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Research paper

Elevated CO_2 counteracts effects of water stress on woody rangeland-encroaching species

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The ubiquity of woody plant expansion across many rangelands globally has led to the hypothesis that the global rise in atmospheric carbon dioxide concentration ([CO_2]) is a global driver facilitating C_3 woody plant expansion. Increasing [CO_2] also influences precipitation patterns seasonally and across the landscape, which often results in the prevalence of drought in rangelands. To test the potential for [CO_2] to facilitate woody plant growth, we conducted a greenhouse study for 150 days to measure CO_2 effects on juveniles from four woody species (*Cornus drummondii C.A. Mey., Rhus glabra L., Gleditsia triacanthos L., Juniperus osteosperma Torr.*) that are actively expanding into rangelands of North America. We assessed chronic water-stress (nested within CO_2 treatments) and its interaction with elevated [CO_2] (800 p.p.m.) on plant growth physiology for 84 days. We measured leaf-level gas exchange, tissue-specific starch concentrations and biomass. We found that elevated [CO_2] increased photosynthetic rates, intrinsic water-use efficiencies and leaf starch concentrations in all woody species but at different rates and concentrations. Elevated [CO_2] increased leaf starch levels for *C. drummondii*, *G. triacanthos*, *J. osteosperma* and *R. glabra* by 90, 39, 68 and 41%, respectively. We also observed that elevated [CO_2] ameliorated the physiological effects of chronic water stress for all our juvenile woody species within this study. Elevated [CO_2] diminished the impact of water stress on the juvenile plants, potentially alleviating an abiotic limitation to woody plant establishment in rangelands, thus facilitating the expansion of woody plants in the future.

***Keywords*:** climate change, elevated CO_2, non-structural carbohydrates, water stress, woody encroachment.

Introduction

Rangelands cover 30% of the Earth's terrestrial surface and provide many ecological services, including forage for grazing animals, floristic diversity and hydrological and biogeochemical cycle regulation (Asner et al. 2004, Ellis and Ramankutty 2008, Dixon et al. 2014). Many grass-dominated rangelands globally are transitioning to either shrublands or woodlands through a process called woody plant encroachment (Briggs et al. 2005, Saintilan and Rogers 2015, Stevens et al. 2017, Venter et al. 2018). Increases in woody plant density and cover in temperate grasslands alter nutrient and water cycles (Gill et al. 2002, Leffler et al. 2002, Brunsell et al. 2013, Yannarell et al. 2014, Logan and Brunsell 2015), change fire regimes (Knapp et al. 2008, Holdo et al. 2009, Wigley et al. 2010), decrease species diversity (Van Auken 2009, O'Connor and Chamane 2012, Ratajczak et al. 2012), and decrease available livestock forage (Anadón et al. 2014). The phenomenon of woody plant encroachment has been attributed to drivers such as changes in land-use and alteration to fire frequency, but these drivers are often localized and do not necessarily explain this global phenomenon. One driver that is global and impacts all rangelands, however, is increased atmospheric CO_2 concentrations (hereafter [CO_2]) (Archer et al. 1995, 2017, Bond and Midgley 2000, Polley et al. 2002*a*).

Atmospheric [CO_2] rose from 280 p.p.m. at the start of the industrial revolution to 414 p.p.m. in 2022, and it is projected to exceed 800 p.p.m. by the end of this century (Collins et al. 2013, Blunden et al. 2018). Rising [CO_2] affects plants directly

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through increased photosynthetic rates, decreased stomatal conductance and improved water-use efficiencies (WUE), which collectively tend to increase rates of plant growth (Bazzaz 1990, Curtis 1996, Curtis and Wang 1998, Leakey et al. 2009). Increased [CO_2] also favors C_3 plants over C_4 plants because of C_3 plants' ability to fix more carbon than C_4 plants at elevated [CO_2], which can lead to increased storage of carbon in the form of non-structural carbohydrates (Curtis and Wang 1998, Bond and Midgley 2000, Leakey et al. 2009, Kgope et al. 2010), although C_4 species can also benefit through increases in WUE (Ainsworth and Long 2004, Morgan et al. 2004). Collectively, these benefits of elevated [CO_2] should favor C_3 woody plants, specifically clonal and resprouting woody plants, over herbaceous species (Ainsworth and Rogers 2007, Clarke et al. 2016), particularly in C_4-dominated grasslands, by facilitating rates of woody plant growth in a disturbance-prone ecosystem (Bond and Midgley 2012, Staver and Bond 2014, Raubenheimer and Ripley 2022).

Much less is known about how elevated [CO_2] may interact with other predicted global changes to influence woody plant encroachment. Droughts are predicted to increase in frequency and intensity in North America and across many rangelands worldwide due to a combination of altered precipitation patterns and warmer temperatures (Collins et al. 2013, Polley et al. 2013, Cook et al. 2015). Decreased soil moisture limits seedling recruitment, juvenile establishment and growth of woody plants (Polley et al. 2002*b*, Hoffmann et al. 2004, Saintilan and Rogers 2015). A key question, however, is how drought will interact with elevated [CO_2] to affect different functional groups of juvenile woody plant encroaching species (e.g., clonal resprouter, resprouter, non-resprouter). Given that elevated [CO_2] can reduce drought stress, it has the potential to ameliorate the deleterious effects of drought on juvenile woody plants and facilitate increased woody plant establishment in rangelands.

Several studies have found that water stress in conjunction with elevated [CO_2] reduced plant growth in resprouting woody plants and increased WUE compared with wetter conditions with elevated [CO_2] (Nackley et al. 2018, Raubenheimer and Ripley 2022); however, all of the species were nitrogen-fixing woody plants. Precipitation and soil moisture are critical determinants of plant growth, and this pattern becomes more apparent with elevated [CO_2] (Hovenden et al. 2019). Yet, reduction in stomatal conductance and increased non-structural carbohydrates in stem tissue while water stressed at higher [CO_2] could be a mechanism for woody plants to survive low soil moistures even with reduced aboveground plant growth (Nackley et al. 2018, Paudel et al. 2018).

