis rates of both *B. gracilis* and *E. elymoides*.

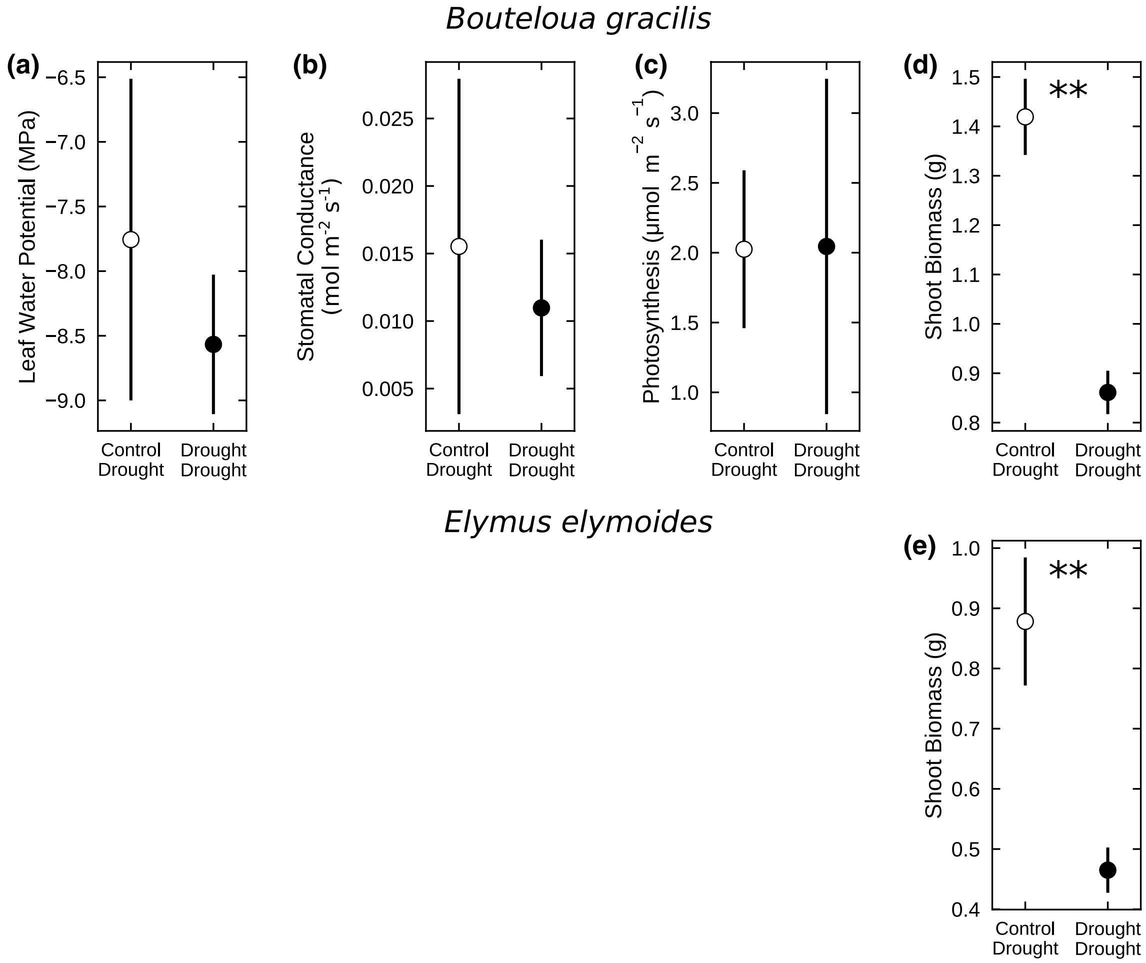

Fig. 5 Leaf water potential (**a**), stomatal conductance (**b**), photosynthesis (**c**), and aboveground biomass (**d**) for *Bouteloua gracilis* for plants during the late period droughts. Aboveground biomass for *Elymus elymoides* during the late period droughts (**e**). Physiological measurements are not shown for *E. elymoides* because they were below detection levels for all measurements. 'Control–Drought' refers to plants exposed to a single, late period drought, and 'Drought–Drought' refers to plants exposed to both early and late period droughts. Points and bars denote means ± 1 SE. The effect of drought is denoted as either moderately (asterisk) or significantly (double asterisk) different

