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Interactive effects of elevated CO₂, warming, reduced rainfall, and nitrogen on leaf gas exchange in five perennial grassland species

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Abstract

Global changes can interact to affect photosynthesis and thus ecosystem carbon capture, yet few multi-factor field studies exist to examine such interactions. Here, we evaluate leaf gas exchange responses of five perennial grassland species from four functional groups to individual and interactive global changes in an open-air experiment in Minnesota, USA, including elevated CO₂ (eCO₂), warming, reduced rainfall and increased soil nitrogen supply. All four factors influenced leaf net photosynthesis and/or stomatal conductance, but almost all effects were context-dependent, i.e. they differed among species, varied with levels of other treatments and/or depended on environmental conditions. Firstly, the response of photosynthesis to eCO₂ depended on species and nitrogen, became more positive as vapour pressure deficit increased and, for a C₄ grass and a legume, was more positive under reduced rainfall. Secondly, reduced rainfall increased photosynthesis in three functionally distinct species, potentially via acclimation to low soil moisture. Thirdly, warming had positive, neutral or negative effects on photosynthesis depending on species and rainfall. Overall, our results show that interactions among global changes and environmental conditions may complicate predictions based on simple theoretical expectations of main effects, and that the factors and interactions influencing photosynthesis vary among herbaceous species.

KEYWORDS

climate change, CO₂, drought, functional groups, global change, grassland, photosynthesis: carbon reactions, stomata

1 | INTRODUCTION

Terrestrial ecosystems will be simultaneously subjected to rising atmospheric carbon dioxide (CO₂) concentration, warmer temperatures, altered precipitation patterns and increased reactive nitrogen (N) cycling over the next century (Intergovernmental Panel on Climate Change, 2013). While the impacts of these global changes on ecosystems have been relatively well studied in single-factor manipulations (J. Song, Wan, Piao, Hui, et al., 2019; J. Song, Wan, Piao, Knapp, et al., 2019), or assessed using observational data (e.g. Hisano, Chen, Searle, & Reich, 2019) or experimental data across environmental variation (e.g. Hovenden et al., 2019),

multi-factor global change field experiments remain necessary to evaluate potential interactive effects among factors. Such experiments are rare, yet have often shown non-additive responses to global changes for plant biomass (Mueller et al., 2016; Reich & Hobbie, 2013; Shaw et al., 2002), photosynthesis (Albert et al., 2011a, 2011b; Blumenthal et al., 2013) and soil resources (Mueller et al., 2016). Only two field experiments other than that presented herein concurrently manipulate CO₂, temperature, rainfall and N (Shaw et al., 2002; J. Song, Wan, Piao, Hui, et al., 2019; J. Song, Wan, Piao, Knapp, et al., 2019).

Plant carbon (C) capture via photosynthesis and subsequent storage allows terrestrial ecosystems to partially offset atmospheric CO₂

rise, a critical ecosystem service, and is controlled by the very environmental factors that are changing globally, including elevated CO₂ (eCO₂), warming, reduced rainfall and increased soil N supply (our experimental treatments). For context, we introduce the expected main effects of these factors on photosynthesis based on theory and evidence, and then describe how each factor might be influenced by some or all of the others. We do not describe every interaction, but rather only those related to the hypotheses we tested (Figure 1).

1.1 | Main effects of eCO₂, increased N supply, reduced rainfall and elevated temperature on photosynthesis

eCO₂ commonly increases rates of leaf-level photosynthesis because enhanced intercellular CO₂ concentration (C_i) leads to reduced photorespiration and increased rates of carboxylation (Ainsworth & Rogers, 2007;

Drake, González-Meler, & Long, 1997; Poorter, 1993). The strongest responses to eCO₂ are generally observed in C₃ species and the lowest in C₄ species, as C₄ species possess a CO₂-concentrating mechanism that renders them less sensitive to increased atmospheric CO₂ (Ainsworth & Rogers, 2007; Leakey et al., 2009). Exposure to eCO₂ can lead to a \approx 50% instantaneous increase in photosynthesis in C₃ species; however, acclimation of this response generally leads to more modest enhancements of \approx 30% (Ainsworth & Long, 2005; Ainsworth & Rogers, 2007; Nowak, Ellsworth, & Smith, 2004).

eCO₂ can also improve leaf water relations and increase soil moisture, representing another mechanism by which eCO₂ may enhance photosynthesis. Elevated external CO₂ concentration allows plants to gain more C per unit of water lost through stomatal openings, and thus stomatal conductance commonly declines in response to eCO₂ without increasing the stomatal limitation of photosynthesis (Ainsworth & Rogers, 2007; Medlyn et al., 2001; Morgan et al., 2004; Pastore, Lee, Hobbie, & Reich, 2019; Wand, Midgley, Jones, & Curtis, 1999).