To test how elevated [CO_2] and water stress impact juveniles of woody encroaching species of different functional groups (clonal resprouting, resprouting and non-resprouting), we conducted a greenhouse experiment to measure the growth and physiology of four woody plant species that are encroaching on North American rangelands in the Great Plains (*Cornus drummondii* C.A. Mey., *Rhus glabra* L., *Gleditsia triacanthos* L.) and the Intermountain West (*Juniperus osteosperma* Torr.). *Cornus drummondii* and *R. glabra* are characterized as clonal resprouting deciduous shrubs, *G. triacanthos* is a resprouting deciduous tree that does not usually form nodules and fix nitrogen in North America (Nippert et al. 2021), and *J. osteosperma* is an evergreen non-resprouting conifer tree (Chesus and Ocheltree 2018). Juvenile woody plants were grown in a factorial combination of [CO_2] (elevated (∼805 p.p.m.) vs ambient (∼414 p.p.m.)) and water availability (well-watered vs chronic water stress). We hypothesized that elevated [CO_2] would increase WUE, through increased photosynthetic rates and decreased stomatal conductance, and ameliorate the effects of water stress on the juvenile woody plants. We also hypothesized that elevated [CO_2] would increase photosynthetic rates and therefore also increase starch storage in stems and roots of the resprouting species resulting in increased total plant biomass, but not the evergreen conifer.

Materials and methods

Study species and experimental design

We grew *C. drummondii*, *R. glabra, G. triacanthos* and *J. osteosperma* in two greenhouse bays of a greenhouse complex at the USDA-ARS Crops Research Laboratory in Fort Collins, CO from November 2016 to April 2017. The greenhouse bays were maintained at a temperature (±SD) of 30 ± 0.7 °C during the day and 21 ± 0.8 °C at night with a 12-h photoperiod. Relative humidity was 20 ± 8% during the day and 25 ± 9% at night. The bays were lit by HID lighting. All plants were grown under ambient [CO_2] (414 ± 31 (SD) p.p.m.v.) and elevated [CO_2] (805 ± 38 (SD) p.p.m.v.), which was controlled by an Argus climate control system (Argus Control Systems LC, Surrey, British Columbia, Canada), and with two soil moisture conditions (chronic water stressed and unstressed). Each of the four treatment combinations (two [CO_2] * two soil moisture conditions) were replicated 10 times per woody plant species.

The experiment was arranged in blocked design with [CO_2] treatments in separate greenhouse bays and the soil moisture treatment nested within the [CO_2] treatment. Because we were limited to one greenhouse bay per [CO_2] treatment, watering treatments were randomized within each [CO_2] treatment. To decrease the likelihood of spurious effects of greenhouse bay, we rotated both the [CO_2] treatments and the plants subject to those treatments between greenhouse bays biweekly (Perry et al. 2013). For the purpose of analysis, we considered pots subjected to different [CO_2] treatments to be true replicates. This approach controls for variation in [CO_2] responses associated with individual plants, their locations within greenhouse bays, and most effects of the bays themselves, but cannot rule out potential spurious effects caused by interactions between greenhouse bay and time (Perry et al. 2013).

Native soil was used for each species to mimic real plant–soil water interactions. For *C. drummondii*, *G. triacanthos* and *R. glabra*, soils were collected at the Konza Prairie Biological Station (KPBS) in the eastern portion of the tallgrass prairie. The soils from KPBS were Irwin silty clay loam. The soil for *J. osteosperma* came from Moffat County in the northwest Colorado. These soils were a loamy sand (Chesus and Ocheltree 2018). The water-stressed treatment for each species was calibrated to its native soil, with plants, prior to the start of the experiment through a dry-down trial where we measured water loss from fully saturated soils. During the dry-down, pots were weighed daily and soil volumetric water content (VWC) was measured using a HydroSense II handheld soil moisture sensor (Campbell Scientific, Logan, UT, USA). We then calculated evapotranspiration (ET) from the daily weights of soils, and plotted it against the corresponding VWC measurements to determine what the lowest and highest VWC values were in conjunction to the lowest and highest ET values based on soil type (loamy sand ET range 0–27%; silty clay loam ET range 10–50%) (Figure S1 available as Supplementary data at *Tree Physiology* Online) We determined that the water stress treatment of junipers in the loamy sand soils would be between 0 and 10% VWC, while the other woody species planted in silty clay loam soils would be a 10–20% VWC. The control water treatment plants had soils watered to full saturation and were not allowed to drop below 15% VWC for the loamy sand soils, and 35% VWC for the silty clay loam soils. All plants were watered once a week, and the water stress plants were watered up to a ‘target weight’, which corresponded to their respective VWC values (fully saturated for control water treatment plants, and for water stress treatment plants 10% for loamy sand soils and 20% for silty clay loam soils). The water stress treatment started on Day 66 and was applied to half of all the plants by species in both [CO_2] treatments until the end of the study (Day 150). The VWC measurements were not taken once the water stress treatment had begun.

Each woody plant species was planted in 30 cm of their respective native soils in 12.7 × 12.7 × 30.5 cm deep square tree pots. None of the woody plant species came from parental lines that were grown in elevated [CO_2] conditions or treatments. *Gleditsia triacanthos* and *R. glabra* plants were grown from seeds collected from KPBS. *Cornus drummondii* was difficult to establish from seed, so first year ramet cuttings were planted. The ramet cuttings were propagated at the Missouri Department of Conservation White State Forestry Nursery and shipped to our greenhouse. *Juniperus osteosperma* seeds were also difficult to germinate (T.W. Ocheltree, personal observation), and so seedlings were transplanted from the field in Moffat County, Colorado. None of the transplanted species had taproots that extended more than 15 cm below the soil surface. *Gleditsia triacanthos* and *R. glabra* seedlings were thinned to just one individual per pot after a month of growth. No fertilizer was ever added to the plants during the experiment, but a pesticide (‘Triple Action’, Ferti-loam) was added to individuals’ leaves when mites were detected, and a fungicide (‘Infuse’, Bonide) was applied to several *R. glabra* plants’ leaves that had mildew during the experiment.

