

RESEARCH ARTICLE

Trading water for carbon in the future: Effects of elevated CO₂ and warming on leaf hydraulic traits in a semiarid grassland

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Abstract

The effects of climate change on plants and ecosystems are mediated by plant hydraulic traits, including interspecific and intraspecific variability of trait phenotypes. Yet, integrative and realistic studies of hydraulic traits and climate change are rare. In a semiarid grassland, we assessed the response of several plant hydraulic traits to elevated CO₂ (+200 ppm) and warming (+1.5 to 3°C; day to night). For leaves of five dominant species (three graminoids and two forbs), and in replicated plots exposed to 7 years of elevated CO₂, warming, or ambient climate, we measured: stomatal density and size, xylem vessel size, turgor loss point, and water potential (pre-dawn). Interspecific differences in hydraulic traits were larger than intraspecific shifts induced by elevated CO₂ and/or warming. Effects of elevated CO₂ were greater than effects of warming, and interactions between treatments were weak or not detected. The forbs showed little phenotypic plasticity. The graminoids had leaf water potentials and turgor loss points that were 10% to 50% less negative under elevated CO₂; thus, climate change might cause these species to adjust their drought resistance strategy away from tolerance and toward avoidance. The C4 grass also reduced allocation of leaf area to stomata under elevated CO₂, which helps explain observations of higher soil moisture. The shifts in hydraulic traits under elevated CO₂ were not, however, simply due to higher soil moisture. Integration of our results with others' indicates that common species in this grassland are more likely to adjust stomatal aperture in response to near-term climate change, rather than anatomical traits; this contrasts with apparent effects of changing CO₂ on plant anatomy over evolutionary time. Future studies should assess how plant responses to drought may be constrained by the apparent shift from tolerance (via low turgor loss point) to avoidance (via stomatal regulation and/or access to deeper soil moisture).

KEYWORDS

carbon dioxide, drought, intraspecific, plant functional type, species, stomata, turgor loss point, warming, water potential, xylem

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1 | INTRODUCTION

To acquire carbon dioxide (CO₂), plants must open their stomata and lose water. Thus, plants cannot simultaneously maximize carbon (C) acquisition and water conservation. The importance of this tradeoff, and its sensitivity to atmospheric CO₂ concentrations, is evident in the concurrence of environmental trends and evolutionary developments over geologic time scales (Haworth et al., 2011; Mueller et al., 2010; Sperry, 2003). For example, as concentrations of atmospheric CO₂ declined between 200 and 60 million years ago, corresponding shifts occurred for leaf anatomical and physiological traits that regulate water transport and loss (de Boer et al., 2012; Feild et al., 2011). Several studies show variation in atmospheric [CO₂] induces integrated shifts in the density, aperture, and maximal conductance of stomata, and these stomatal responses to [CO₂] are thought to be similar whether considering geologic, evolutionary timescales or phenotypic responses of individuals (Franks et al., 2012; Franks & Beerling, 2009). But, due to emphasis on other plant traits and functions, many prior studies of elevated CO₂ (eCO₂) offer limited insights into the effects of eCO₂ on plant–water relations (Ainsworth & Rogers, 2007; Leakey et al., 2009; Wullschleger et al., 2002). A holistic understanding of how eCO₂ modifies plant–water relations requires studies that assess hydraulic traits more directly and broadly (Becklin et al., 2017; Domec et al., 2017; Wullschleger et al., 2002), including anatomical traits of stomata and xylem and other traits that reflect the costs of water loss (e.g., indices of drought tolerance for tissues).

Understanding how anatomical traits of stomata and xylem respond to eCO₂ and resulting climate shifts is important for several reasons (Matthews & Lawson, 2019). First, those anatomical responses can identify the mechanisms underlying the effects of eCO₂ and climate change on leaf gas exchange, plant productivity and transpiration (Ainsworth & Rogers, 2007; Domec et al., 2017; Franks et al., 2012). Second, anatomical traits can be used to estimate maximal rates of gas exchange and transpiration where and when those processes have not been measured (Franks & Beerling, 2009; McElwain et al., 2015). Third, because the anatomy of xylem and stomata affects other plant functions, such as evaporative cooling and tolerance of cold and dry conditions (Domec et al., 2017; Gutschick, 1999; Zanne et al., 2018), knowing the effects of climate change on these anatomical traits can provide broader insight than leaf gas-exchange measurements.

Drought tolerance in plants is multidimensional and is derived from diverse anatomical, morphological, and biochemical traits. For example, narrower leaf veins and smaller xylem vessels have been positively correlated with resistance to embolism and maintenance of hydraulic function in leaves (Scoffoni et al., 2017). The ability to maintain leaf function during drought is also strongly correlated with the leaf water potential at turgor loss (or “turgor loss point,” which itself is largely determined by leaf osmotic potential) (Bartlett et al., 2012). Effects of eCO₂ and warming on such indices of drought tolerance must be documented (Domec et al., 2017; Wullschleger et al., 2002) to predict how plants will respond to shifts in drought

frequency and/or severity that are coincident with changing [CO₂] and temperature (Naumann et al., 2018). Studies of how climate change affects turgor loss point may be especially informative; this trait is rapidly becoming one of the most widely measured indicators of drought tolerance, and more useful to describing plant functional strategies (Bartlett et al., 2016; Blackman, 2018; Griffin-Nolan et al., 2019; Májeková et al., 2021; Ocheltree et al., 2020; Sun et al., 2020; Wilcox et al., 2021). Only a few experiments have measured the response of turgor loss point to CO₂ or warming for herbaceous plants (Li et al., 2020; Wullschleger et al., 2002).

