







Lower grass stomatal conductance under elevated CO₂ can decrease transpiration and evapotranspiration rates despite carbon fertilization

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Funding information

This work was supported in part by a research grant from Science Foundation Ireland (SFI) under Grant Numbers ICRA-G-SFI 13/RC/2092 and 13/RC/2092_P2 and is co-funded by iCRA-G industry partners. This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement 840799.

Abstract

Anthropogenic increase in carbon dioxide (CO₂) affects plant physiology. Plant responses to elevated CO₂ typically include: (1) enhanced photosynthesis and increased primary productivity due to carbon fertilization and (2) suppression of leaf transpiration due to CO₂-driven decrease in stomatal conductance. The combined effect of these responses on the total plant transpiration and on evapotranspiration (ET) has a wide range of implications on local, regional, and global hydrological cycles, and thus needs to be better understood. Here, we investigated the net effect of CO₂-driven perennial ryegrass (*Lolium perenne*) physiological responses on transpiration and evapotranspiration by integrating physiological and hydrological (water budget) methods, under a controlled environment. Measurements of the net photosynthetic rate, stomatal conductance, transpiration rate, leaf mass per area, above-ground biomass, and water balance components were recorded. Measured variables under elevated CO₂ were compared with those of ambient CO₂. As expected, our results show that elevated CO₂ significantly decreases whole-plant transpiration rates (38% lower in the final week) which is a result of lower stomatal conductance (57% lower in the final week) despite a slight increase in aboveground biomass. Additionally, there was an overall decline in evapotranspiration (ET) under elevated CO₂, indicating the impact of CO₂-mediated suppression of transpiration on the overall water balance. Although studies with larger sample sizes are needed for more robust conclusions, our findings have significant implications for global environmental change. Reductions in ET from ryegrass-dominated grasslands and pastures could increase soil moisture and groundwater recharge, potentially leading to increased surface runoff and flooding.

KEYWORDS

biomass, climate change, controlled chamber experiments, elevated CO₂, evapotranspiration, perennial ryegrass

Sate Ahmad, Charilaos Yiotis, and Weimu Xu, equal contributions.

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INTRODUCTION

Plants play a major role in the hydrological cycle as global transpiration (T) accounts for 61% of terrestrial evapotranspiration (ET), with the ratio of T to ET ranging from 51% in dryland ecosystems to 70% in tropical rainforests (Schlesinger & Jasechko, 2014). Through the exchange of water, carbon, and energy, plants are closely coupled with their surrounding atmosphere (Zhan et al., 2022). As such, changes in climatic conditions and atmospheric composition can directly affect plant functioning, which in turn can cause changes in the ambient environment (Poorter et al., 2022). Although plant responses to elevated CO₂ vary, typically elevated CO₂ alters plant physiology and development in two primary ways: (1) enhanced photosynthesis and increased primary productivity due to carbon fertilization and (2) suppression of leaf transpiration rate as a result of an elevated CO₂-driven decrease in stomatal conductance (Lockwood, 1995) and density (McElwain & Chaloner, 1995; Woodward, 1987). Both these processes can directly or indirectly alter the hydrological cycle (Steinthorsdottir et al., 2012), including the water balance at different spatial and temporal scales (Gedney et al., 2006).

Water use efficiency (WUE), which expresses the trade-off between carbon assimilation and water used or released as transpiration (Fatichi et al., 2022), has been observed to increase over the last decades as a result of increasing CO₂ (Soh et al., 2019; Zhang et al., 2022), although some (Li et al., 2023) have argued that the increase has stalled since the year 2001. An increase in WUE can be either caused by the carbon fertilization effect, which is the increase in photosynthesis and vegetation productivity driven by increasing atmospheric CO₂ concentration (Wang et al., 2020) or by decreased transpiration rates caused by CO₂-mediated stomatal suppression or a combination of both. Gains in WUE in response to rising CO₂ have been found to depend on plant traits (Mndela et al., 2022), leaf habit and biome type (Soh et al., 2019). Globally the CO₂ fertilization effect has dominated the WUE trend over semi-arid regions, while the WUE trend in tropical forests was driven primarily by transpiration suppression (Zhang et al., 2022). However, earlier studies found no evidence of a carbon fertilization effect in terms of tree growth despite a substantial increase in WUE (Peñuelas et al., 2011; van der Sleen et al., 2015), indicating a strong effect of transpiration suppression. A review (Walker et al., 2021) synthesizing a range of evidence for CO₂-fertilization concludes that while there is high confidence that global primary productivity and WUE have increased during the historical period, there is low to medium confidence in the magnitude and low confidence in how much such changes are attributable to increasing CO₂ due to the complex nature of the problem.

Higher productivity resulting in higher biomass accumulation increases plant water demand and thus transpiration (Briggs & Shantz, 1913; Yi & Yano, 2023) and can also lead to an increase in ecosystem evapotranspiration (Ahmad et al., 2021). However, if increased biomass results in a larger canopy or a higher leaf area, on the one hand, it may increase rainfall interception (Aston, 1979) and thus interception evaporation, while on the other, it may reduce soil evaporation as higher Leaf Area Index (LAI) limits light transmission (Ahmad

et al., 2020; Seo & Kim, 2021). Therefore, carbon fertilization can have both negative and positive effects on ecosystem evapotranspiration and overall catchment hydrology. As carbon fertilization is constrained by several factors such as soil nutrient availability (Lenka & Lal, 2012) and terrestrial water storage (Humphrey et al., 2018), over recent decades there has been a global decline in the contribution of carbon fertilization to plant photosynthesis (Wang et al., 2020). Such a decline is likely to increase the relative importance of how total evapotranspiration is affected via CO₂-mediated transpiration effects. If transpiration suppression results in an overall decrease in evapotranspiration, it can increase surface runoff along with associated streamflow and flooding risk (Gedney et al., 2006; Yang et al., 2019). While it is widely agreed that increased CO₂ usually causes a decrease in stomatal conductance (transpiration suppression), few studies (Purcell et al., 2018; Urban et al., 2017) have found a positive effect of elevated CO₂ on stomatal conductance under certain environmental conditions thereby challenging this 'over-simplistic notion' (Purcell et al., 2018, p.1137). Furthermore, studies of CO₂ effects on temperate grasslands, ecosystems that are responsible for storing large quantities of carbon (up to 8 t C ha⁻¹ yr⁻¹, Jones & Donnelly, 2004), are limited. Studies carried out on temperate grasslands have been mostly based on free air CO₂ enrichment (FACE) experiments, the vast majority of which have not included night-time CO₂ enrichment due to high costs, which can potentially lead to research artifacts (Allen et al., 2020; Baker et al., 2022). Hence, further studies are required to understand how the effect of CO₂ on plant transpiration may translate into changes to total evapotranspiration at a larger scale. This can be achieved with controlled environment experiments on model plant species (e.g., perennial ryegrass) that are designed to incorporate both plant physiological and ecohydrological approaches.