Leaf-level gas exchange

Instantaneous leaf-level-gas exchange measurements were made on four individuals of each woody plant species per [CO_2] and water stress treatment every 2 weeks from 10:00 to 16:00 h for the full duration of the experiment (Days 92–149). Gas exchange measurements were made on fully expanded leaves using a Li-6400XT portable photosynthesis system, with red/blue light source and the CO_2 injector (LI-COR Inc., Lincoln, NE, USA). Leaves that did not fill the Li-6400XT cuvette were photographed on a 1 cm^2 grid paper using an iPhone 5c camera (8 MP). Leaf area was estimated from the photographs using ImageJ software (Schneider et al. 2012), and corrected in the gas exchange file. The light source of the Li-6400XT was set to a light intensity of 1200 $\mu mol\ m^{-2}\ s^{-1}$ and the CO_2 concentrations were adjusted to match the appropriate greenhouse CO_2 treatments (400 p.p.m., 800 p.p.m.). Leaf temperatures within the cuvette were on average at 33.6 (±1.5) °C. Intrinsic water-use efficiency (iWUE) was calculated post-hoc as the photosynthetic rate divided by stomatal conductance to yield iWUE expressed as $\mu mol\ CO_2\ mol^{-1}\ H_2O$.

Biomass and tissue preparation

At the end of the experiment all plant tissues were harvested and prepared for further analyses. Leaves and stems were harvested, separated and placed in labeled plastic bags. Roots were harvested by gently emptying the pot into a sieve and rinsing with water until all soil particles were removed. All tissues were then microwaved for 90 s to halt enzymatic activity for starch quantification (Landhäusser et al. 2018). After all tissues were microwaved, they were oven dried at 60 °C for 48 h. Biomass was recorded separately for leaves, stems and roots. After biomass was recorded, samples were ground and stored in glass vials in a −20 °C freezer prior to starch analysis.

Starch analysis

For starch analysis, 20 mg of each plant tissue type had the simple sugars extracted according to the ethanol extraction method (Hendrix 1993). The ethanol-extracted tissue samples were immersed in 1 ml of water in a 2 ml microcentrifuge tube and autoclaved for 1 h at 135 °C. Samples were then dried at 60 °C. Starch concentrations were quantified using the Total Starch Assay Kit (Total Starch Assay Kit, Megazyme Co., Ireland). The plant tissues were digested in 1 ml of α-amylase, boiled for 20 min and then cooled for 10 min, followed by an addition of

15 μl amyloglucosidase. The samples were mixed in a shaking water bath at 50 °C for 45 min. Samples were then plated out in 96-well plates and then 200 μl of GOPOD (glucose oxidase/peroxidase with O-dianisidine) reagent (provided in Total Starch Assay Kit) was added to the samples. After 20 min of incubation at room temperature, absorbance was read at 510 nm on a 96-well plate reader spectrophotometer. Starch concentrations were quantified using a standard curve from maize starch (provided in Total Starch Assay Kit) that was digested and analyzed at the same time as the other plant tissues. A positive control sample of *C. drummondii* root tissue with known concentrations of starch was used to verify the precision of this protocol (O'Connor et al. 2020). To compare carbon allocation among treatments, all tissue-specific starch concentrations were multiplied by each individual's respective tissue-specific biomass.

Data analysis

All analyses were conducted for each woody species to address the physiological effects of [CO_2] and water stress on each trait measured. We first analyzed photosynthesis using an ANCOVA that included stomatal conductance as the covariate with [CO_2] and the water stress treatments as fixed-effect variables. The covariate and both fixed-effects variables were analyzed as a full factorial. Included in this model is a nested random effect of the individuals sampled within each block to account for covariation between multiple measurements on the same individual. To help determine how much of an effect [CO_2] had on photosynthesis we did a log–log analysis with stomatal conductance using the smatr R package (Warton et al. 2012). In addition, we analyzed repeated measures linear mixed models of woody plant photosynthetic rates and iWUE as response variables, with fixed-effect variables as [CO_2] × water stress interactions. We included a nested random effect of individuals sampled within each block, specifying a continuous AR1 covariance structure.

Tissue-specific biomass and starch were analyzed as type 3 ANCOVAs. The fixed effects for the analyses were [CO_2] treatment, water stress treatment and [CO_2] * water stress interactions with mean water loss prior to the water stress treatment as a covariate (a proxy for pre-treatment differences in plant size). All statistical model assumptions were met. Statistical significance was set at an a priori α of 0.05. All figures and statistical analyses were done in Program R with R Studio (R Studio Team 2019, R Core Team 2020). R packages used for all statistical analyses and graphics can be found in Supplemental Citation Section available as Supplementary data at *Tree Physiology* Online.

Results

Leaf gas exchange and water-use efficiencies

Photosynthesis at a given level of stomatal conductance (g_s) increased with elevated [CO_2] (significant $g_s \times CO_2$ interaction), and this was consistent for all species (Figure 1). Two species, *G. triacanthos* and *R. glabra*, also showed a significant stomatal conductance-by-[CO_2]-by-water stress interaction, which means that with elevated [CO_2] and water stress photosynthesis maintained high rates with lower stomatal conductance (Figure 1; Table S1 available as Supplementary data at *Tree Physiology* Online). This pattern of high rates of photosynthesis maintained with low stomatal conductance is made apparent through a log–log analysis of photosynthesis and stomatal conductance, where the slopes are significantly different for both *G. triacanthos* and *R. glabra* with elevated [CO_2] and the interaction or elevated [CO_2] and water stress (Table S2 available as Supplementary data at *Tree Physiology* Online).