However, we reject our second hypothesis that either grass species would exhibit physiological acclimation to a second drought. Here, we found no evidence of acclimation of leaf water potential, stomatal conductance, or photosynthesis of two semi-arid grasses of differing photosynthetic pathways to repeated droughts. In fact, the impacts of a second drought on grass physiology and production mirrored the impacts of a single, late period drought. These results suggest that the dominant species of the shortgrass steppe respond to drought in a cumulative manner, which simplifies predictions of the effects of multiple drought events on ecosystem function of the shortgrass steppe.

Grasses of different photosynthetic pathways often adopt different strategies for resisting or tolerating drought. Water stress forces C_3 grasses to close their stomata, minimizing water loss while also reducing intracellular CO_2 concentrations and inhibiting photosynthesis (Flexas and Medrano 2002). C_4 grasses, on the other hand, maintain high stomatal conductance rates during periods of water stress, albeit still suffering reduced photosynthetic rates as a consequence of decreased water supply (Pearcy and Ehleringer 1984; Ward et al. 1999; Ripley et al. 2010; Taylor et al. 2010). Our experiment confirms previous observations for C_4 grasses; *B. gracilis* did not adjust stomatal conductance in response to early period water stress but did lower photosynthesis rates and, as a result, suffered decreased biomass production. *Elymus elymoides*, on the other hand, appeared to increase stomatal conductance and photosynthesis rates in response to water stress, contradicting numerous studies that report drought-induced reductions in both aspects of C_3 physiology (Kemp and Williams III 1980; Ward et al. 1999; Flexas and Medrano 2002; Ripley et al. 2010; Taylor et al.

2010). The discrepancy between our results and previous studies is best explained by our timing of gas exchange measurements. We reported gas exchange measurements from the wettest day immediately following watering because stomatal conductance and photosynthesis rates of *E. elymoides* on the driest day were below detectable levels. It is likely that gas exchange of *E. elymoides* declines severely during water stress but can quickly rebound above non-stressed levels upon re-watering, even in the midst of long-term drought. The ability of *E. elymoides* to quickly recover photosynthesis following desiccation likely results from changes in leaf pigmentation, as water-stressed *E. elymoides* leaves took on a deep purple hue (Lemoine, Griffin-Nolan, Lock *pers. obs.*). Foliar chlorophyll and carotenoid concentrations of many grass species increase during drought (Taylor et al. 2010; Cenzano et al. 2013), potentially enabling rapid recovery following re-watering after an early period drought.

Physiological recovery following water deficits might be an important component of ecosystem resilience to drought, potentially enabling primary production to match or surpass pre-drought levels. In this study, post-drought re-watering facilitated nearly complete recovery of leaf water potential, stomatal conductance, and photosynthesis rates of two dominant Colorado shortgrass species. Indeed, many species possess the ability to regain physiological function; gas exchange of trees often returns to normal levels following alleviation of water stress (Galle et al. 2011; Yan et al. 2017), though some species experience more permanent physiological changes if drought induces severe physiological stress, like xylem cavitation (Galle et al. 2011; Skelton et al. 2017). Arid- and semi-arid grasses, including *B. gracilis*, also achieve physiological parity with unstressed plants following re-watering (Sala et al. 1982; Ward et al. 1999; Luo et al. 2011). Yet recovery of physiological function does not necessarily translate into recovery of primary production. Leaf height and biomass of early drought *B. gracilis* and *E. elymoides* remained suppressed throughout the experiment. Such biomass reductions can persist for several years following extreme drought in the shortgrass steppe, leading to substantial lags in primary production under increased precipitation and, eventually, long-term shifts in community composition (Lauenroth and Sala 1992; Rondeau et al. 2013, 2018). The disconnect between recovery of physiological function and biomass production suggests that ecosystem resilience to early period drought require more than recovery of leaf water potential and gas exchange.