Across experimental studies, there are inconsistent effects of climate change on indices of plant drought tolerance or anatomical traits of stomata and xylem (e.g., Yan et al., 2017). Effects of eCO₂ on these traits are often small, species specific, and differ among plant functional types (Ainsworth & Rogers, 2007; Domec et al., 2017; Gerald et al., 2016; Franks et al., 2012; Leakey et al., 2009; Marchi et al., 2004; Matthews & Lawson, 2019; Miranda-Apodaca et al., 2018; Taylor et al., 2018). Effects of warming on these traits are also variable, but more rarely assessed (Matthews & Lawson, 2019; Yan et al., 2017). When combined, elevated CO₂ and warming might have counteracting impacts on hydraulic traits (Becklin et al., 2017; Yan et al., 2017), because eCO₂ alone and warming alone can have opposing effects on the limitation of leaf function by [CO₂] and water availability (Matthews & Lawson, 2019). For example, eCO₂ typically increases soil moisture while reducing stomatal conductance and maximal rates of carboxylation (Leakey et al., 2009; Morgan et al., 2004), while higher temperatures can have the opposite effects (Bunce, 2000; Turner, 1991; Xu et al., 2013). In other words, eCO₂ alone typically reduces joint limitation by C and water (relative to other factors), perhaps diminishing the benefits of anatomical features that maximize conductance of water through xylem and stoma; conversely, warming alone can exacerbate joint limitation by C and water. Due to the dearth of factorial experiments and relevant trait measurements in past experiments, very little is known about the combined, interactive effects of eCO₂ and warming on drought tolerance or anatomical hydraulic traits.

In this study, we assess the effects of eCO₂ and warming on leaf hydraulic traits of five common species in the mixed-grass prairie of North America. This prairie is one of the most extensive grasslands in North America and is valuable for supporting livestock production and maintaining biodiversity (Samson et al., 2004). Biogeochemical processes in the mixed-grass prairie and other North American grasslands are primarily water limited (Hsu et al., 2012), which could elevate the importance of hydraulic traits in shaping plant functions. By utilizing a multi-year field experiment with factorial, open-air treatments, our assessment of the response of hydraulic traits to climate change is novel and realistic compared to prior studies.

Our overarching hypothesis is that, by reducing substrate limitation of photosynthesis, eCO₂ should induce trait shifts along the water for C tradeoff that indicate diminished returns on investment in water transport and loss to support C-acquisition (*sensu de Boer et al., 2012, 2016*). For example, we expect eCO₂ to induce coordinated reductions in the fraction of leaf area allocated to stomata and

the dimensions of leaf xylem vessels (Gerald et al., 2016; Haworth et al., 2018), because lower rates of water transport and loss are required to maintain photosynthetic rates when atmospheric $[\text{CO}_2]$ is higher. Furthermore, because eCO_2 boosts soil moisture in this prairie (Blumenthal et al., 2018), we expect eCO_2 to reduce indices of leaf-level drought tolerance, reflecting diminishing returns on investment in drought tolerance due to partial alleviation of water limitation by eCO_2 . Conversely, because warming reduces soil moisture (Blumenthal et al., 2018) and increases evaporative demand, warming in this prairie could have opposing effects on plant-water relations (compared to eCO_2), namely, warming could increase leaf-level drought tolerance, the fraction of leaf area allocated to stomata, and the size of xylem vessels. Yet, warming effects might be smaller than those of eCO_2 (Matthews & Lawson, 2019) due to the lack of direct effects of warming on C-limitation of leaf functions.

2 | MATERIALS AND METHODS

2.1 | Experiment

The Prairie Heating and Carbon dioxide Enrichment (PHACE) experiment was located west of Cheyenne, Wyoming (USA) in an intact mixed-grass prairie (41°11'N, 104°54'W, 1930m.a.s.l.). Mean annual precipitation is 397 mm and mean annual temperature is 8°C (1984–2013; GHCND:USW00024018). The experiment was conducted in a pasture with a history of light grazing by cattle and native herbivores, but cattle were excluded during the experiment (2005–2013) (Mueller et al., 2016). Experimental plots were distributed among two blocks with slightly different soil types. Surface soils (0–15 cm) are sandy loams consisting of $\geq 55\%$ sand and $\leq 20\%$ clay, with more clay ($\leq 38\%$) and less sand ($\geq 42\%$) in deeper layers. There were five replicate plots for each treatment, including eCO_2 alone (~ 600 ppm), warming alone ($+1.5^\circ\text{C}$ during the day and $+3^\circ\text{C}$ at night), eCO_2 plus warming, and plots with ambient climate. Free-air CO_2 enrichment technology was used for the eCO_2 treatments and infrared heaters were used for the warming treatments (Kimball et al., 2008; LeCain et al., 2015). Soil moisture was measured hourly as volumetric water content using frequency domain reflectometry sensors (Sentek EnviroSCAN) buried at two depths (10 and 20 cm) (Blumenthal et al., 2018).