Accounting for time since the start of the water stress treatment, photosynthetic rates increased in plants grown in elevated [CO_2] for all species (Figure 2; Table S3 available as Supplementary data at *Tree Physiology* Online). Water stress reduced photosynthetic rates (from Day 92, first measurement, to Day 149) for *G. triacanthos* ($P = 0.005$), but not *C. drummondii*, *J. osteosperma* or *R. glabra* (Table S3 available as Supplementary data at *Tree Physiology* Online). With no [CO_2] × water stress treatment interaction effect on photosynthetic rates for any of the woody plant species (Table S3 available as Supplementary data at *Tree Physiology* Online).

Intrinsic water-use efficiency (iWUE $= A/g_s$) during the sampling period was higher for all species with elevated [CO_2] than the ambient [CO_2] treatment (Figure 2; Table S3 available as Supplementary data at *Tree Physiology* Online). Water stress improved the iWUE of *J. osteosperma* ($P = 0.004$), but not of *C. drummondii*, *G. triacanthos* or *R. glabra* (Table S3 available as Supplementary data at *Tree Physiology* Online). Elevated [CO_2] and water stress increased iWUE more for *C. drummondii* and *G. triacanthos* (i.e., significant [CO_2] × water stress interaction), while there was no [CO_2] × water stress treatment interaction for *J. osteosperma* and *R. glabra* (Figure 2; Table S3 available as Supplementary data at *Tree Physiology* Online).

Tissue-specific total starch

Total leaf starch increased with elevated [CO_2] for all species (Figure 3). *Cornus drummondii* total leaf starch increased by 90%, *G. triacanthos* total leaf starch increased by 39%, *J. osteosperma* total leaf starch increased by 68% and *R. glabra* total leaf starch increased by 41% (Table S4 available as Supplementary data at *Tree Physiology* Online). Water stress decreased total leaf starch for all woody species (Figure 3). *Cornus drummondii* had a 38% reduction in total leaf starch, *G. triacanthos* total leaf starch reduced by 50%, *J. osteosperma* total leaf starch was reduced by 52%, and *R. glabra* total leaf starch was reduced by 8% (Table S4 available as Supplementary data at *Tree Physiology* Online). There were no treatment

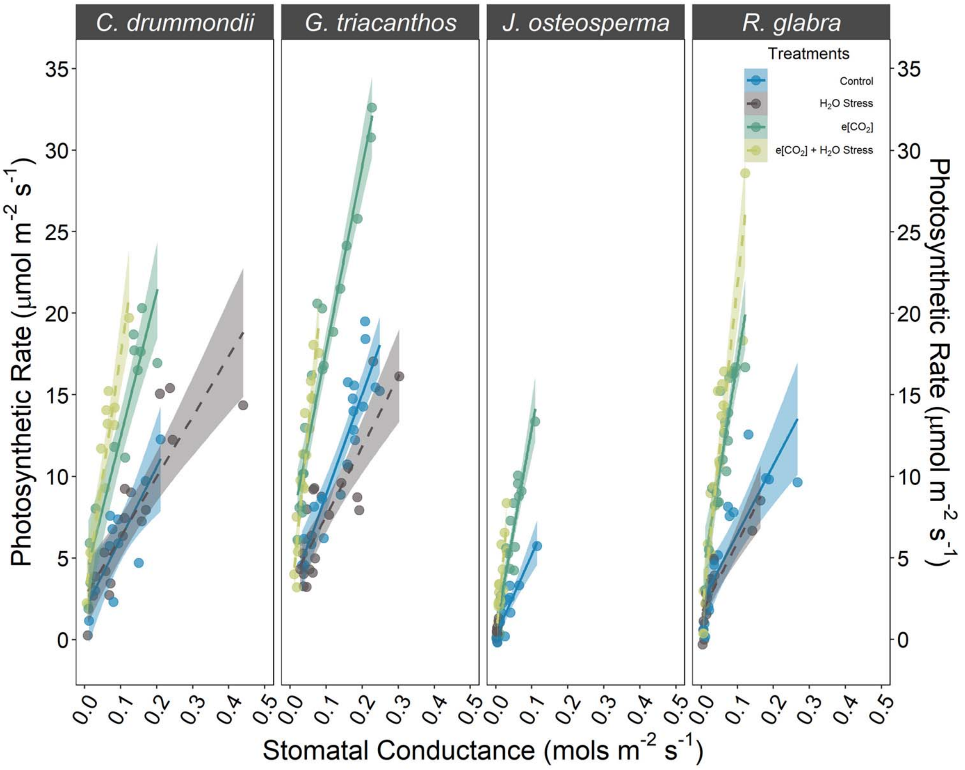

Figure 1. Instantaneous photosynthetic rate and stomatal conductance for each woody plant species (*Cornus drummondii*, *Gleditsia triacanthos*, *Juniperus osteosperma*, *Rhus glabra*). [CO_2] and water stress treatments are represented by different colors and line types (solid = not water stress, dashed = water stressed). The color polygons surrounding the lines of best fit represent 95% confidence intervals.