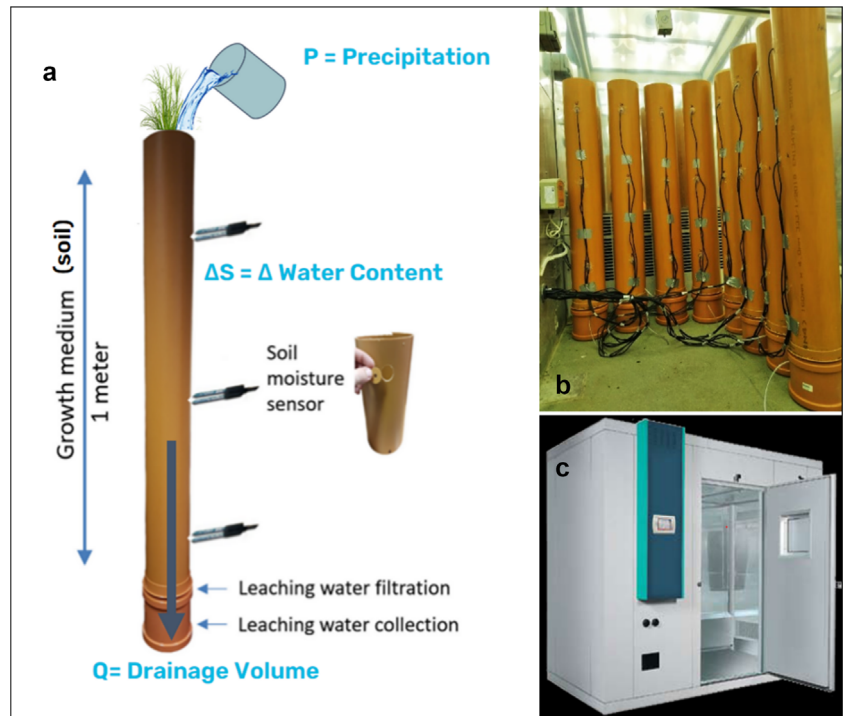
Here, we investigate the effect of elevated CO₂ on perennial ryegrass (*Lolium perenne*), a dominant species of temperate grassland ecosystems (Blackmore et al., 2015) and pastures as well as a commercially important forage crop, in terms of (1) leaf-area based and whole-plant transpiration, (2) actual evapotranspiration, and (3) above-ground biomass accumulation by comparing plants grown under ambient CO₂ levels (400 ppm) with those grown under elevated CO₂ levels (800 ppm) in controlled environment chambers. Our work advances the current understanding of the net effect of CO₂-driven plant physiological responses on transpiration and evapotranspiration by integrating physiological and hydrological methods (water budget), under a controlled environment (chamber experiments).

MATERIAL AND METHODS

Controlled-environment experiment

The experiment was carried out at the Experimental Atmospheres and Climate (PEAC) lab at University College Dublin (UCD) in 2018. The experiment, which was carried out for 18 weeks, included a total of 12 tube pots (1.1 m long, Figure 1a) with 2 treatments and 6 replicates per treatment growing in 4 walk-in Conviron BDW40 controlled-

FIGURE 1 (a) Tube-pot (column) setup along with hydrological variables measured. (b) Tube pots instrumented with soil moisture sensors. (c) Conviron BDW40 growth chamber.



environment chambers (Conviron, Winnipeg, MB, Canada). Three replicates per treatment were placed in one chamber to eliminate any potential chamber effect (Porter et al., 2015). The chambers control light temperature, humidity, CO_2 , and O_2 within 5% of the corresponding set points. Perennial ryegrass (*L. perenne* ‘Boyne’), a characteristic temperate grassland species (C3 grass), was grown under a 16 h/8 h simulated day/night program following Yiotis et al. (2021): 05:00–06:00 h, dawn; 06:00–09:00 h, light intensity progressively rising from $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ to $600 \mu\text{mol m}^{-2} \text{s}^{-1}$; 09:00–17:00 h, midday light intensity at the canopy level of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$; 17:00–20:00 h, light intensity decreases from $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ to $300 \mu\text{mol m}^{-2} \text{s}^{-1}$; 20:00–21:00 h, dusk. CO_2 concentration in two of the chambers was set at 400 ppm (ambient, labeled as ASP), while the other two were running at 800 ppm of CO_2 (elevated, labeled as CSP), which is the projected atmospheric concentration for 2074–75 (shared socioeconomic pathway [SSP] 5–8.5) and 2091–92 (SSP3–7.0), according to Meinshausen et al. (2020). However, since one of the CSP treatment plants was visibly unhealthy and did not grow properly, the sample size of the CSP (treatment), had to be reduced to five. The maximum day- and minimum night temperatures used in the chambers were 23°C and 18°C , respectively, with the relative humidity in all chambers controlled throughout the simulated day/night cycle at 65%. The cultivar Boyne was selected based on its high CO_2 fertilization response reported by Yiotis et al. (2021).

The lower end of each pot was covered with Terram T1000 Geotextile (pore diameter = $75 \mu\text{m}$), which only allowed excess water to leak out of the pot. Overlying the geotextile, a 5 cm-thick layer of quartz gravel was added to prevent clogging of the geotextile. Buckets were placed underneath the pots to collect the leachate and were covered to prevent evaporation loss. Drums containing filtered water

were placed in the chambers. Small air pumps were used to promote the mixing of CO_2 with water, which was then used to water the pots.