2.2 | Sampling

In the final year of the experiment (2013), after 8 years of eCO_2 and 7 years of warming, we measured hydraulic traits of leaves from five of the most common species: *Artemisia frigida* (a C3 subshrub), *Bouteloua gracilis* (a C4 grass), *Carex duriuscula* (a C3 sedge), *Pascopyrum smithii* (a C3 grass), and *Sphaeralcea coccinea* (a C3 forb). These species have divergent phenologies (Blumenthal et al., 2020; Reyes-Fox et al., 2014). For example, leaf out typically occurs earliest for *Carex duriuscula* and latest for *Bouteloua gracilis*, while

leaf senescence occurs earliest for *Carex duriuscula* and latest for *Artemisia frigida*. Across all 20 plots, the five focal species accounted for 33–97% of the above-ground biomass harvested in the final year of the experiment, with a mean value of 68%, a median value of 72%, and a standard deviation of 18%. Traits were measured once in late June or early July, except pre-dawn leaf water potentials, which were measured in both early June (DOY 164) and early July (DOY 191). Substantial rain events did not occur between the earliest and latest sampling events (Figure 1), and the weather was cool and dry

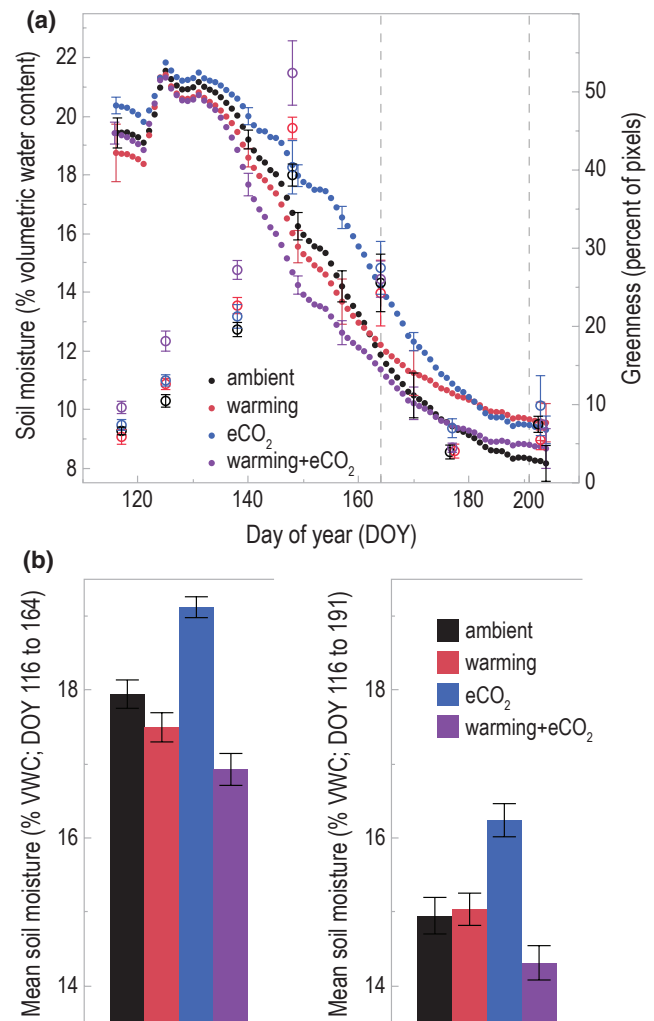


FIGURE 1 Volumetric water content of surface soils (5–25 cm) during the growing season in which leaf sampling occurred. Panel (a) shows the daily trajectory of soil moisture prior to and between sampling events for leaf water potential, which occurred on day of year (DOY) 164 and 191 (dashed vertical lines). Weekly to biweekly measures of plot greenness are also shown in panel (a) using larger, open symbols (Zelikova et al., 2015). Panel (b) shows mean soil moisture integrated over two different time periods relevant to plant sampling. Error bars are based on standard error and are shown only for select dates in panel (a). Mixed models that account for spatial and temporal autocorrelation of daily VWC during the two periods show evidence of an interaction between the eCO_2 and warming treatments ($p = .01$ for both time periods). For DOY 164 and 191 only, the p values for the CO_2 * temperature interaction were 0.02 and 0.11.

compared to previous growing seasons and climate normals (Mueller et al., 2016); the mean temperature during the growing season was -12.5°C in 2013, the year of sampling, and -15.5°C in 2012, which was the warmest growing season of the 30-year period that ends in 2013. During the sampling period, leaf senescence was evident for many but not all individuals of *Carex duriuscula*, and for some but not most individuals of the other four species. Above-ground biomass and greenness were lower in the sampling year (2013) than in any prior year of the experiment, reflecting the effect of two consecutive growing-seasons with low precipitation and soil moisture (Mueller et al., 2016; Zelikova et al., 2015).

2.3 | Trait measurements

Traits were measured following standard protocols (Pérez-Harguindeguy et al., 2013; Sack et al., 2010). Pre-dawn water potentials (Ψ) were measured on two separate dates, day of year (DOY) 164 and 191 (Figure 1). On these dates, leaves were collected from 1 to 3 individuals per plot for each species between the hours of 1:30 and 3:30 AM. The leaves were sealed in plastic bags and stored in a dark cooler until water potentials were measured by Scholander-type pressure chambers within 8 h of sampling. Within species, there was no relationship between measurement time and water potential. Measures of leaf drought tolerance and anatomical leaf traits were performed for an additional sample of 1–3 individuals per plot for each species. For each species, all or most leaf samples used for drought tolerance and anatomical traits were acquired for all plots in a single day, with the first and last leaves collected on DOY 169 and 186, respectively, and just a few samples collected after DOY 181. Measures of turgor loss point and osmotic potential at full turgor, two similar indices of leaf drought tolerance, were derived from pressure–volume curves representing water loss and Ψ over time as leaves dehydrated. Pressure–volume curves were recorded following overnight rehydration of leaf tissues. For the two grass species, obtaining complete pressure–volume curves for every plot sometimes required a second or third sampling (e.g., due to leaf breakage from repeated Ψ measurements). For these two species, we used data from five experimental plots to compare observations of turgor loss point from the earliest and latest sampling events, and we observed similar means for each event (Figure S1).