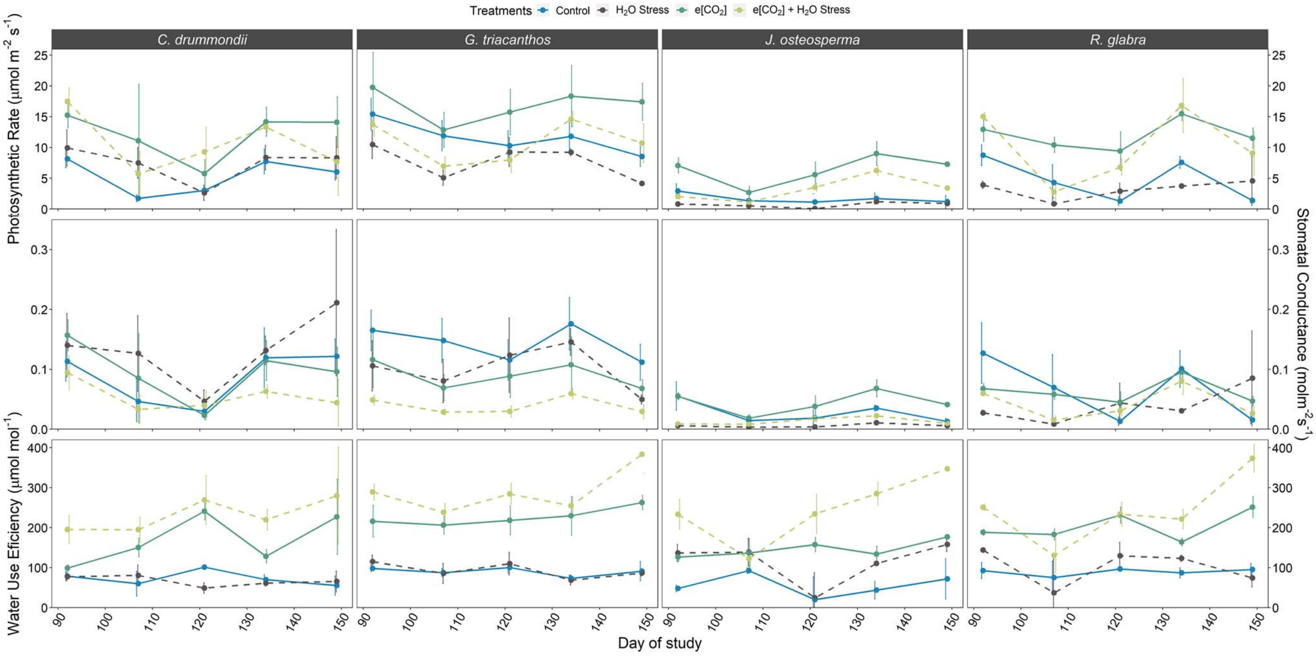

Figure 2. Photosynthetic rates (A_{net}), stomatal conductance (g_s), and iWUE (A_{net}/g_s) results of each species by treatment measured approximately every 2 weeks. Woody plant species (*Cornus drummondii*, *Gleditsia triacanthos*, *Juniperus osteosperma*, *Rhus glabra*) are listed by columns. [CO_2] and water stress treatments are identified by different colors and line type (solid = not water stress, dashed = water stressed). Water stress began on Day 66 in the greenhouses and ended on Day 150. Each point and vertical line is the calculated treatment mean and corresponding standard error.

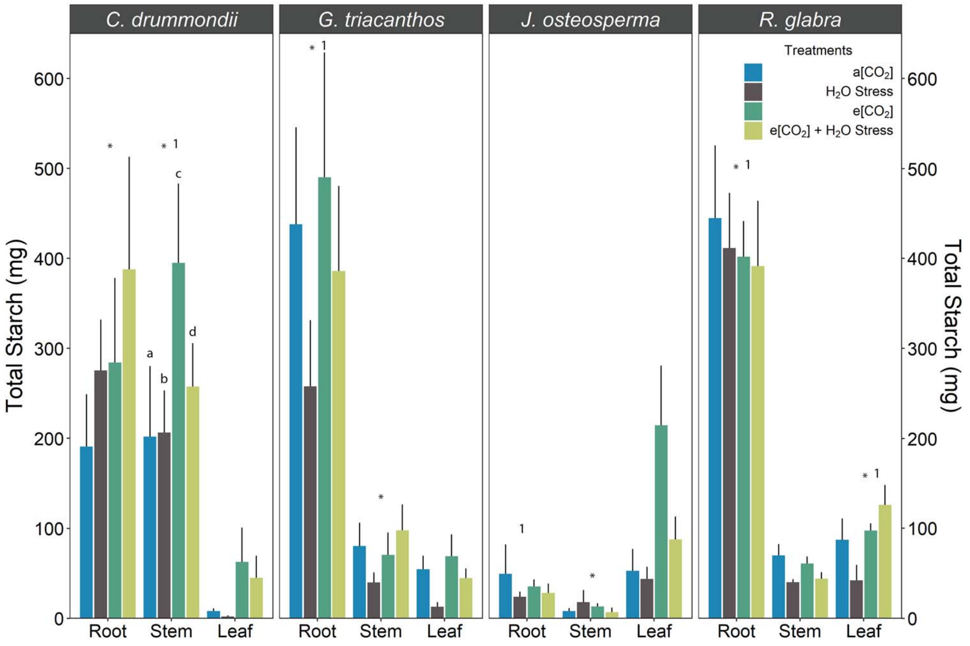

Figure 3. The raw means of total starch content (mg) of each woody plant species and tissue type for the different treatment combinations (±SE). Woody plant species (*Cornus drummondii*, *Gleditsia triacanthos*, *Juniperus osteosperma*, *Rhus glabra*) are separated by column panels, and [CO$_2$] and water stress treatments are differentiated by color. Significance of treatment main effects and interactions are: 1 difference between [CO$_2$] treatment, * difference between water stress treatment, and lower-case letters indicate differences between [CO$_2$] and water stress treatments. Significance was determined at a $P = 0.05$.

interactions between water stress and elevated [CO$_2$] for total leaf starch in any of the woody plants.