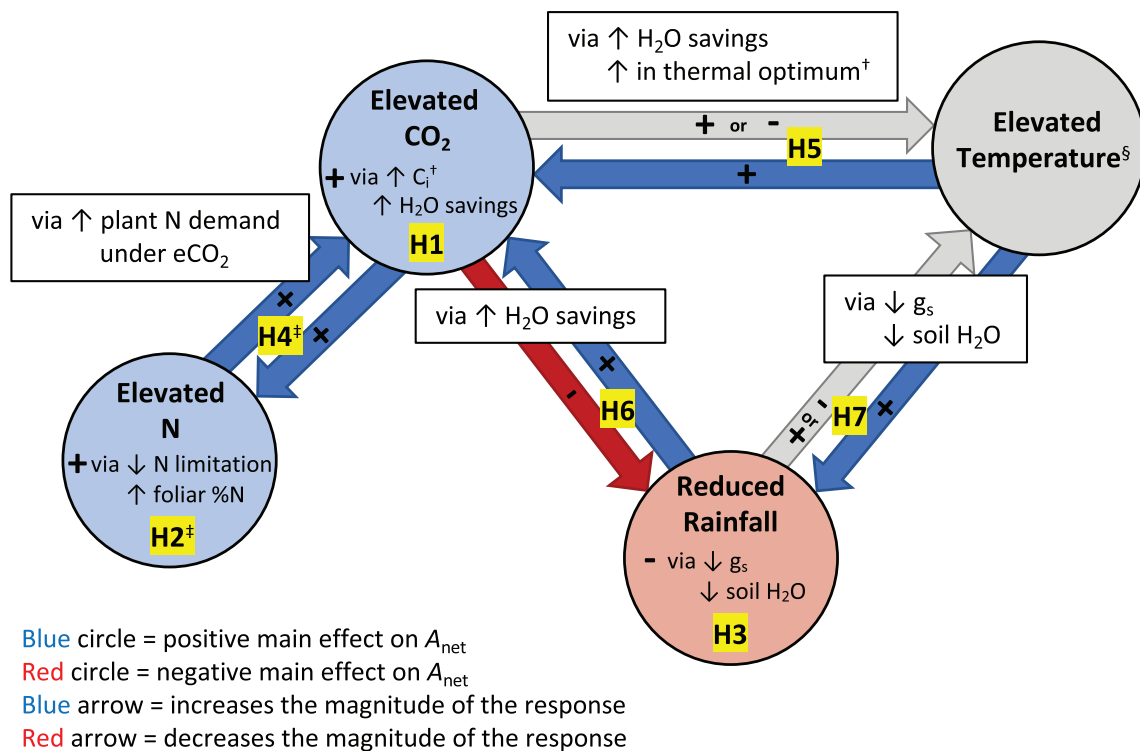


FIGURE 1 Conceptual diagram of main and interactive effects of eCO₂, elevated soil N, reduced rainfall and elevated temperature on leaf net photosynthesis (A_{net}). Hypotheses tested in this study are depicted as “H1”–“H7.” The circles represent hypothesized main effects, with blue colour indicating a positive and red colour a negative main effect of the factor on photosynthesis (H1–3). Hypothesized interactions among factors are depicted using arrows (H4–7); a blue arrow coming from a factor signifies that it will increase the magnitude of the response of photosynthesis to the factor on the “receiving end” (whether negative or positive), while a red arrow coming from a factor signifies that it will decrease the magnitude of the response of photosynthesis to the factor on the receiving end (whether negative or positive). Note that for the grey arrows, we expected eCO₂ to increase a positive response or decrease a negative response to elevated temperature and for reduced rainfall to decrease a positive response or increase a negative response to elevated temperature. We list potential mechanisms within the circles for the main effects and within boxes for the interactions as rationale for our hypotheses, but note these mechanisms were not directly tested here. [†] = primarily in C₃ species; [‡] = except in legumes; [§] = Without knowing the thermal optimum of photosynthesis for each species, there was no clear basis for hypothesizing the main effect of temperature; however, we do test hypotheses of how other factors interact with elevated temperature (H5, H7)

Therefore, eCO₂ often increases plant water-use efficiency (WUE) and soil moisture (Adair, Reich, Trost, & Hobbie, 2011; Jackson, Sala, Field, & Mooney, 1994; Morgan et al., 2004; Nelson et al., 2004; Niklaus, Spinnler, & Körner, 1998), though increased leaf area under eCO₂ can offset soil water savings (Centritto, Lee, & Jarvis, 1999; Nowak et al., 2004). Unlike the enhancement in photosynthesis caused by increased C_i, enhancements via water savings may be common among C₄ species, as C₄ species often show eCO₂-induced reductions in stomatal conductance similar to those of C₃ species (Ghannoum, Caemmerer, Ziska, & Conroy, 2000; Pastore et al., 2019; Wand et al., 1999).