A sandy topsoil mix (20% quartzite-based sand) was used as a growth medium without additional feeding throughout the experiment in order to simulate unmanaged grassland conditions. Two harvest cuttings were performed just above the base of the plants (to allow regeneration) on weeks 12 and 18 since the initiation of the experiment. Immediately following the first harvest, an addition of 8 g of fresh grass clippings were added as mulch into the respective pots, where they were harvested.

Hydrological variables and weekly ET calculation

Leachate and drainage volume

The pots received a relatively high hydraulic loading in the first few weeks (until week 5) before the soil moisture content reached equilibrium. Afterward, 500 ml (water input/precipitation) was added to each pot directly on the top of the soil, twice a week for the rest of the experiment. Every Monday, prior to the water addition, leachate accumulated in the collection buckets during the previous week was pumped into a graduated cylinder and their volumes were recorded. Precipitation amounts and drainage volume were aggregated at the weekly timescale.

Calculation of volumetric water content

Volumetric water content (VWC) was recorded using soil moisture sensors (Decagon EC5 sensors, currently Meter EC5) at three

different depths (10, 45, and 85 cm) at 1-minute intervals. All sensors were calibrated in the growth medium using standard methods as described by the supplier before the columns were packed. Absolute volumetric water content, i.e. the absolute fraction of water volume in a given soil-water mix volume around the sensor, was measured. EC5 sensors have a small volume of influence, approximately .3 L, and have an accuracy of at least $\pm 2\%$ with soil-specific calibration (Decagon Devices, Inc., 2016). While we cannot fully exclude the influence of edge effects in the column, all sensors were positioned in a similar manner, making the results among these sensors comparable. However, in several columns, disruption with soil moisture sensors was detected after only a few weeks, thereby reducing the number of columns with complete sets of accurate soil moisture data across the full monitoring period down to four: two with plants (and soil) under ambient CO_2 and two under elevated CO_2 .

To derive weekly changes in VWC (ΔS), the VWC values at midnight of the first day of each week for all depths were used (see Supplementary Information: S11: Figure A). The VWC at the surface and at the bottom of the column were assumed to be the same as that of 10 cm and at 85 cm, respectively. ΔS for each depth and for each week was calculated and interpolated using natural cubic splines for the entire soil column. An example of column A4SP1 (chamber 4, ambient) and week 14 is shown in Figure 2.

Area under curve was calculated using R with the help of the AUC() function from the DescTools package (Signorell et al., 2022) with "spline" as the method, which uses cubic spline interpolation. This AUC value (in percentage) was then multiplied by the volume of the soil to get the ΔS in cm^3 for the total soil column at the end of each week (Figure 3).

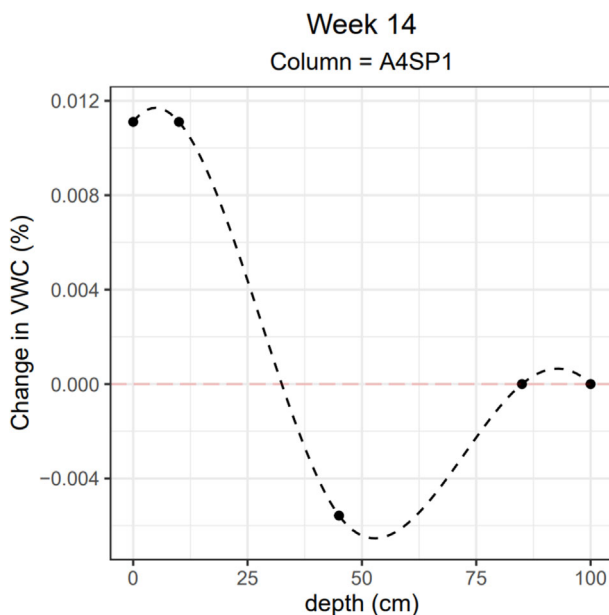


FIGURE 2 Variation in VWC (ΔS) according to depth over week 14 (column A4SP1).

Calculation of actual evapotranspiration

Weekly evapotranspiration (ET) from each column was quantified based on the water balance approach (equation 1). In general, the water balance of an ecosystem can be divided into (1) precipitation, (2) surface runoff, (3) recharge to groundwater, and (4) evapotranspiration, with the balance equating to the change in water content of the soil – see Equation 1.

$$P = ET + Q \pm \Delta S + \text{SRO} \quad (1)$$

where ET = evapotranspiration, P = precipitation or water added, Q = discharge, and SRO = surface runoff.

As there is no surface runoff in the experiment, the water balance equation for each column across each week is expressed by:

$$ET = P - Q \pm \Delta S \quad (2)$$

As there is no surface runoff in the experiment, it is not included in the water balance equation. The ET values which are in weekly rates, were then divided by 7 to get a week-averaged daily ET rate. As with all water balance approaches, the key assumption is that water bound into the plant, is negligible, compared to the values of other water balance components.

Plant properties

Aboveground dry biomass, leaf mass per area, aboveground leaf mass fraction, and total leaf area.

The effect of carbon fertilization can be measured in several ways and can manifest as higher photosynthetic rate, higher leaf mass per unit area, or higher accumulation of dry biomass. Following the two harvests of all aboveground biomass on weeks 12 and 18, the fresh

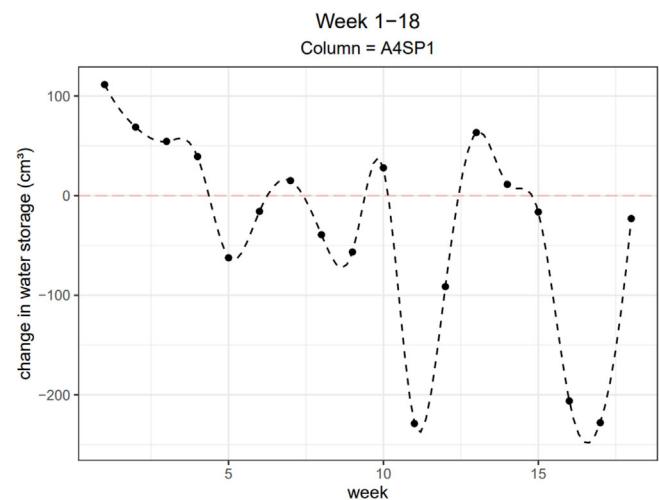


FIGURE 3 Change in water storage over time for the full length of the soil column (A4SP1).