Within 1–3 days of sampling, rehydrated and refrigerated leaves were preserved for measurement of anatomical traits by placement in a solution of ethanol (50%), propionic acid (5%), formalin (10%), and water (Ruzin, 1999). Microscopy was used to measure stomatal density, stomatal length and width, and the dimensions of xylem vessels. Imprints of stoma were obtained by brushing clear nail polish on the leaf surface and removing the dry imprint with clear tape. Imprints were then attached to a microscope slide for imaging and measurement. Imprints of abaxial leaf surfaces were of insufficient quality to count and measure abaxial stoma (e.g., because abaxial surfaces were too hairy or stoma were between veins). Following de Boer et al. (2016), the fraction of leaf epidermis allocated to stomata (f_{gc})

was estimated “directly” by multiplying stomatal density by stomate size (area) and dividing by the sampled leaf area. Due to uncertainty about the precision of our measurements of stomatal width, we also estimated the area of individual stomata and f_{gc} using allometry (as length divided by 2 for forbs and length divided by 4 for graminoids, following Franks and Beerling (2009) and Taylor et al. (2012)). Across all species and plots, the “direct” and allometric estimates of stomate area and of f_{gc} were tightly correlated (R_2 of 0.92 and 0.58, respectively). Within species, the correlations between allometric and directly measured f_{gc} all had R_2 greater than 0.78. For measuring xylem vessel dimensions, leaf cross-sections were stained with safranin-O and imaged with a fluorescent scope. A cross-section of the entire leaf blade was used for grasses and the petiole was measured for forbs. In both cases, the leaf area apical to the cross-section was measured to account for changes in vessel diameter with position on the leaf (Ocheltree et al., 2012).

2.4 | Statistical analyses

We used least-squares ANOVA to assess the effects of treatments in a factorial design, including the main effects of eCO_2 and warming alone and their interaction. Species identity was included as a fixed effect, along with interactions between “species” and each treatment. Block was included as a random effect. For some anatomical traits, we only sampled *Artemisia frigida* in 16 of the 20 plots, so this species was excluded prior to modeling treatment effects on those traits. For models of xylem vessel dimensions that were standardized by leaf area, *Pascopyrum smithii* was also excluded from the analysis due to missing leaf area data. For all models reported below, each species included in the model was sampled in at least 4 of the 5 plots of each treatment. Lastly, to assess the role of soil moisture in mediating treatment effects on these leaf traits, for the model structures described above we added volumetric soil moisture (integrated from 5 to 25 cm deep and over various time periods): if effects of warming and/or CO_2 treatments persisted despite inclusion of soil moisture as a covariate, we concluded those treatments likely had “direct” effects on leaf traits (i.e., beyond effects that could have been mediated by treatment effects on soil moisture).

3 | RESULTS

3.1 | Soil moisture

Volumetric water content in surface soils (5–25 cm) declined steadily from the beginning of the growing season ($\sim 20\%$ on DOY 120) to the first sampling of leaf traits ($\sim 13\%$ on DOY 164), and then continued to decline until the end of leaf sampling ($\sim 9\%$ on DOY 191; Figure 1a). Consistent with earlier reports, treatment effects on soil moisture were dynamic over time (Blumenthal et al., 2018; Mueller et al., 2016). eCO_2 alone had a persistently positive effect on soil moisture that became stronger as spring

transitioned to summer, boosting water content from ~12% in ambient plots to 14% in plots with eCO₂ and ambient temperature for the first sampling of leaves (DOY 164; Figure 1a). Warming alone reduced soil moisture early in the growing-season (but by less than 0.5%), had no effect during the first leaf sampling, and then increased soil moisture by the final sampling of leaves (DOY 191; Figure 1a); thus, the time-integrated effects of warming alone shifted from small and negative for the period pre-dating the first leaf sampling to neutral for the period pre-dating the final sampling (Figure 1b). Lastly, the effect of eCO₂ plus warming shifted over the growing season from roughly neutral to strongly negative, reducing soil moisture from ~16% to ~14% by DOY 150 (2 weeks before leaf sampling began). From the start of leaf sampling to the end, soil moisture in the combined eCO₂ and warming plots went from slightly lower than ambient to slightly higher than ambient (Figure 1a). When integrated over periods that pre-dated sampling, the combined effects of eCO₂ and warming on soil moisture were non-additive at the beginning and end of the leaf sampling period; this is evident in the lack of a positive CO₂ effect on warmed plots (Figure 1b).

3.2 | Overview of leaf traits

For all traits, differences among species were generally larger than the differences among treatments. For the traits shown in Figures 2 and 3, the maximal difference among species' means in ambient conditions was never less than 100% of the mean for the species with the lowest trait value. Conversely, when averaged across all five species, the maximal difference between the trait means of ambient plots and each of the three treatments was never more than 50% of the trait value in ambient plots. Across all traits, treatment effects were often small to negligible and the effects of eCO₂ were more apparent and substantial than those of warming. Generally, the effects of eCO₂ varied with species identity, but not with the presence or absence of warming (Table 1).

3.3 | Leaf Ψ

The sedge (*Carex duriuscula*) and C4 grass (*Bouteloua gracilis*) had the lowest water potentials measured pre-dawn (Ψ_{pd}). In ambient plots, leaf Ψ_{pd} declined by at least 1 MPa for all species between the June and July sampling events (Figure 2). In June and July, eCO₂ increased leaf Ψ_{pd} , especially for graminoid species. For the C3 grass (*Pascopyrum smithii*), leaf Ψ_{pd} in plots with eCO₂ were half as negative as in ambient plots. For the two grass species, there was also evidence that eCO₂ moderated the decrease in Ψ_{pd} that occurred as soils dried between June and July. In June only, eCO₂ and warming effects were non-additive ($p = .01$): the increase in leaf Ψ_{pd} due to eCO₂ was smaller in warmed plots than in ambient temperature plots. One of the forbs, *Sphaeralcea coccinea*, exhibited minimal sensitivity to eCO₂ and the least change in Ψ_{pd} from June to July.