Increased soil N supply can also enhance plant C capture because the key photosynthetic enzymes and pigments contain substantial amounts of N and many ecosystems are N-limited. Thus, photosynthesis often increases with foliar N concentration in a variety of ecosystems (Reich, Walters, & Ellsworth, 1997). Increased N supply via fertilization has been shown to enhance photosynthesis in various grassland species, including several C₃ and C₄ grasses and forbs, but to reduce photosynthesis in N-fixing legumes (Pastore et al., 2019; Reich et al., 2003).

Reduced water availability due to changes in precipitation patterns and/or increasing temperatures may also impact photosynthesis. To reduce water loss, stomatal conductance can decrease in response to a decline in soil moisture and/or an increase in vapour pressure deficit (VPD, a measure of atmospheric dryness which increases exponentially with temperature at constant water vapour concentration); however, reduced conductance can cause CO₂ uptake to synchronously decline (Berry & Bjorkman, 1980; Chaves, Maroco, & Pereira, 2003; Damour, Simonneau, Cochard, & Urban, 2010; Gunderson et al., 2002). In other words, warming and reduced rainfall treatments may each induce stomatal closure and thus cause concomitant reductions in photosynthesis due to CO₂ substrate limitation. Plants can also acclimate to low water availability in a multitude of ways, such as by accumulating solutes, decreasing leaf area, increasing leaf thickness, increasing the root:shoot ratio, or shedding leaves to reduce transpiration (Chaves et al., 2003; Reddy, Chaitanya, & Vivekanandan, 2004).

Although increasing temperatures can affect photosynthesis via changes in VPD or soil moisture, as described above, warming can also enhance or reduce photosynthesis depending on whether leaf temperature is below or above the thermal optimum of a species (Berry & Bjorkman, 1980; Sage & Kubien, 2007). If leaf temperature is below the thermal optimum, an increase in temperature could directly alleviate biochemical limitations of photosynthesis, whereas an increase beyond the thermal optimum could reduce photosynthesis. However, plants grown at elevated temperature may acclimate to maintain or increase photosynthetic rates at the new, higher temperature (Way & Yamori, 2014). Thus, potential effects of elevated growth temperatures on leaf physiology, like increases in the thermal optimum, greater heat tolerance of enzymes, and increased electron transport capacity (Berry & Bjorkman, 1980; Hikosaka, Ishikawa, Borjigidai, Muller, & Onoda, 2005; Kattge & Knorr, 2007; Sage & Kubien, 2007; Way & Yamori, 2014), may alter the relationship of photosynthesis and temperature (Way & Yamori, 2014).

1.2 | The influence of increased N supply, reduced rainfall and elevated temperature on responses to eCO₂

Greater acclimation to eCO₂ often occurs when N availability is low (Ainsworth & Rogers, 2007), as photosynthetic rates are strongly coupled to leaf N concentration (Reich et al., 1997). If plants are N-limited under eCO₂, C supply may exceed demand and the resulting accumulation of photosynthetic end-products (i.e. leaf nonstructural carbohydrates) signals plants to physiologically adjust by down-regulating the activity or production of the enzyme RuBisCO and reducing maximum carboxylation velocity (V_{cmax}) and chlorophyll content, resulting in reduced foliar N concentration (Drake et al., 1997; Stitt & Krapp, 1999; Urban, 2003). Therefore, responses to eCO₂ may be greater or sustained over a longer duration at high compared to low soil N availability, although this is not always found (Pastore et al., 2019).

Reduced rainfall and elevated temperatures may influence the response of photosynthesis to eCO₂ because of the effects of eCO₂ on plant water use and soil moisture described above. The water-savings benefit (i.e. improved leaf water relations and soil water conservation under eCO₂) may be particularly important in water-limited conditions, such as when low rainfall and/or soil drying associated with warmer temperatures reduce soil moisture, leading to greater eCO₂ responses in these conditions (Blumenthal et al., 2013; LeCain, Morgan, Mosier, & Nelson, 2003; Volk, Niklaus, & Körner, 2000). However, it is also possible that plants that are primarily water-limited may not be able to use additional CO₂, thereby eliminating eCO₂ responses (Reich, Hobbie, & Lee, 2014).