plant material was put in separate, labeled paper sampling bags and was dried in a Binder FED 720 drying oven (Binder GmbH, Tuttlingen, Germany) at 70 °C for 3 days. After the samples were dried, the aboveground dry biomass (DW_{ab}) was measured with a PG5002-S balance (Mettler Toledo, Columbus, OH, USA). Three fully developed, mature tillers were sampled from each plant for the estimation of the aboveground leaf dry mass fraction (LMF_{ab} , i.e., the tiller-based leaf dry mass/DW ratio), and the leaf mass per area (LMA). The three tillers were first defoliated, and their leaf area was measured with an AM300 Leaf Area Meter (ADC BioScientific Ltd, Hertfordshire, UK). The leaves and stems from each plant were then put in separate paper envelopes, dried, and weighed. Based on the tiller-based values of leaf area (LA_t) and leaves' (DW_{l-t}) and stems' (DW_{s-t}) dry weight, we calculated the LMF_{ab} and the LMA of the study plants as:

$$LMA = DW_{l-t} / LA_t \quad (3)$$

where LMA is in $g\ m^{-2}$, DW_{l-t} in g and LA_t in m^2 and,

$$LMF_{ab} = DW_{l-t} / (DW_{l-t} + DW_{s-t}) \quad (4)$$

where LMF_{ab} is in $g\ g^{-1}$ and DW_{s-t} in g.

Subsequently, the total fresh leaf area (LA_{total}) of each study plant was estimated as:

$$LA_{total} = DW_{ab} \times LMF_{ab} \times LMA^{-1} \quad (5)$$

where LA_{total} is in m^2 and DW_{ab} in g.

Stomatal conductance, rate of transpiration, and whole-plant transpiration rate

On weeks 12 and 18, just prior to the first and second harvest, respectively, spot measurements of net photosynthesis (A_{op}), stomatal conductance (g_{sop}), and the transpiration rate (T_{op}) were performed in the chambers on three fully expanded, third leaf blades from the top of three tillers per individual plant. The measurements were taken between 09:00 h and 12:00 h with a CIRAS-2 gas analyzer (PP-Systems, Amesbury, MA, USA) attached to PLC6 (U) cuvette fitted with a 1.7-cm² measurement window and a red/white light LED unit. During the measurements, light intensity ($600\ \mu\text{mol}\ m^{-2}\ s^{-1}$), cuvette temperature (23 °C), cuvette humidity (65%), and reference CO₂ (400 or 800 $\mu\text{mol}\ \text{mol}^{-1}$ for the ASP and CSP treatment plants, respectively) were controlled at values virtually identical to those experienced by the plants in the chambers. Light saturation of perennial ryegrass photosynthesis requires intensities typically unattainable in growth chambers, especially considering that the blades are not orientated vertically to the direction of light. The maximum light intensity attainable in the BDW40 chambers is in the range of 1,000 $\mu\text{mol}\ m^{-2}\ s^{-1}$. However, setting the light intensity close to this value considerably increases the thermal load in the chambers and stretches their ability to control temperature. Thus, we set the light intensity to 600 μmol

$m^{-2}\ s^{-1}$, which is a standard midday light intensity used in many previous chamber studies (e.g. Yiotis et al., 2021; Yiotis & McElwain, 2019).

The flow rate during the measurements was set at 200 $\text{cm}^3\ \text{min}^{-1}$ and the average vapor pressure deficit with the blades clamped in the cuvette was $.96 \pm .11$ kPa. The ryegrass blades only partially covered the area of the measurement window, thus, upon clamping them in the cuvette, the light unit was removed and photographs of them were taken. Based on these photographs, the actual measured leaf areas were calculated using the ImageJ software (Schneider et al., 2012). Finally, the estimated areas were used to recalculate the original data. Since the cuvette conditions closely mimicked those of the chambers, the CO₂ and H₂O concentration differences between the cuvette and the surrounding atmosphere were minimal and no post-measurement corrections for leaks were needed. Nevertheless, we regularly ensured that the difference between the reference CO₂ concentration of the analyzer and the CO₂ concentration of the empty and closed cuvette was ~zero. Although the preparation for each spot measurement (i.e., gas analyzer positioning, blade clamping, and photographing) was fairly quick at 2–3 minutes, care was taken so that there was no build-up of CO₂ in the chambers during the measurements, which could lead to a dynamic partial closure of stomata. To this aim, the measurements were taken from a laptop connected to the gas analyzer and placed outside the chambers. Further, when in the chambers, the personnel performing the measurements always exhaled through the small openings on the chamber walls used for the cable connection of the gas analyzer to the laptop. Upon clamping each blade, stabilization of the A_{op} , g_{sop} , and T_{op} values typically took 1–2 minutes, upon which point no signs of adjustments to cuvette conditions were observed.

Assuming that all leaves of each individual plant receive an irradiance roughly equal to that measured at the canopy level and transpire at a rate equal to the average rate previously determined through measurements on three separate leaves of the same plant (see also Yiotis et al., 2021 for another example of this approach), the measured values of T_{op} and the calculated values of LA_{total} were used to approximate the in-situ total rate of transpiration (T_{total}) of each study plant as:

$$T_{total} = T_{op} \times LA_{total} \quad (6)$$

where T_{total} is in $\text{mmol}\ \text{H}_2\text{O}\ s^{-1}\ \text{plant}^{-1}$ and T_{op} is in $\text{mmol}\ \text{H}_2\text{O}\ m^{-2}\ s^{-1}$.

Statistical approach

All analyses were carried out in RStudio version 2022.2.03 (RStudio Team, 2022). To check for statistical differences between treatment (CSP) and control (ASP), linear mixed effect modeling was implemented utilizing the lme4 package (Bates et al., 2015) with CO₂ as a fixed and chamber as a random factor. It should be noted that while the chamber effect was significant on week 12, on week 18 it was insignificant.