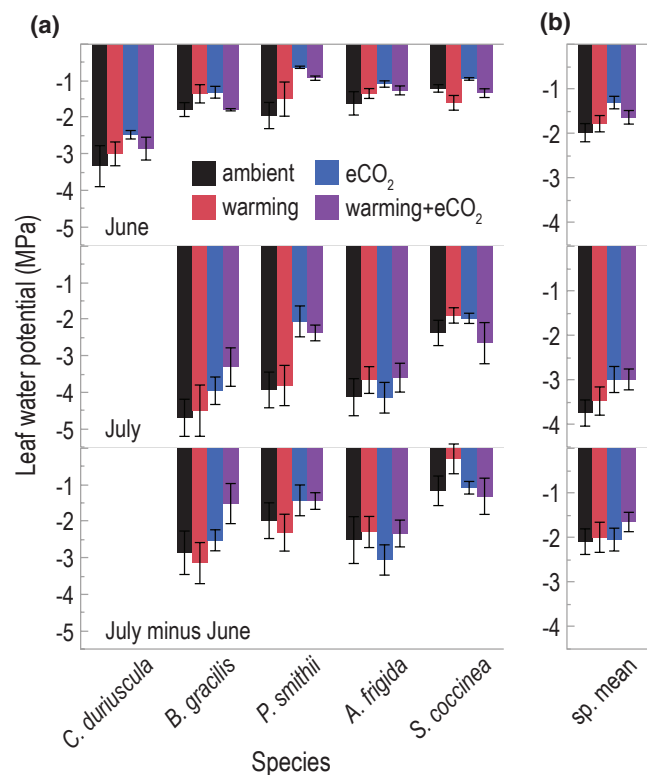


FIGURE 2 Mean leaf water potentials (pre-dawn) for different treatments, species, and sampling periods. The bottom panel shows how much pre-dawn water potentials declined from the first (June) to the second (July) sampling period, mirroring the decline of soil moisture observed in all treatments (Figure 1). The left panel (a) shows treatment means separately for each species, while the right panel (b) shows treatment means averaged across all species. See Table 1 for the modeled effects of species and treatments and their interactions.

Although leaf Ψ_{pd} was much lower for the later sampling event for which soil moisture was also lower (Figure 2), variability in Ψ_{pd} within species was only weakly positively correlated with plot-level variability in soil moisture in June ($R^2 = 0.04$, $p < .001$) and July ($R^2 = 0.02$, $p = .16$). The effects of eCO₂ on Ψ_{pd} remained apparent and species-specific when soil moisture for the day of leaf sampling was included as a covariate (Table S1). Similarly, Ψ_{pd} in warmed and ambient temperature plots remained indistinguishable ($p > .2$) when soil moisture was included as a covariate.

3.4 | Turgor loss point and osmotic potential

These related indices of leaf-level drought tolerance were tightly correlated across species and treatments ($R^2 = 0.94$, $p < .001$, $n = 99$). As for leaf Ψ_{pd} , the sedge *C. duriuscula* had the lowest values for turgor loss point and osmotic potential at full turgor, while the two forbs had higher, and similar, values for these traits (Figure 3). For the graminoid species, leaves exposed to eCO₂ had turgor loss points and osmotic potentials that were ~10 to 30% less negative than in ambient [CO₂] plots, indicating potentially diminished drought tolerance due to eCO₂. Within-species variability in turgor

TABLE 1 Effects of species and treatments on leaf traits based on least-squares ANOVA models

Model term	Dependent variable									
	Pre-dawn Ψ (DOY 164)	Pre-dawn Ψ (DOY 191)	Δ pre-dawn Ψ (DOY 164 to 191)	Osmotic potential at full turgor	Turgor loss point	Stomate density	Stomate size (meas.)	f_{gc} (meas.)	Xylem vessel area	Stand. Xylem vessel area
Species	****	****	****	****	****	****	****	****	****	****
CO ₂	****	***	****	****	****	****	****	**	****	****
Species*CO ₂	*	**	*	****	***	***	++	*	*	*
Temperature				*		+	+	*	*	+
Species*temp.									**	
CO ₂ *temp.	**					+				
Sp.*CO ₂ *temp.						**				
n	98 (5 sp.)	78 (4 sp.)	76 (4 sp.)	99 (5 sp.)	99 (5 sp.)	73 (4 sp.)	74 (4 sp.)	73 (4 sp.)	76 (4 sp.)	57 (3 sp.)
R ²	0.69	0.52	0.44	0.92	0.89	0.90	0.92	0.52	0.64	0.68

Note: ****($p < .001$); ***($p < .01$); **($p < .05$); *($p < .1$); ++ and + show when $p < .15$ and $p < .20$, respectively, for interaction terms only, following Mueller et al. (2016) and Stehman and Meredith (1995). These additional thresholds are reported for interaction terms because in factorial designs with low n , such as this experiment, risk of Type II errors is likely much greater than that of Type I errors (Smith et al., 2002) and interpretation of main effects is conditional upon the interaction (Stehman & Meredith, 1995). The modeled effects for stomate size and f_{gc} that were measured using allometry (based on measured stomatal length) were similar to those shown here (based on measurements of both length and width), except the CO₂*species interaction terms had p values greater than 0.2, and for stomate size the allometric version showed weak evidence of a CO₂*temperature interaction ($.1 < p < .2$). Blue shading indicates CO₂-effects, red shading indicates temperature-effects, and purple shading indicates interactive effects between CO₂ and temperature.

loss point and osmotic potential was not correlated with variability in soil moisture among plots ($p > .2$); this relationship was assessed for mean soil moisture integrated over three time intervals of different length, all preceding leaf sampling (DOY 100 to 160, DOY 100 to 148, and DOY 117 to 148; for reference, greenness of ground cover did not exceed 10% until after DOY 117 and peak greenness was observed at DOY 148 [Zelikova et al., 2015]). Thus, as for leaf Ψ_{pd} , the effects of eCO₂ on leaf turgor loss point and osmotic potential remained apparent and species specific when soil moisture was included as a covariate (Table S1).