Warming can also influence responses to eCO₂ via effects of temperature on photosynthetic physiology. In C₃ species, photosynthesis increases more with increasing leaf temperature when CO₂ is elevated, and thus the eCO₂ response of photosynthesis may be greater at higher temperatures (Long, 1991). This is because the thermal optimum for photosynthesis in C₃ species shifts upwards as CO₂ rises since C loss via photorespiration increases exponentially with rising temperature but declines with CO₂ rise (Long, 1991). A meta-analysis of CO₂ × temperature studies (mostly in controlled environments, that is, greenhouses and growth chambers) showed that relative photosynthetic enhancement (i.e. percent increase) under eCO₂ was indeed greatest at higher temperatures in C₃ species, likely due to reduced photorespiration (Wang, Heckathorn, Wang, & Philpott, 2012).

1.3 | The influence of eCO₂ on responses to increased N supply

Plant demand for N commonly increases under eCO₂ to support enhanced rates of photosynthesis and growth (Stitt & Krapp, 1999). As a result, N limitation may develop or strengthen under eCO₂ (Reich et al., 2006; Stitt & Krapp, 1999). Thus, photosynthesis may respond more to increased N supply under eCO₂.

1.4 | The influence of reduced rainfall and eCO₂ on responses to elevated temperature

Both positive and negative effects of warming on photosynthesis may occur over time through the mechanisms described above, and this may partly depend on precipitation. For instance, because warming can cause or intensify water stress, studies have shown that warming has more positive effects on photosynthesis when soil moisture is less limiting and more negative effects at low soil moisture (Arend, Brem, Kuster, & Günthardt-Goerg, 2013; Dikšaitytė, Viršilė, Žaltauskaitė, Januškaitienė, & Juozapaitienė, 2019; Reich et al., 2018). However, other studies have failed to find interactive effects of warming and precipitation on rates of photosynthesis (Rodgers, Hoepfner, Daley, & Dukes, 2012; B. Song, Niu, & Wan, 2016; Wu, Dijkstra, Koch, Peñuelas, & Hungate, 2011). eCO₂ may increase positive (or decrease negative) responses of photosynthesis to warming by increasing water savings and, in C₃ species, also the thermal optimum, as described above.

1.5 | The influence of elevated temperature and eCO₂ on responses to reduced rainfall

If reduced rainfall has a negative effect on photosynthesis, further drying due to warming may amplify these negative effects. In contrast, eCO₂ may help to offset declines in photosynthesis due to reduced rainfall because eCO₂ improves water savings.

1.6 | Objectives and hypotheses

Due to the rarity of multi-factor global change field experiments and limited comparisons of multiple species, much uncertainty remains regarding how concurrent global change factors may interact to affect leaf gas exchange. To our knowledge, the only other field experiments that have factorially manipulated CO₂, temperature, rainfall and N are the Jasper Ridge Global Change Experiment in a California grassland dominated by annual grasses and forbs (Shaw et al., 2002), and an open-top chamber experiment in a temperate steppe ecosystem of the Mongolian Plateau, where the warming treatment is only applied at night (J. Song, Wan, Piao, Hui, et al., 2019; Song, Wan, Piao, Knapp, et al., 2019); to our knowledge, neither has reported leaf gas exchange responses to these factorial treatments. Thus, our objective was to evaluate leaf gas exchange responses of perennial grassland species belonging to four functional groups (C₃ and C₄ grasses, N-fixing legumes and non-leguminous forbs) to individual and interactive global changes. To do so, we leveraged a sub-experiment within the greater BioCON (Biodiversity × CO₂ × Nitrogen) free-air CO₂ enrichment (FACE) experiment, in a strongly N-limited central Minnesotan grassland (Reich et al., 2001; Reich et al., 2014; Thakur et al., 2019). Within this sub-experiment (TeRaCON, temperature × rainfall × CO₂ × N), 9-species mixtures were factorially exposed to four global change factors at two levels each: CO₂ (ambient and + 180 ppm), above and

belowground temperature (ambient and ≈ + 2.5°C), rainfall (ambient and ≈ -45% summer rainfall) and N supply (ambient and + 4 g N m⁻² year⁻¹).