Although early period drought reduced biomass production of both *B. gracilis* and *E. elymoides* by ≈ 60%, late period drought did not strongly affect biomass production of either species. For example, *B. gracilis* biomass declined by only 19% during a single, late period drought, suggestive of reduced sensitivity to soil moisture deficits for older *B. gracilis* plants. One potential explanation for reduced sensitivity to the late period drought is that the late period drought was 6 weeks shorter than the early period drought. Longer droughts often produce greater reductions in photosynthesis and stomatal conductance than brief droughts (Ripley et al. 2010), such that the late period drought may not have been of sufficient duration to match early period declines in biomass and physiological function. In many cases, however, declines in photosynthesis and gas exchange asymptote after 2–4 weeks (Poulson et al. 2006; Hein et al. 2016), such that the late period drought may have captured the extent of physiological damage to *B. gracilis* and *E. elymoides*. Isolating the effects of drought timing vs. drought duration remains an interesting avenue of future research.

Reduced sensitivity to late period drought might also occur because of ontogenetic shifts in plant resource allocation throughout the growing season for perennial grasses. The dominant C_4 grass of the tallgrass prairie, *Andropogon gerardii*, is also most sensitive to early period droughts (Avolio et al. 2018). Declining sensitivity to water stress for older *A. gerardii* results from the fact that photosynthesis and growth rates naturally decline ontogenetically over the course of the growing season, thereby limiting the capacity for drought-imposed reductions for late period *A. gerardii* (Dietrich and Smith 2015). It is likely that reduced sensitivity of *B. gracilis* to late drought reported here results from a similar mechanism; late period *B. gracilis* had undergone an ontogenetic decline in relative growth rates and possessed little capacity for biomass reductions. Yet the mechanism for increased tolerance to late period drought likely varies with photosynthetic pathway. The C_3 grass *E. elymoides* experienced only slight, non-significant declines in biomass during the late period drought (similar to *B. gracilis*), but maintained high stomatal conductance and photosynthetic rates throughout the experiment. The lack of a late period reduction in *E. elymoides* biomass is due to ontogenetic dieback of living tissue. Similar to many C_3 grasses, *E. elymoides* achieves maximum biomass in April or May and has almost entirely senesced by August (James et al. 2008; Baughman et al. 2016). Thus, sensitivity to a second drought must be assessed earlier in the season for C_3 grasses like *E. elymoides* compared to C_4 species like *B. gracilis*.

Reduced sensitivity to a single, late period drought was not matched by evidence of acclimation against a second, late period drought. Leaf physiology did not differ between single or recurrent drought, and the biomass reduction imposed by a second drought was nearly identical to the biomass reduction imposed by a single, late period drought for both *B. gracilis* and *E. elymoides*. Similarly, *Robinia pseudoacacia* and *Amorpha fruticosa* exhibited no drought acclimation and were in fact more sensitive to the second drought than the first (Yan et al. 2017). Early drought also increased mortality and decreased biomass for herbaceous species exposed to a second late period drought more than plants exposed to a single, late drought (Wang et al. 2017). In fact, increased sensitivity to late period drought appears to be at least as common as acclimation against multiple droughts (Zavalloni et al. 2008; Dreesen et al. 2014). Given the inconsistent evidence for drought acclimation among prior studies, it is not surprising that we failed to detect drought acclimation in the drought-adapted, semi-arid grasses examined here.

Semi-arid grasslands are particularly sensitive to variation in precipitation (Huxman et al. 2004; Wilcox et al. 2017), and both inter- and intra-annual variability in precipitation are expected to increase in the future (IPCC 2014). It is increasingly important to understand how ecosystems will respond to repeated drought events, especially if dominant plant species exhibit an acclimatized response that mitigates the consequences of a second drought. Our results suggest that late period droughts do exert weaker effects on biomass production of two dominant shortgrass species, but that the weaker effects are due to ontogenetic changes in plant physiology as opposed to physiological acclimation against multiple droughts. As a consequence, current ecosystem models that incorporate grass phenology and seasonal physiology should provide accurate predictions of primary production under future climates.

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