3.5 | Leaf anatomy

Together, stomatal size and density shape the integrated function and allocation cost of stomata, as reflected in the fraction of leaf epidermis allocated to stomata (f_{gc}) (de Boer et al., 2016). Elevated CO₂ reduced f_{gc} for the C4 grass *B. gracilis* (by ~30%) and for the forb *S. coccinea* (by ~10%), with minimal effects on other species (Figure 3). These effects of eCO₂ on f_{gc} were a function of reductions in both stomatal density and size (Figure S2; Table 1). Warming had a small positive effect on f_{gc} that was not statistically dependent on either species or [CO₂], though its effect was largest for the sedge *C. duriuscula* and the C3 grass *P. smithii*. Models with soil moisture as a covariate showed some evidence of species-specific effects of soil moisture on stomatal anatomy; but, as for the other traits, the species-specific effects of eCO₂ remained apparent even after accounting for the statistical effects of variability in soil moisture among plots (Table S1). The strongest modeled effects of soil moisture were observed for stomatal density of *S. coccinea* (negative correlation), stomatal size of *P. smithii* (negative correlation), and f_{gc} of *B. gracilis* (positive correlation) and *S. coccinea* (negative correlation).

The size of individual xylem vessels, as indicated by their cross-sectional area, was unaffected by eCO₂ and increased by warming (Figure S3), making this the only measured leaf trait that was more sensitive to warming than to eCO₂. However, like the effects of eCO₂ on other traits, the effects of warming on xylem vessel size were strongly species specific. The warming effects were most apparent for the C3 sedge (*C. duriuscula*) and one of the forbs (*S. coccinea*), and minimal to negligible for the C4 grass (*B. gracilis*). The size of sampled leaves was also larger in warmed plots for *C. duriuscula* and *S. coccinea* (Figure S3), and when xylem vessel size was standardized by the projected surface area of sampled leaves, there is evidence of a negative effect of warming for the C3 sedge (*C. duriuscula*; Figure 3 and S3).

4 | DISCUSSION

4.1 | Overview

The results were generally consistent with our overarching hypothesis: elevated [CO₂] (eCO₂) caused hydraulic traits to shift toward values indicative of reduced investments in carbon-acquisition,

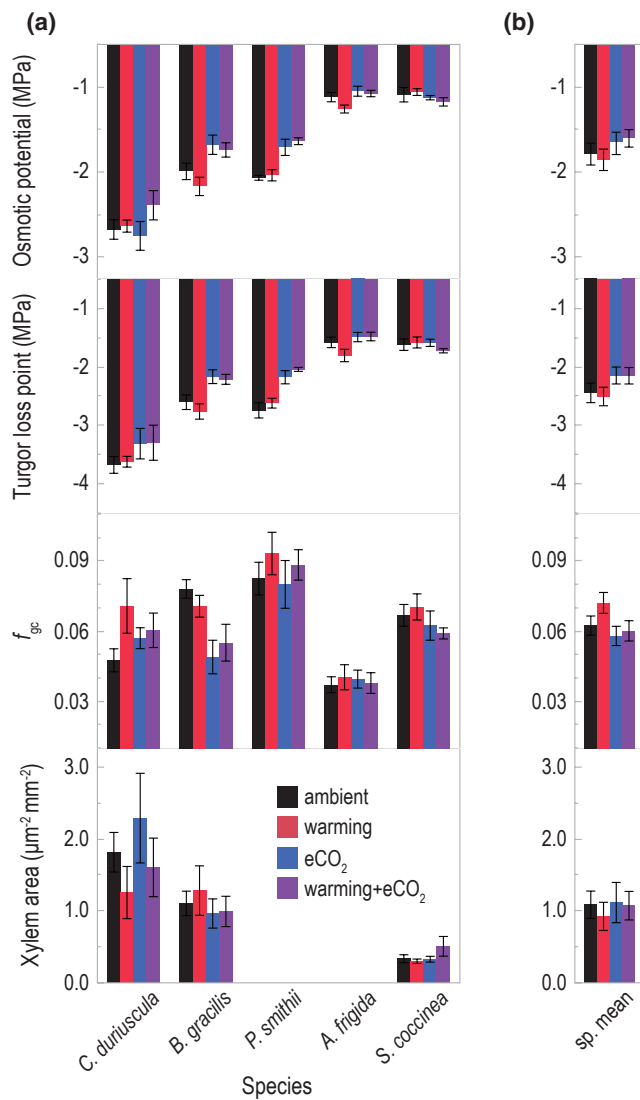


FIGURE 3 Mean values of leaf traits for different species and treatments. The panels on the left (a) show treatment means separately for each species, while the panels on the right (b) show treatment means averaged across three to five species. Xylem area was measured as the cross-sectional area of individual vessels. See Table 1 for the modeled effects of species and treatments and their interactions.