Although these four global change factors could potentially be involved in a high number of 2-, 3-, and 4-way interactions with responses that could vary among species, developing hypotheses for all possible interactions would be unwieldy and not especially useful. Therefore, given the complexity of this experiment, we focus our hypotheses here on individual global change factors and particular potentially important interactions that may manifest via the mechanisms described above (Figure 1). By “photosynthesis,” here, we refer to leaf net photosynthesis measured at light saturation, growth CO₂ concentration and 25°C. We also note that our hypothesized interactions can be meaningfully interpreted in both directions, that is, the effect of “factor A” depends on the level of “factor B,” and the effect of “factor B” also depends on the level of “factor A.” We hypothesized that:

- 1 **CO₂:** eCO₂ will increase photosynthesis, primarily in C₃ compared to C₄ species.
- 2 **N:** Increased soil N supply will increase photosynthesis, except in legumes.
- 3 **Rainfall:** Reduced rainfall will decrease photosynthesis.
- 4 **CO₂ × N:** The response of photosynthesis to eCO₂ will be more positive under increased compared with ambient N supply, except in legumes.
- 5 **CO₂ × Temperature:** The response of photosynthesis to eCO₂ will be more positive under elevated compared with ambient temperature.
- 6 **CO₂ × Rainfall:** The response of photosynthesis to eCO₂ will be more positive under reduced compared with ambient rainfall.
- 7 **Temperature × Rainfall:** The response of photosynthesis to elevated temperature will be less positive (or more negative) at reduced compared with ambient rainfall.

Note that we excluded a hypothesis for the main effect of temperature because responses of photosynthesis to elevated temperature are difficult to predict without knowing the thermal optimum of photosynthesis. However, we were able to form hypotheses about interactions with temperature based on the potential mechanisms described above (e.g. reduced rainfall should negatively impact responses of photosynthesis to elevated temperature, but this could manifest as a decreased positive response or the development/strengthening of a negative response to elevated temperature).

2 | METHODS

2.1 | Site description and experimental design

This work was conducted at the TeRaCON experiment (Thakur et al., 2019) within the Cedar Creek Ecosystem Science Reserve in East Bethel, Minnesota, USA, part of the Cedar Creek Long Term

Ecological Research (LTER) program. TeRaCON is composed of 48 2×2 m plots chosen with a stratified random approach (to balance treatments and locations among experimental blocks) from 64 plots within the greater BioCON experiment that were initially planted with nine perennial grassland species. The BioCON experiment (Reich et al., 2001; Reich et al., 2014) was established in 1997 on secondary successional grassland and is characterized by sandy, N-poor soils derived from a glacial outwash plain. Climate is continental, with mean January temperature of -11°C , mean July temperature of 22°C and mean annual precipitation of 660 mm year^{-1} . The TeRaCON experiment is a complete factorial design with two temperature (ambient and $\approx +2.5^{\circ}\text{C}$ soil and surface warming), two growing-season rainfall (ambient and $\approx -45\%$), two atmospheric CO_2 (ambient and $+180 \text{ ppm}$), and two soil N (ambient and $+4 \text{ g N m}^{-2} \text{ year}^{-1}$) treatments. Each of the 16 treatment combinations is replicated in three plots. In 1997, plots were planted with nine species randomly selected from a pool of 16 possible species belonging to four herbaceous plant functional groups: C_3 grasses: *Agropyron repens*, *Bromus inermis*, *Koeleria cristata*, *Poa pratensis*; C_4 grasses: *Agropyron gerardii*, *Bouteloua gracilis*, *Schizachyrium scoparium*, *Sorghastrum nutans*; non-leguminous forbs: *Achillea millefolium*, *Anemone cylindrica*, *Asclepias tuberosa*, *Solidago rigida*; and N-fixing legumes: *Amorpha canescens*, *Lespedeza capitata*, *Lupinus perennis*, *Petalostemum villosum*. Here, we focus on four species, including *A. repens*, *A. gerardii*, *S. rigida*, *L. capitata*, and *L. perennis*. These species were chosen because they are representative of each of the four functional groups and were available in all treatment combinations. Two legumes were chosen because the contrasting reproductive phenologies between the two legume species make for an interesting comparison (i.e. *L. perennis* achieves peak growth and sets seed earlier in the growing season than other species).

CO_2 enrichment and N fertilization treatments began in 1998. Plots occur within six 20-m diameter free-air CO_2 enrichment (FACE) rings, each containing 61 2×2 m plots. Half of the rings receive ambient air and half receive air enriched in CO_2 during daylight hours throughout the growing season. Within each ring, half of the plots receive an N fertilization treatment, applied as slow-release ammonium nitrate in three equal fractions over the growing season. The rainfall reduction treatment began in 2007 and is administered to half of the 48 plots that compose the TeRaCON experiment. Portable 2×2 m rainout shelters are used to intercept select rain events throughout the growing season to achieve a $\approx 45\%$ rainfall reduction ($16.4 \pm 1.5 \text{ cm}$ of rainfall removed on average from May 1 to August 10 each year, with standard error representing variability among years); intercepted rain is moved away from plots via gutters. Rainout shelters are manually installed only just before and during rain events to minimize their influence on the plots. The warming treatment began in 2012 and is also administered to half of the 48 plots that compose the TeRaCON experiment. Heating is applied during the growing season using infrared lamps aboveground (four per plot) and vertical rods placed in the soil to 1 m depth (3 m^{-2}). Realized mean increases in temperature imposed by the warming treatment