RESULTS

Transpiration rates and stomatal conductance

While T_{total} of plants under elevated CO_2 was not significantly different ($p = .425$) from those under ambient CO_2 on week 12, significantly lower rates ($p = .004$) were observed for plants under elevated CO_2 on week 18 (Figure 4a). Similarly, under elevated CO_2 , T_{op} (i.e. the transpiration rate per unit leaf area) was significantly lower ($p = .003$) on week 18, although not on week 12 (Figure 4b). We need to note, however, that even though the differences on week 12 were not statistically significant, CSP plants still displayed, on average, lower values of T_{op} and T_{total} than the ASP plants. g_{sop} on both weeks 12 and 18 is significantly lower in plants growing under elevated CO_2 compared to control plants (Figure 4c). It is, therefore, apparent that there was a significant effect of elevated CO_2 on g_{sop} leading to a suppression of transpiration.

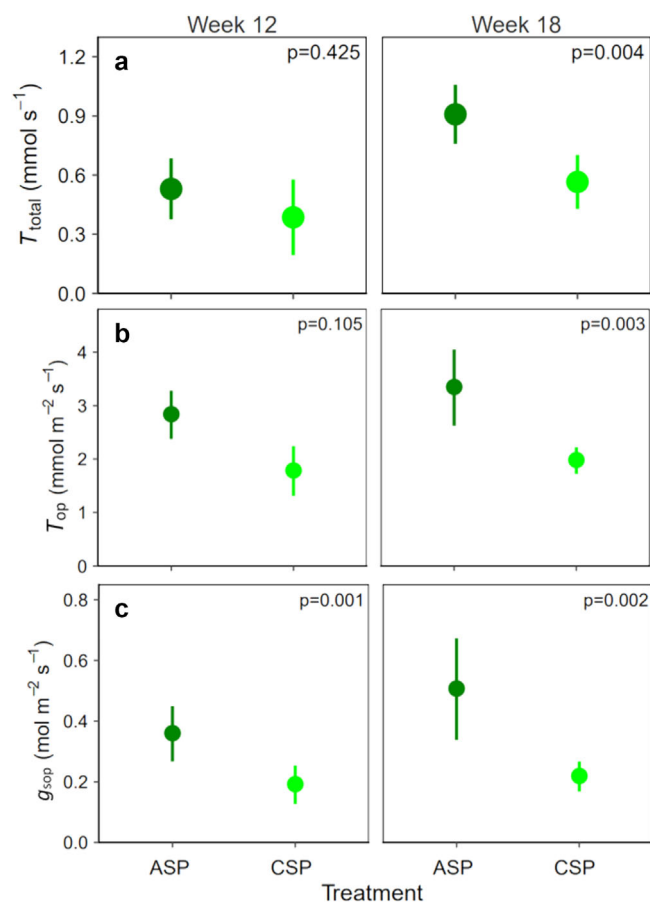


FIGURE 4 (a) Mean whole-plant transpiration rates (T_{total}), (b) mean transpiration rate per unit leaf area (T_{op}) and (c) operational stomatal conductance (g_{sop}) according to treatment and experimental weeks. Error bars indicate standard deviation ($n = 6$ for ASP and $n = 5$ for CSP).

Carbon fertilization effects

Photosynthetic rates on both weeks 12 and 18 show no significant differences between ASP and CSP, indicating no clear enhancement due to higher CO_2 (Figure 5a). This could be due to variations in the rate of photosynthesis under within the treatments. However, although the differences were not statistically significant, CSP plants always showed higher photosynthetic rates, on average.

LMA is not significantly different between the two treatments on week 12, yet, on week 18, plants under elevated CO_2 show significantly higher LMA, though only at 10% significance level ($p = .075$, Figure 5b). Although the mean aboveground biomass is higher under elevated CO_2 compared to accumulation under ambient CO_2 on week 12, the difference is not significant. However, on week 18, aboveground biomass under elevated CO_2 is significantly higher ($p = .0099$) compared to plants under ambient CO_2 (Figure 5c), indicating carbon fertilization effects through higher quantity of carbon assimilation.

Actual evapotranspiration and leachate generation

Over the course of the experimental weeks, all columns show changes in ET. From week 5 to week 12, there is a general trend of increasing ET, after which there is a rapid decrease on weeks 13 and 14, as a result of harvesting aboveground biomass (Figure 6). Afterward, as the plants continue to grow again, there is again a general trend of increasing ET, at a faster rate than at the initial stages of plant development. For a total of 8 weeks (out of the 14 weeks), both columns under elevated CO_2 show lower ET, compared to either of the two columns under ambient CO_2 . Further, cumulative ET (added over the 14 weeks) for columns under elevated CO_2 was lower than for those under ambient CO_2 (Figure 7).

When ET values of all four columns are plotted against the T_{total} values of the corresponding plants for weeks 12 and 18 (Figure 8), the Pearson R^2 shows strong positive correlations for both weeks 12 and 18, ($R^2 = .65$, and $.96$, respectively), although significant only for week 18 ($p = .022$) owing to the small sample size.

Plant growth leads to higher transpiration on a whole-plant basis. As a result, less water should drain out from the bottom of the column (leachate production). A scatterplot of leachate volume versus whole plant transpiration (Figure 9) shows a significant negative correlation ($R^2 = .439$, $p < .001$), between the two variables, indicating that the physiological measurements of in situ T_{op} and our approach of estimating T_{total} based on T_{op} and LA_{total} yields data reflective of water balance components.

DISCUSSION

Evapotranspiration (ET) is an important part of the hydrological cycle at the local, regional, and global scales. Any changes to ET would in turn alter soil moisture and water tables, as well as runoff generation.