Total stem starch increased 38% with elevated [CO$_2$] for *C. drummondii*, all other woody species did not have a significant increase in total stem starch due to elevated [CO$_2$] (Figure 3; Table S4 available as Supplementary data at *Tree Physiology* Online). Water stress reduced total stem starch by 22 and 9% for *C. drummondii* and *G. triacanthos*, respectively, while *J. osteosperma* had an increase in total stem starch by 16% with water stress (Figure 3; Table S4 available as Supplementary data at *Tree Physiology* Online). Only *C. drummondii* had an interaction between the [CO$_2$] and water stress treatments, with elevated [CO$_2$] increasing stem starch more without water stress than with water stress (Table S4 available as Supplementary data at *Tree Physiology* Online).

Effects of [CO$_2$] and water stress on total root starch concentration varied by species. *Gleditsia triacanthos* increased total root starch by 31% with elevated [CO$_2$], while *R. glabra* had a 9% reduction in total root starch with elevated CO$_2$ (Figure 3; Table S4 available as Supplementary data at *Tree Physiology* Online). Water stress increased total root starch in *C. drummondii* by 40% (Table S4 available as Supplementary data at *Tree Physiology* Online), and decreased it in *G. triacanthos*, *J. osteosperma* and *R. glabra* by 29, 39, and 6%, respectively (Table S4 available as Supplementary data at *Tree Physiology* Online). There was no interaction effect between [CO$_2$] and water stress for total root starch for any of the woody plants.

Biomass

Leaf biomass decreased in water stressed plants of all woody species except *J. osteosperma*, and increased in response to [CO$_2$] in *G. triacanthos* and *R. glabra* (Figure 4; Table S5 available as Supplementary data at *Tree Physiology* Online). Stem biomass decreased in *G. triacanthos* and *R. glabra* due to water stress (Figure 4; Table S5 available as Supplementary data at *Tree Physiology* Online). *Cornus drummondii* stem biomass increased with elevated [CO$_2$], primarily in the absence of water stress ([CO$_2$] × water stress interaction; Figure 4; Table S5 available as Supplementary data at *Tree Physiology* Online). Root biomass decreased because of water stress for all woody plants but *J. osteosperma*, and increased with [CO$_2$] for *C. drummondii*, *G. triacanthos* and *R. glabra* (Figure 4; Table S5 available as Supplementary data at *Tree Physiology* Online). Water stress decreased total biomass for all woody species except *J. osteosperma*, while [CO$_2$] increased total biomass for *C. drummondii* and *G. triacanthos* (Figure 4; Table S5 available as Supplementary data at *Tree Physiology* Online). *Rhus glabra* total biomass decreased with [CO$_2$] (Figure 4; Table S5 available as Supplementary data at *Tree Physiology* Online).

Discussion

Between 2005 and 2009 the Natural Resources Conservation Service (NRCS) alone spent roughly \$122 million on woody plant control programs, not including \$2 million spent on

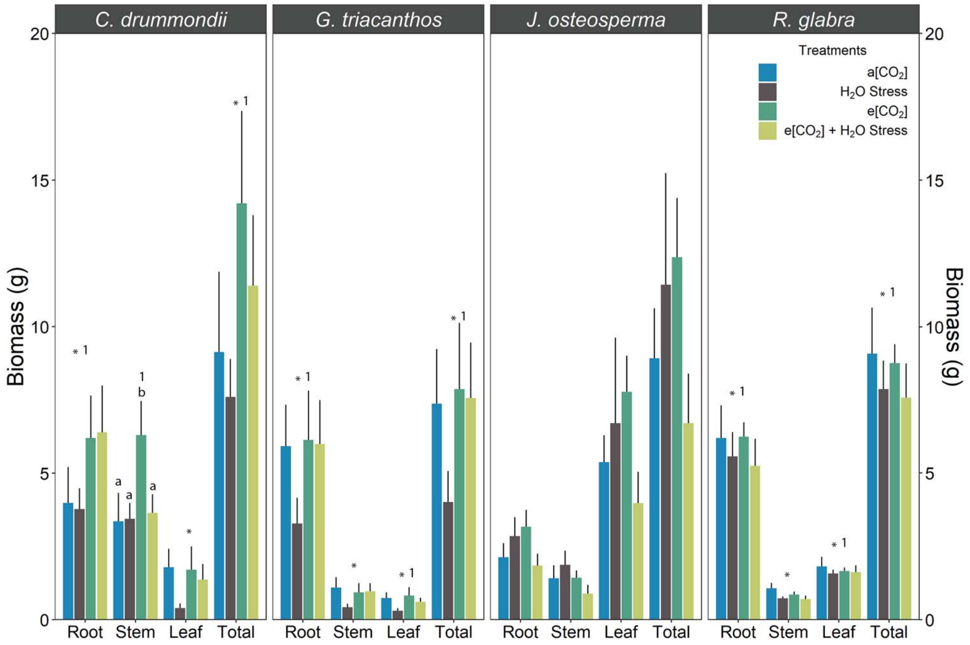

Figure 4. The raw means of biomass (g) of each woody species by tissue type and totaled for each treatment combination (±SE). Woody plant species (*Cornus drummondii*, *Gleditsia triacanthos*, *Juniperus osteosperma*, *Rhus glabra*) are separated by column panels, and CO_2 and water stress treatments differentiated by color. Significance of treatment main effects and interactions are: 1 difference between [CO_2] treatment, * difference between water stress treatment, and lower-case letters indicate differences between [CO_2] and water stress treatments. Significance was determined at a $P = 0.05$.

prescribed fire programs, which are often used to control woody fuels in rangelands (Tanaka et al. 2011). Woody encroachment by native species such as *Juniperus* spp., *Cornus drummondii*, *Gleditsia triachanthos* and *Rhus glabra* have increased their extent on the landscape between 0.1 and 2.3% cover annually across rangelands in North America (Barger et al. 2011). This expansion by native woody plants is not linear and suggests that changes in drivers (i.e., CO_2), filters (recruitment limitation from drought or disturbance) or both have changed (Bond and Midgley 2012, Archer et al. 2017, Case et al. 2020). Our data suggest that juvenile woody plants increase rates of growth as [CO_2] increases, providing them a mechanism to bypass a recruitment filter (water stress). This ability to bypass a recruitment filter is extremely beneficial in disturbance- prone communities (e.g., grasslands and savannas), because rapid growth in height improves chances of escaping flame lengths of fire, which is a filter known as the fire trap (Holdo et al. 2014, Nippert et al. 2021). The mechanisms behind these increased growth rates and biomass were higher rates of photosynthesis, both in absolute terms and per unit of stomatal conductance (increased WUE), which results in increased photosynthates and more total leaf starch.