compared with ambient temperature for May through September over the 7 years were $2.33 \pm 0.06^{\circ}\text{C}$ aboveground and $2.61 \pm 0.03^{\circ}\text{C}$ belowground to 1 m, with standard errors representing variability among all months across the 7 years.

2.2 | Leaf gas exchange

Leaf net photosynthesis and stomatal conductance were measured using LICOR 6400 portable infrared gas exchange systems (Li-Cor Inc, Lincoln Nebraska) with program OPEN 6.1.3–4 equipped with a 6,400-02B red/blue external light source set to $1,500 \mu\text{mol m}^{-2} \text{ s}^{-1}$ photosynthetically active radiation (PAR). Measurements were made on upper, fully expanded leaves of similar ontogenetic stage typically between 08:00 and 15:00 local time between June 12 and July 27 of 2018. Measurements were made six times within a plot for each combination of species \times temperature \times rainfall \times CO_2 \times N throughout the growing season on sunny days. Measurements of a plot were made at random times of day on separate days for different plants in order to capture potential variability. Rates were determined at light saturation (mean PAR \pm SE: $1498 \pm 1 \mu\text{mol m}^{-2} \text{ s}^{-1}$) near ambient humidity (mean chamber VPD \pm SE: $1.49 \pm 0.02 \text{ kPa}$) with cuvette block temperature set to 25°C (realized mean block temperature \pm SE: $25.6 \pm 0.06^{\circ}\text{C}$) and at approximately the CO_2 concentration in which leaves were grown (mean sample chamber $[\text{CO}_2] \pm$ SE for ambient CO_2 treatment: $405.9 \pm 0.4 \mu\text{mol mol}^{-1}$; mean sample chamber $[\text{CO}_2] \pm$ SE for elevated CO_2 treatment: $589.9 \pm 0.4 \mu\text{mol mol}^{-1}$). Intrinsic water-use efficiency (iWUE) was calculated as the rate of leaf net photosynthesis divided by stomatal conductance. C_i and leaf-to-air vapour pressure deficit (VPD_L) were also obtained as part of the leaf gas exchange measurements.

We chose to measure rates of gas exchange within a relatively modest range of cuvette block temperatures across temperature treatments to best understand the effects of the elevated temperature treatment on photosynthetic capacity. Importantly, leaf temperature during gas exchange measurements did not vary by temperature treatment as measured by an internal fine wire thermocouple touching the leaf at the bottom of the LICOR cuvette (mean leaf temperature \pm SE at ambient and elevated temperature, respectively: $28.1 \pm 0.1^{\circ}\text{C}$ and $28.2 \pm 0.1^{\circ}\text{C}$). Thus, we determined whether the elevated temperature treatment influenced leaf responses at a common measurement temperature.

The projected area of the portion of each leaf used for leaf gas exchange measurements was determined using IMAGEJ (National Institutes of Health, Bethesda, MD) and was used to standardize rates of leaf gas exchange on a per area basis. These leaf portions were then oven-dried at 65°C for at least 48 hr before weighing. Specific leaf area (SLA) was calculated as the ratio of leaf area to dry mass. Each leaf portion for the first four measurement dates for each species was then ground and analysed for foliar C and N concentrations on an Elemental C-N Analyzer (ECS 4010 CHNSO Analyzer, Costech

Analytical Technologies Inc., Valencia, CA). Leaves from the final two measurement dates of each species were not analysed for CN because absolute rates of photosynthesis began to decline, indicating the commencement of senescence, which could alter foliar tissue chemistry.

V_{cmax} (maximum rate of carboxylation) was estimated using the one-point method of De Kauwe et al. (2016) and the Bernacchi, Singaas, Pimentel, Portis Jr, and Long (2001) set of temperature dependencies. Related equations can be found in de Kauwe et al. (2016). \hat{V}_{cmax} was not calculated for the C₄ species, *A. gerardii*, as the equation only applies to C₃ photosynthesis and measurements of C_i do not approximate CO₂ concentration at the site of carboxylation (C_c) in C₄ species. We acknowledge that this method may underestimate V_{cmax} in some species and, therefore, may not be useful for comparing estimates among species in all cases (Burnett, Davidson, Serbin, & Rogers, 2019); however, here we are interested primarily in treatment responses of V_{cmax} rather than differences in absolute rates among species.