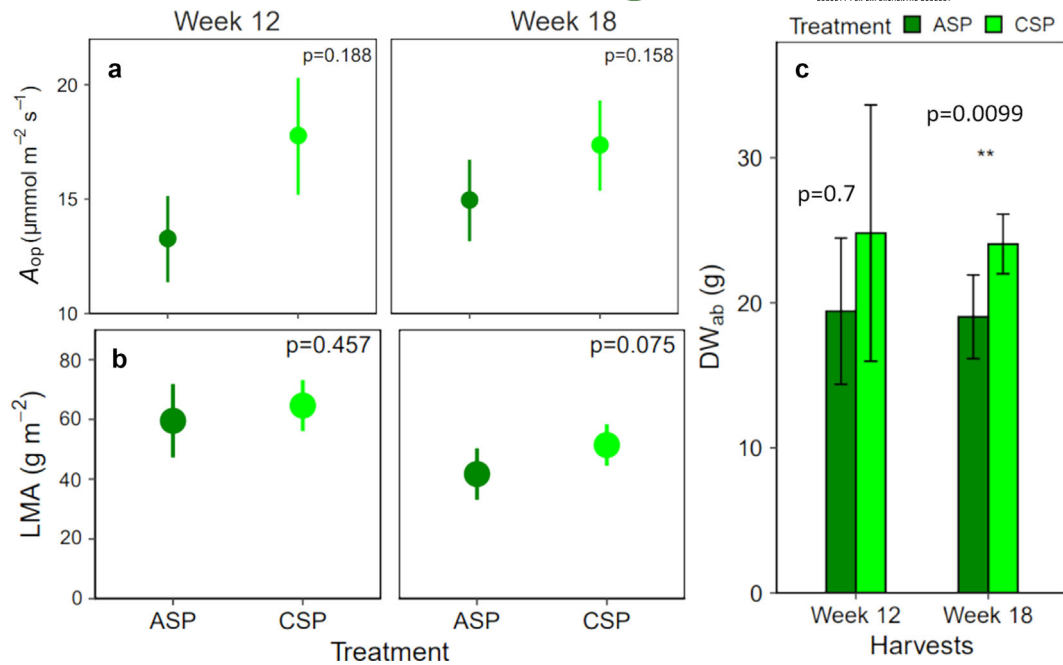
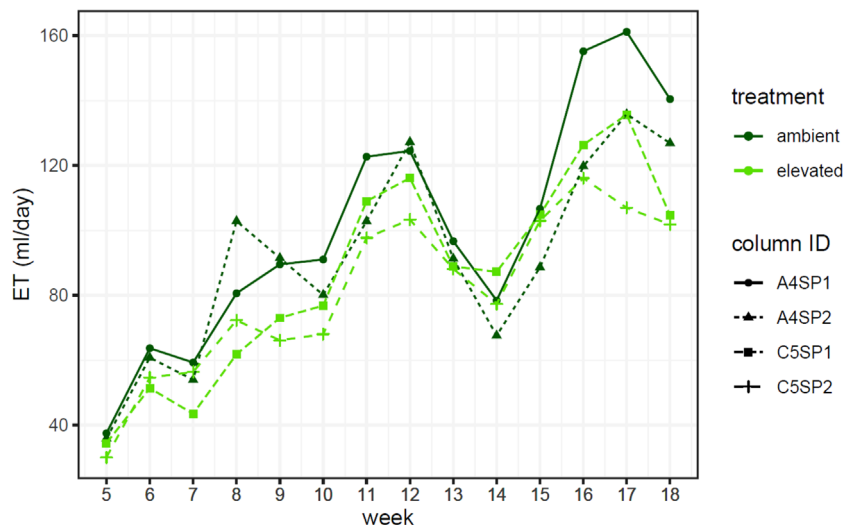


FIGURE 5 (a) In situ rate of net photosynthesis (A_{op} in $\mu\text{mol m}^{-2} \text{s}^{-1}$), (b) leaf mass per unit area (LMA, g m^{-2}) and (c) above ground biomass, dry weight (DW_{ab} in g) under elevated and ambient CO_2 for weeks 12 and 18 ($n = 6$ for ASP and $n = 5$ for CSP). Error bars represent standard deviation.

FIGURE 6 Scatterplot of mean daily ET, estimated on an experimental week basis, under ambient, and elevated CO_2 .



Changes in plant transpiration (T) result in a simultaneous change in total ET in an ecosystem. The magnitude of this effect would strongly depend on what portion of ET is comprised by transpiration.

Our results show a strong positive correlation between T_{total} , determined from plant physiological measurements and actual ET quantified using the water-balance approach on weeks 12 and 18, although the correlation is only significant for week 18 (Figure 8). Lower cumulative values of actual ET (weeks 5–18) are observed under elevated CO_2 , compared to plants under ambient CO_2 . The decline in ET rate is consistent with the results from lysimeter experiments carried out on montane grasslands, which also found that ET

decreases under elevated CO_2 (Vremec et al., 2023). Another study (Bernacchi et al., 2007) carried out on soybean fields, also found lower ET (derived via residual energy balance) under elevated CO_2 as a result of CO_2 -mediated decline in stomatal conductance, despite large increases in photosynthesis rates.

Whole-plant T_{total} as well as per unit area T_{op} rates are also significantly lower on week 18, under elevated CO_2 . Stomatal conductance (g_s) also showed lower values under elevated CO_2 relative to ambient CO_2 . Therefore, under a controlled environment experiment, where only CO_2 concentrations are different between treatments, the decrease in cumulative actual ET appears to be driven by a strong

decrease in whole plant T_{total} rates (38% lower on week 18) which in turn is a result of lower g_{sop} (47% and 57% lower on weeks 12 and 18, respectively), compared to plants under ambient CO_2 levels. Stomatal adjustment is a well-known plant physiological response to elevated CO_2 . Stomata are vital doors that regulate gas exchange between vegetation and the atmosphere, i.e., CO_2 entering from the atmosphere and water vapor exiting from plants into the atmosphere (Woodward, 1987). To adapt to changing environmental conditions, plants need to modulate stomatal development and behavior to optimize CO_2 and water exchange through the leaf epidermis (Lawson et al., 2014). Such stomatal adjustment is usually achieved through changing stomatal density and/or by dynamically regulating stomatal conductance (via guard cells). A higher CO_2 concentration generally causes reductions in stomatal density and/or stomatal opening and therefore leads to reductions in leaf transpiration as well as overall canopy or ecosystem ET (Bernacchi & VanLoocke, 2015; Xu et al., 2016). To address the limitations of a small sample size for the

determination of water balance-based ET ($n = 2/\text{treatment}$), we correlated leachate data with T_{total} data, which showed a significant negative correlation (Figure 9), indicating that the main water balance component required to quantify ET responds to changes in physiological measurements of T . Hence, this corroborates the finding of the near-perfect correlation between ET and T_{op} (Figure 8).