Elevated [CO_2] and water stress improves stomatal regulation

Our four woody-encroaching plant species (*C. drummondii*, *G. triacanthos*, *J. osteosperma*, *R. glabra*) displayed greater intrinsic WUE (A/g_s) with elevated [CO_2] due to increased photosynthetic rates and water conservation through decreased stomatal conductance (Figures 1 and 2), which agrees with results from other studies and meta-analyses with regards to elevated [CO_2] (Curtis 1996, Curtis and Wang 1998, Polley et al. 2003, Leakey et al. 2009, Kelly et al. 2016). Elevated [CO_2] increases photosynthetic rates in C_3 plants through higher [CO_2], and carboxylation despite reductions in enzymatic kinetics (Hamerlynck et al. 2000, Lambers et al. 2008, Leakey et al. 2009, Kelly et al. 2016). We found that with the water stress and elevated [CO_2] treatment *C. drummondii*, *G. triacanthos* and *R. glabra* all trended towards higher photosynthetic rates with reduced stomatal conductance likely because of an increased gradient between intercellular [CO_2] (C_i) and the elevated [CO_2] of the atmosphere (eC_a) (Figure 1; Figure S2 available as Supplementary data at *Tree Physiology* Online).

Cornus drummondii and *G. triacanthos* were the only species in which elevated [CO_2] increased iWUE more under chronic water stress than under well-watered conditions (Figure 2). Increased iWUE in these two woody species with the elevated [CO_2] and chronic water stress treatment provides strong supporting evidence for the idea that elevated [CO_2] mitigates the deleterious effects of water stress from juvenile woody encroaching species in rangelands (Polley et al. 1996, Morgan et al. 2007). Currently, drought or water stress is one of the limiting factors of woody plants establishment and growth in some rangelands around the world (Barger et al. 2011, Nackley et al. 2018, Case et al. 2020). If juvenile woody plants can overcome this physiological barrier through elevated [CO_2], this could improve both their recruitment and recovery from disturbance.

Tissue-specific starch dynamics

Our results for tissue-specific starch storage with elevated [CO_2] were not as expected based on previous literature. We did not see the resprouting woody plant roots act as a sink for starch when grown under elevated [CO_2] as reported in other studies. Several studies in South Africa and Australia have reported that resprouting woody plants grown at elevated [CO_2] had an increase in starch and other non-structural carbohydrate (NSC) concentrations in root tissues compared with ambient [CO_2] (Bond and Midgley 2000, Kgope et al. 2010, Clarke et al. 2016). These increases in NSC storage in roots can be explained through increased photosynthetic rates, which allow roots to increase their carbon sink potential (Martínez-Vilalta et al. 2016). In our study, we observed increased rates of photosynthesis for all our woody species under elevated [CO_2], but that did not translate to increased total root starch in all of the woody species. There were trends of increased total root starch in *C. drummondii* and *G. triacanthos*, two woody resprouters, while there was no trend for increased total root starch in *R. glabra*, a resprouter or *J. osteosperma*, a non-resprouter. While the expected pattern of increased root starch was not observed, potentially because these were juvenile plants that were still allocating carbon for growth, elevated [CO_2] did increase total leaf starch for all woody species. The increased total leaf starch is likely due to increased photosynthetic rates, and may translate to increased root or stem carbohydrate storage at the end of the growing season or potentially because the root sink was suppressed by water stress.

Total leaf starch was also reduced by water stress. Lower starch concentrations associated with water stress could be a result of a breakdown of starch molecules to a more soluble sugar molecule (i.e., sucrose, fructose, glucose) that would increase the concentration of osmolytes in the tissues to help mitigate the effects of water stress (Graves et al. 1991, Martínez-Vilalta et al. 2016, Pausas et al. 2016). Overall, all our species saw a decrease in total leaf starch due to water stress, but an interesting finding was that *R. glabra* had increased total leaf starch concentrations with the combination of water stress and elevated [CO_2], which could result from an imbalance in carbon source–sink dynamics. *Rhus glabra*'s increase in leaf starch concentration while being water stressed and exposed to elevated [CO_2] might be an example of [CO_2] directly mitigating the effects of water stress.

Water stress influenced the sink dynamics for woody tissues more than elevated [CO_2] during the experiment. Water stressed *C. drummondii* plants had higher starch concentrations in root tissue and *G. triacanthos* had higher stem starch concentrations compared with the unstressed individuals (Figure 4), which could be a mechanism for the plant to prepare for new leaf and shoot growth when soil water conditions improve (Clarke et al. 2013, Pausas et al. 2016, Klimešová et al. 2018). In contrast, water- stressed *R. glabra* plants decreased the starch concentration in their stems, which has been reported in other woody plants in drought studies (Anderegg and Anderegg 2013, Rosas et al. 2013, Maguire and Kobe 2015).