2.3 | Volumetric soil water content

Volumetric soil water content (VWC, m³ H₂O m⁻³ soil) was measured (a) hourly for each plot and (b) instantaneously at the time of leaf gas exchange measurements for the area directly surrounding each plant used. The former was measured using time-domain reflectometry (TDR; CS650 Water Content Reflectometers, Campbell Scientific, Inc., Utah). One TDR sensor in each plot measured the average soil moisture from 0 to 20 cm depth every hour. The latter was measured using a Portable Soil Moisture Probe (ML2x ThetaProbe, Delta-T Devices Ltd., Cambridge, UK), which measures a cylindrical ≈ 75 cm³ volume of soil (6 cm deep with a diameter of 4 cm) and was calibrated to Bio-CON soil. Three instantaneous soil VWC measurements were made concurrently with each leaf gas exchange measurement surrounding the base of the plant and averaged. We compared both measurements of VWC, which were positively correlated based on simple linear regression ($r = 0.81$, $p < .0001$). We found that plot-level VWC measurements corroborated observations based on VWC measurements

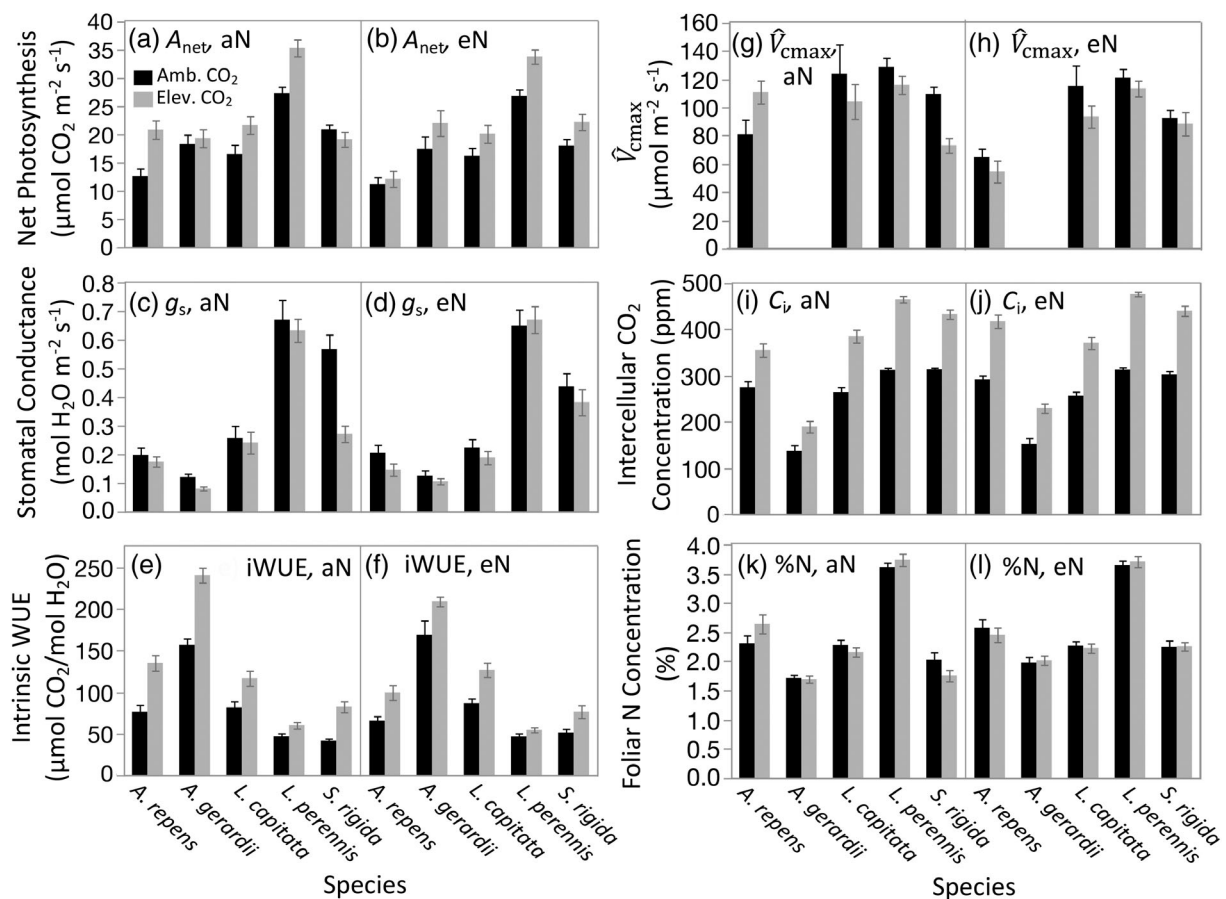


FIGURE 2 Leaf-level physiology under ambient (black) and elevated (grey) CO₂ at contrasting N levels (aN = ambient N, eN = elevated N) in five perennial grassland species: (a and b) leaf net photosynthesis (A_{net} , $n = 480$); (c and d) stomatal conductance (g_s , $n = 480$); (e and f) intrinsic water-use efficiency (iWUE, $n = 480$); (g and h) estimated maximum carboxylation capacity (\hat{V}_{cmax} , $n = 383$); (i and j) intercellular CO₂ concentration (C_i , $n = 477$); (k and l) foliar N concentration (%N, $n = 320$). Error bars represent standard error. Results of statistical analyses are reported in Tables 1 and 2. Species \times CO₂ \times N interactions were not detected by analyses for iWUE and C_i ; however, all variables are presented similarly for comparison due to their relationship with or potential influence on A_{net}

from the soil directly surrounding the plant during leaf gas exchange measurements. Thus, we only report VWC measured for each plant in conjunction with leaf gas exchange here.

2.4 | Data analysis

Multiple measurements within a plot, spanning a growing season, were taken on different individuals and included in analyses. For analyses of photosynthesis, stomatal conductance, $iWUE$, \hat{V}_{cmax} and C_i , we used linear mixed-effects models including main effects of species, temperature, rainfall, CO_2 , N and up to three-way interactions of the above. Ring was nested within CO_2 as a random effect. We also