At the weekly scale, ET increases until the first harvest is carried out on week 12, at which point ET decreases again, and then quickly increases at a faster rate than the initial weeks. This is because the rate of plant growth after the first harvest is much more rapid and is thus coupled with an increasing rate of ET (increasing plant water demand). All tiller bases give rise to new growth simultaneously, facilitated by the already developed root system, which can access water from a larger soil volume and a higher depth than initially.

Upon comparing three different indicators of carbon fertilization effects of elevated CO_2 , we found that there are no significant differences in photosynthesis rates on either of the weeks (12 and 18) that

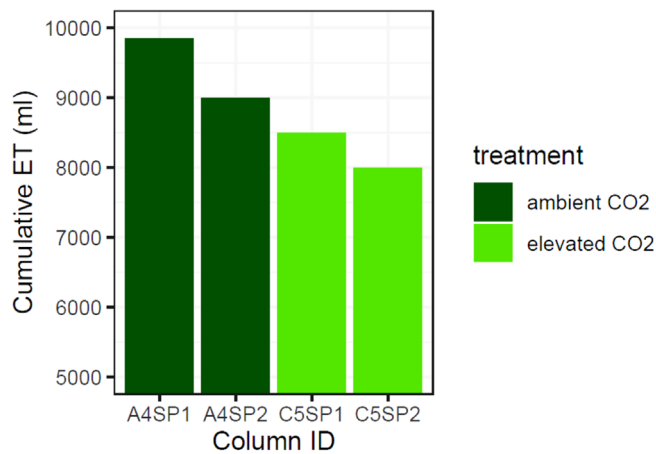


FIGURE 7 Bar chart of cumulative ET (5 to 18 weeks) under ambient and elevated CO_2 .

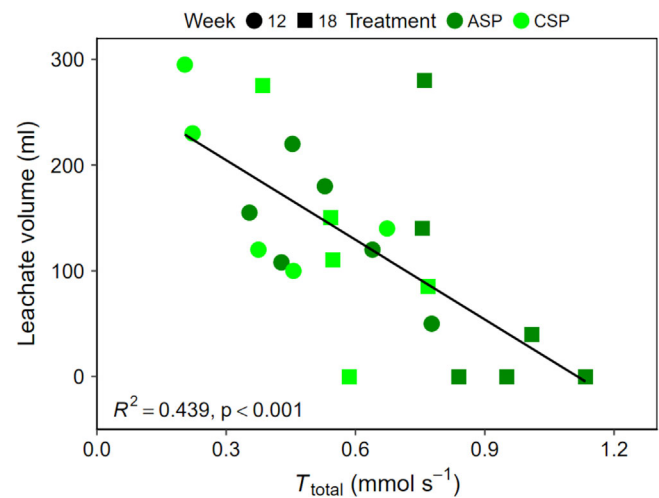


FIGURE 9 Scatterplot of leachate volume versus whole transpiration rates according to weeks and treatments.

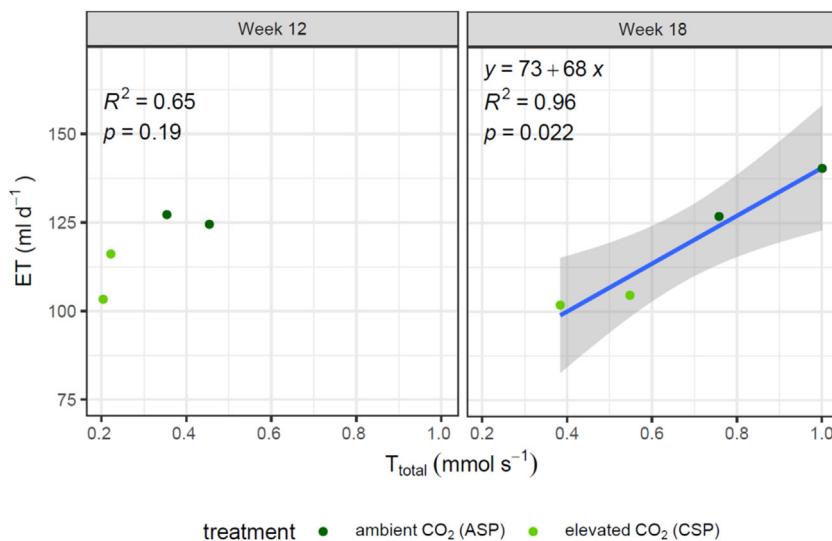


FIGURE 8 Scatterplots of evapotranspiration rates (ET, in ml d^{-1}) versus whole plant transpiration rates (T_{total} mmol s^{-1}) for weeks 12 and 18 are shown in four individual columns (two per treatment).



measurements were carried out. The measurements of photosynthesis reveal only a snapshot of carbon assimilation in action and may not reflect the actual cumulative effect that higher CO₂ may have on overall biomass accumulation. Hence, it is likely that plant structural/morphological parameters may be better indicators of the cumulative effect of carbon fertilization on our plants. Indeed, leaf mass per area (LMA) showed a slight increase in response to higher CO₂, although significant only at a 10% risk level. This is consistent with previous studies which also found that LMA increases under higher CO₂ in a perennial C3 plant (*Polygonum cuspidatum*, Ishizaki et al., 2003) as well as in perennial ryegrass of the same cultivar ('Boyne', Yiotis et al., 2021) used in our study. One explanation why the impact of CO₂ on LMA was marginal rather than stark, is that, in our experiment, in order to mimic unmanaged grasslands, no external inputs of nutrients were added. Nutrient availability strongly influences the effect of elevated CO₂ on plant growth (Ainsworth & Long, 2005, 2021; Jiang et al., 2020; Soussana & Lüscher, 2007; Stitt & Krapp, 1999), and thus under potential nutrient limitation, any maximum enhanced fertilization effect may not have been realized. Furthermore, the chamber effect observed on week 12 (but not week 18) may have played a role in the absence of statistically significant differences for many of the parameters (such as T_{op}, T_{total}, DW_{ab}) recorded on week 12 as well as for the absence of significant correlation between T_{total} and ET for the same week.