water transport, and drought-tolerance. Furthermore, as expected based on prior studies (Gerald et al., 2016; Habermann, Dias de Oliveira, et al., 2019; Habermann, Martin, et al., 2019; Haworth et al., 2015; Knapp et al., 1994; Lauber & Körner, 1997; Maherali et al., 2002; Marchi et al., 2004; Matthews & Lawson, 2019; Miranda-Apodaca et al., 2018; Wang et al., 2020), the effects of eCO₂ varied from negligible for some species, especially the forbs, to moderate for other species, especially the grasses. Direct effects of eCO₂ and warming on hydraulic traits appeared to be more important than indirect effects mediated by treatment impacts on soil moisture. This is apparent in the observation that treatment effects on leaf water potentials, drought tolerance and anatomy did not

simply mirror treatment effects on soil moisture. The CO₂*warming interaction was typically not significant for leaf traits, with a main effect of eCO₂ being most common (Table 1), but the CO₂*warming interaction was typically significant for soil moisture, with generally sub-additive effects for the combined eCO₂ + warming treatment (Figure 1; see also Blumenthal et al., 2018; Mueller et al., 2016). In other words, while the eCO₂ alone and eCO₂ + warming treatments generally had the most divergent soil moisture (Figure 1), these treatments often had similar values for leaf traits (Figures 2 and 3). Similarly, warming had no apparent effect on pre-dawn water potentials for both sampling periods, despite a strong positive effect of warming on surface soil moisture during the later sampling (Figures 1 and 2). With the exception of xylem vessel dimensions, the effects of warming on hydraulic traits were less evident than the effects of eCO₂, suggesting these hydraulic traits are more sensitive to direct manipulations of carbon limitation than to manipulations of temperature or indirect effects of global change on water availability. Similarly, an earlier study from this experiment showed the effects of eCO₂ on leaf gas exchange were larger and more common than the effects of warming (Sage et al., 2020).

4.2 | Evolutionary vs. phenotypic responses to eCO₂

Over millenia to millions of years, reconstructed shifts in eCO₂ correspond with widespread changes in the allocation of leaf epidermal tissues to hydraulic architecture, including stomata and leaf veins (de Boer et al., 2012; Feild et al., 2011; Franks & Beerling, 2009). The comparably small and sometimes neutral responses of leaf hydraulic anatomy to 7 years of eCO₂ in this study can be partly understood by considering the role of stomatal aperture in mediating plant responses to environmental change (Haworth et al., 2011; Matthews & Lawson, 2019). Specifically, by closing their stomata more frequently and/or opening them less widely, plants can adjust C-acquisition and water transport and loss without modifying hydraulic architecture. Meta-analyses of FACE studies have suggested that while reductions in stomatal conductance in response to eCO₂ are common and often substantial, corresponding shifts in stomatal architecture appear to be less common and weaker (Ainsworth & Rogers, 2007). For three of the five species in this study, Sage et al. (2020) measured leaf gas exchange during two prior years of the same experiment. The forb *S. coccinea* reduced stomatal conductance due to eCO₂ (up to 40% lower than in ambient CO₂ plots), contrasting with the much smaller reductions we observed for the fraction of leaf surface allocated to stomata (f_{gc} ; Figure 2). For the other forb in this study, *A. frigida*, eCO₂ had no apparent effect on stomatal conductance (Sage et al., 2020) or f_{gc} . The only graminoid species included in both studies, *P. smithii*, had up to 65% lower stomatal conductance in eCO₂ plots (depending on the year and season), with no apparent effect of eCO₂ on f_{gc} later in the experiment.

Why might the species in this study have smaller or neutral responses of stomatal architecture to $e\text{CO}_2$ as compared to responses of stomatal conductance? In ecosystems with substantial variability in temperature and precipitation within and across years, like this semiarid grassland, a 50% increase in $[\text{CO}_2]$ is not sufficient to eliminate temperature and/or water stress during short-term hot and dry periods (e.g., droughts and heatwaves). During such times, a substantial investment in hydraulic architecture, and in leaf-level drought tolerance, is likely required to maintain basic leaf functions and vitality, even with higher atmospheric $[\text{CO}_2]$. For example, perhaps reduced f_{gc} or smaller xylem vessels would be costly during sunny, warm days when evaporative demand and photosynthetic potential is high, even when $e\text{CO}_2$ makes water somewhat less limiting; this could negate or partly counteract the potential benefits of reduced investment in hydraulic architecture. Thus, species adapted to such variable climates might also have conservative anatomical responses to altered environmental conditions. The work of Haworth et al. (Haworth et al., 2013, 2015) is relevant here; they suggested that species with stronger short-term adjustments of stomatal aperture to $e\text{CO}_2$ might be less likely to also adjust stomatal density or other aspects of stomatal architecture. If such a trade-off is strong and persists across taxonomic and biogeographic scales, many plants in the mixed-grass prairie and other semiarid ecosystems might have little change in f_{gc} as $[\text{CO}_2]$ increases, because the climate might favor species capable of using adjustments of stomatal aperture to enhance water-use efficiency and drought resistance (Ocheltree et al., 2020). For the mixed-grass prairie community in this study, data on leaf-gas exchange of common species suggest that stomatal conductance of all species varies substantially within and across years (coincident with variability in weather), with rates of conductance reduced by 40–80% during dry periods (Ocheltree et al., 2020; Sage et al., 2020). In contrast to this and other field studies, the controlled conditions in chamber and glasshouse experiments might facilitate “unrealistic” expression of phenotypic plasticity of hydraulic architecture.