Few effects of water stress and [CO_2] on biomass

Elevated [CO_2] caused an increase in total and tissue-specific biomass (roots, stems, leaves) for the resprouting woody species (Figures 4 and 5). We did observe a [CO_2] effect with increased root biomass for the resprouting species *C. drummondii*, *G. triacanthos* and *R. glabra*. The most notable increase was in root biomass for *C. drummondii* and *G. triacanthos*. This pattern of increased total biomass but especially root biomass with elevated CO_2 is what is expected and has been reported by other studies investigating resprouting woody species (Polley et al. 1999, Kgope et al. 2010, Souza et al. 2019). Interestingly, both *R. glabra* and *J. osteosperma* biomass did not respond as expected to elevated [CO_2]. A study in Australia found mixed results with total biomass between multiple resprouting and non-resprouting species (Clarke et al. 2016). The researchers found that some non-resprouting woody plants had increased total biomass with elevated [CO_2], while the resprouting species did not have an increase in total biomass (Clarke et al. 2016). However, Clarke et al. did find that the resprouting woody species allocated more biomass belowground. Another study from Brazil found that it took more than 1 year for juvenile woody plants to show increased biomass accumulation due to elevated [CO_2] (Souza et al. 2019).

Our study observed the typical drought response of reduced total and tissue-specific (roots, stems, leaves) biomass, but only for the three resprouting woody species—*C. drummondii*, *G. triacanthos* and *R. glabra* (Figures 4 and 5). This response of decreased biomass is a consistent pattern across many ecosystems (Maguire and Kobe 2015, Nackley et al. 2018, Souza et al. 2019). What is interesting is the lack of response from *J. osteosperma* biomass—total or tissue-specific—with regards to chronic water-stress. Potentially, *J. osteosperma*'s lack of biomass reduction is because it is a slow-growing drought-adapted species (Linton et al. 1998, Anderegg and Anderegg 2013), and the duration of the experiment was not long enough to capture the pattern. Overall, the increased biomass response of *C. drummondii* and *G. triacanthos* to elevated [CO_2] confirms that CO_2 helps these two woody species with or without water stress. Even more remarkable is the fact that elevated [CO_2] ameliorated the reduction of biomass during water stress in *G. triacanthos*.

Conclusion

We tested how four different woody plant species that are encroaching on rangelands of the USA were affected by elevated

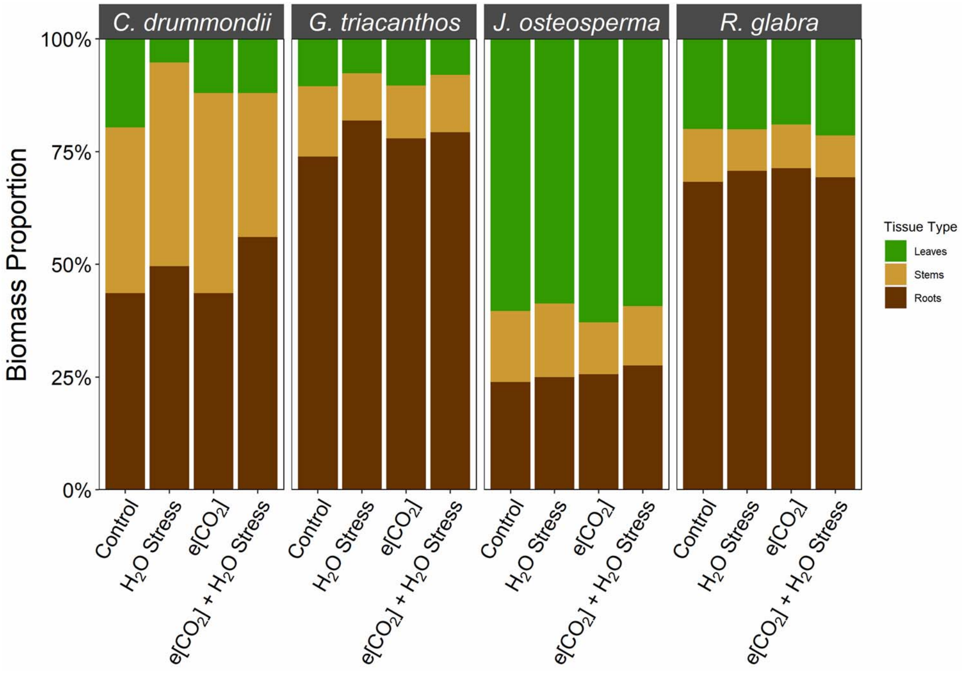

Figure 5. Tissue specific biomass proportion for each of the woody species (*Cornus drummondii*, *Gleditsia triacanthos*, *Juniperus osteosperma*, *Rhus glabra*) based on the experimental treatments.

[CO_2] and chronic water stress in their native soils. Our results showed that these woody plants have the potential for large increases in photosynthetic rates and iWUE with elevated [CO_2], which translate into increases in starch at the leaf level, and in some cases, at the whole-plant level. However, the mixed effect of [CO_2] on biomass in our experiment demonstrates the need to understand each species and that generalizations often might lead to misleading conclusions about elevated [CO_2] impacts on specific species. Moreover [CO_2] × water stress interactions for iWUE demonstrated that, for some species, the strongest benefits of [CO_2] might be present under dry conditions. Even though our plants were grown in a non-competitive environment the physiological responses could be moderated based on competition with C_3 or C_4 grasses present in their respective grassland matrices (Manea and Leishman 2019, Raubenheimer and Ripley 2022). Regardless, each of these reported responses to elevated [CO_2] should mitigate the negative effects of chronic water stress and may allow populations of these woody plants to persist through escaping the fire trap, for the resprouting species, and expand in their respective rangelands in the future if active management is not implemented.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Data availability statement

All data used in this manuscript is available. DOI: 10.6073/pasta/4f9d285275ba5458fee8161d7132341d

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Conflict of interest

None declared.

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Declarations

Mention of a proprietary product does not constitute a guarantee or warranty of the product by USDA, Colorado State University, Kansas State University, or the authors and does not imply its approval to the exclusion of other products. The USDA is an equal opportunity provider and employer.

Authors' contributions

R.C.O. conceived, designed and executed this study, did the statistical analyses, and wrote the manuscript. D.M.B., T.W.O., and J.B.N. helped in experimental design, writing, and statistical advise.

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