In terms of biomass accumulation, we were only able to record the aboveground dry biomass (DW_{ab}), due to the very large, interspersed network of fine roots, which were not possible to separate from the soil for determining belowground biomass accurately. Nevertheless, DW_{ab} was significantly higher under elevated CO₂ compared to the control, indicating a carbon fertilization effect. This is in agreement with previous studies involving both controlled chambers (Yiotis et al., 2021) and FACE experiments (Brinkhoff et al., 2019) which found significant increases in ryegrass dry biomass driven by increased CO₂. Although aboveground biomass (in dry weight) under elevated CO₂ was significantly higher for the final harvest, the potential effect on transpiration demand did not offset the effects of CO₂-mediated transpiration suppression on overall evapotranspiration. This brings to the fore the question of whether similar counteracting effects can be expected in the field or at larger spatial scales (e.g., landscapes and catchments)?

A large-scale modeling study over Eurasia found that between 1982 to 2014, the positive contribution of CO₂-induced increase in LAI to transpiration was offset by the CO₂-induced decreases in transpiration (Zhang et al., 2021). A study on a temperate montane grassland (consisting mostly of C3 grasses) as part of a mini-FACE experiment, found no effect of elevated CO₂ (696 ppm against an ambient CO₂ level of 418 ppm) on LAI, yet found a substantial decreasing effect on stomatal conductance leading to an overall decrease in ecosystem evapotranspiration (Vremec et al., 2023). However, a different modeling study on a different temperate grassland, also utilizing FACE experimental data, found an opposite net effect, i.e. an increased total aboveground dry biomass under elevated CO₂ (~480 ppm), compared to biomass under ambient CO₂ (~395 ppm),

increased overall transpiration rates to such an extent that it counteracted the water saving effects of CO₂-induced stomatal suppression (Kellner et al., 2017). Yet, a different FACE study found that decreased stomatal conductance in soybeans under elevated CO₂ (550 ppm versus 375 ppm) is strongly coupled with an overall decline in ecosystem evapotranspiration despite carbon fertilization effects (Bernacchi et al., 2007). Thus, with such uncertainty of the net effects of elevated CO₂ on transpiration and evapotranspiration, scaling up of key processes from leaf to catchments is critical, and must be integrated with approaches across various disciplines (hydrology, botany, soil science, ecophysiology).

As with all lab experiments, our study has limitations in terms of how closely it can simulate actual field conditions, which FACE is much better at. For example, interception losses have not been quantified, as water was added directly onto the soil surface to ensure that all plants receive the same amount of water. Nevertheless, as mentioned earlier, FACE experimental setups usually do not include nighttime CO₂ enrichment, which is not the case in our experimental setup. Furthermore, with the help of a controlled chamber experiment, we have the advantage of controlling potentially confounding factors such as temperature and humidity, enabling the collection of highly accurate hydrological data for each plant as well as arriving at more robust conclusions. However, we acknowledge that our sample size is very small, especially with regard to the water budget component of the experiment, owing to the malfunctioning of soil moisture sensors. Therefore, future studies should explore the role of roots in disrupting moisture sensors to improve soil moisture measurements in experimental settings.

In conclusion, our findings indicate that elevated CO₂ leads to a reduction in stomatal conductance, resulting in suppressed T rates in perennial ryegrass and thus a reduced rate of total evapotranspiration. The study highlights the complex interplay between carbon fertilization and T suppression in response to elevated CO₂ levels. While carbon fertilization can lead to higher biomass accumulation, increasing transpiration demand, such increased transpiration can be offset by the suppression effect of elevated CO₂ on stomatal conductance. Such was the case in this study, where despite increased dry biomass of ryegrass (an indicator of carbon fertilization effect), there was a net decrease in transpiration leading to reduced overall evapotranspiration. Reduced evapotranspiration can lead to increased water availability, especially in poorly drained soils, which may raise the risk of waterlogging and flooding. Our study underscores the need to consider the combined effects of carbon fertilization and transpiration suppression when assessing the impacts and implications of rising atmospheric CO₂ levels on runoff generation and flooding risks. Future research should incorporate a full factorial design where both carbon dioxide and vapor pressure deficit (combined impact of humidity and temperature) are manipulated, as well as include multiple grassland species. Research in this area is helpful in understanding the direct impact of elevated CO₂ along with climatic impacts on plant physiology and its implications for water management, flood risk, and ecosystem sustainability in the face of ongoing climate change.

AUTHOR CONTRIBUTIONS

SA, CY, and WX contributed equally to the manuscript. **SA:** Formal analysis, Writing—original draft, Writing—reviewing & editing, Visualization, Software, Investigation. **CY:** Conceptualisation, Supervision, Methodology, Formal analysis, Investigation, Data curation, Writing—reviewing & editing, Visualization, Software. **WX:** Conceptualisation, Supervision, Methodology, Formal analysis, Investigation, Data curation, Writing—reviewing & editing, Visualization. **JK:** Formal analysis, Data curation, Visualization, Software, Writing—reviewing & editing, Investigation. **LG:** Conceptualisation, Supervision, Writing—reviewing & editing, Fund acquisition, Project administration. **JM:** Conceptualisation, Supervision, Writing—review & editing, Funding acquisition, Project administration.

ACKNOWLEDGMENTS

The authors would like to thank Bredagh Morah for practical help throughout the experiment and Antonietta Knetge for proofreading the final version of the manuscript. Open access funding provided by IReL.

CONFLICT OF INTEREST STATEMENT

No conflict of interest declared.

DATA AVAILABILITY STATEMENT

Data will be made available on request.

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SUPPORTING INFORMATION

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How to cite this article: Ahmad, S., Yiotis, C., Xu, W., Knappe, J., Gill, L., & McElwain, J. (2024). Lower grass stomatal conductance under elevated CO₂ can decrease transpiration and evapotranspiration rates despite carbon fertilization. *Plant Direct*, 8(10), e70013. <https://doi.org/10.1002/pld3.70013>