4.3 | Community scale and ecosystem scale processes

Knowledge of the physiological responses of these five species to $e\text{CO}_2$ and warming is key to understanding how climate change has shifted plant community composition and ecosystem function in this mixedgrass prairie. *Bouteloua gracilis*, the lone C4 species in this study and one of the most abundant species in the community, was the only species that reduced allocation of leaf area to stomata (f_{gc} ; Figure 3). Although gas-exchange measurements are not available for confirmation (but see LeCain et al., 2003), the integrated effects of $e\text{CO}_2$ on stomatal density, size, and f_{gc} of *B. gracilis* (Figure S1) suggest this species adopted a more conservative water-use strategy, and that its anatomical responses to $e\text{CO}_2$ help explain the positive effects of $e\text{CO}_2$ on soil moisture (Figure 1) (Blumenthal et al., 2018). Conversely, the common C3 species in this study showed smaller or neutral responses of stomatal anatomy to $e\text{CO}_2$. Gas-exchange measurements from earlier years of this experiment show

stomatal conductance of the grass *P. smithii* and forb *S. coccinea* was sometimes reduced by 50% under $e\text{CO}_2$, and other times minimally affected (Sage et al., 2020). Collectively, these results suggest the physiological responses of *P. smithii* and *S. coccinea* also contributed to higher levels of soil moisture under $e\text{CO}_2$, but these species maintained a more flexible strategy for water-use and C-acquisition by altering stomatal aperture rather than architecture. This strategy could be advantageous during warm and wet time periods, and sufficient during typical dry periods. Thus, the contrasting responses and strategies of *B. gracilis* and *P. smithii* could help explain why *B. gracilis* became less abundant in $e\text{CO}_2$ plots as this experiment progressed (Mueller et al., 2016) (Figure S4).

Compared to the two forbs in this study, the three graminoids had more negative values for pre-dawn water potentials (Ψ_{pd}), turgor loss points, and/or osmotic potentials at full turgor (Figures 2 and 3), perhaps revealing a strategy to use shallower, more transient water resources in conjunction with greater tolerance of desiccation (see also Blumenthal et al., 2020; Ocheltree et al., 2020). Furthermore, while leaf Ψ_{pd} and the two proxies for leaf-level drought tolerance of the forbs were insensitive to the treatments, for the graminoids, leaf Ψ_{pd} , turgor loss points, and osmotic potential were all less negative in $e\text{CO}_2$ plots. This pattern hints at a potential shift in drought resistance strategies for these three common graminoid species. Specifically, less negative leaf Ψ_{pd} in $e\text{CO}_2$ plots likely indicates reduced levels of water stress (and during a time when soil moisture was generally low; Figure 1). The water content of deeper soil layers is higher and more stable over time in this ecosystem (Blumenthal, unpublished; Lauenroth et al., 2014), so one possible explanation for graminoids' altered Ψ_{pd} under $e\text{CO}_2$ is a shift to utilizing deeper soil moisture. Less negative values of turgor loss point and osmotic potential are indicative of a reduced ability of leaves to remain active and viable during meteorological droughts. Together, these patterns in Ψ_{pd} , turgor loss point, and osmotic potential can be interpreted as a potential shift away from physiological drought tolerance and toward physiological drought avoidance as a consequence of $e\text{CO}_2$. For example, less negative turgor loss points could place greater emphasis on adjustments to stomatal aperture as a means of conserving water and avoiding physiological drought (Sun et al., 2020). Such a shift toward drought avoidance by these dominant graminoids could help explain the increased temporal stability of aboveground production due to $e\text{CO}_2$ in this experiment (Zelikova et al., 2014). But, if the less negative turgor loss points of the graminoids under $e\text{CO}_2$ are not counter-balanced by other forms of drought tolerance or avoidance, these species could become more sensitive to drought, especially extreme droughts that could negate some means of drought avoidance (e.g., use of deep soil water). To clarify the impacts of $e\text{CO}_2$ and warming on plant–water relations, and project the impacts of drought in a warmer world with higher $[\text{CO}_2]$, scientists must conduct more integrative studies of drought-resistance traits and their sensitivity to climate change, in this prairie and other ecosystems. Future work could assess whether plants that use deeper layers of soil moisture, perhaps including the forbs in this study (Ocheltree et al., 2020), are less likely to adjust their drought resistance strategies in response to climate change.

4.4 | Conclusions

Our study suggests that, for the extensive mixed-grass prairie of North America, shifts in the relative abundance of species due to climate change might have larger implications for drought resistance than the physiological responses of individual species. This conclusion is consistent with other studies, even in much different ecosystems, which also suggest changes to community composition due to climate change are likely to yield similar or larger impacts on ecosystem functions than the impacts of climate change on physiological traits of individual species (Langley & Hungate, 2014). Other studies in both mixed-grass and short-grass prairies show that, among dozens of common species and for present climate conditions, graminoids and forbs have divergent phenotypes for several hydraulic traits (Blumenthal et al., 2020; Ocheltree et al., 2020). Thus, shifts in the abundance of these functional types could have the most impact on plant water-relations within these semiarid ecosystems. Future studies should assess whether the hydraulic traits of different plant functional groups have distinct responses to climate change, as we observed for the limited sample of species in this study.

AUTHOR CONTRIBUTIONS

KEM, TWO, DMB, and JAK conceived the hypotheses and designed the sampling strategy and trait measurements. DMB, DGW, and EP designed and maintained the experiment. KEM, TWO, JAK, and JB conducted the sampling and trait measurements. KEM wrote the manuscript and performed the statistical analyses. All authors revised the draft manuscript.

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CONFLICT OF INTERESTS

The authors have no conflict of interest to report.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available via Dryad at <https://doi.org/10.5061/dryad.2280gb5vn>

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SUPPORTING INFORMATION

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