included VWC and VPD_L as continuous predictor variables, and each was \log_{10} -transformed. Initially, we included all interactions up to three-way of the continuous predictor variables and main categorical factors; however, we then removed any interactions including continuous variables for which $p > .1$ except those that were relevant to our main hypotheses or two-way interactions that were necessary to keep because of related three-way interactions that were retained.

For analyses of SLA, %N and VWC, we used linear mixed-effects models including main effects of species, temperature, rainfall, CO_2 , N, all two-way interactions of the above and all three-way interactions that included species. Ring was nested within CO_2 as a random effect. For SLA, we enforced a lower bound of zero on the range

Effect	df^a	A_{net} $p > F$	g_s $p > F$	$iWUE$ $p > F$	\hat{V}_{cmax} $p > F$	C_i $p > F$
Species	4	<.0001	<.0001	<.0001	<.0001	<.0001
CO_2	1	.12	.56	.02	.44	.005
N	1	.03	.30	.23	.004	.09
Rain	1	.006	.23	.14	.009	.04
Temp	1	.42	.95	.28	.40	.75
Species \times CO_2	4	.77	.50	.25	.32	.002
Species \times N	4	.04	.80	.11	.02	.08
Species \times rain	4	.31	.55	.72	.49	.81
Species \times temp	4	.22	.66	.98	.37	.94
$CO_2 \times$ N	1	.70	.59	.17	.39	.23
$CO_2 \times$ rain	1	.18	.04	.06	.45	.32
$CO_2 \times$ temp	1	.11	.05	.21	.80	.57
N \times rain	1	.15	.05	.11	.78	.19
N \times temp	1	.57	.94	.57	.47	.69
Rain \times temp	1	.98	.42	.14	.16	.12
Species \times $CO_2 \times$ N	4	.003	.04	.88	.02	.63
Species \times $CO_2 \times$ rain	4	.04	.14	.74	.93	.38
Species \times $CO_2 \times$ temp	4	.27	.85	.50	.45	.37
Species \times N \times rain	4	.83	.48	.02	.75	.41
Species \times N \times temp	4	.25	.33	.70	.12	.79
Species \times rain \times temp	4	.002	.03	.12	.03	.36
$CO_2 \times$ N \times rain	1	.66	.25	.14	.43	.41
$CO_2 \times$ N \times temp	1	.63	.61	.14	.84	.82
$CO_2 \times$ rain \times temp	1	.36	.84	.29	.61	.20
N \times rain \times temp	1	.70	.31	.14	.17	.38

Note: $p < .1$ bolded to draw attention to the most important terms in the model. Italicized p values indicate values for which false discoveries are least likely; to control for multiplicity, we used the Benjamini–Hochberg false discovery rate controlling procedure and indicate in italics all factors for which we can reject the null hypothesis based on the B–H thresholds with $q = 0.1$. Raw p values are shown.

Note: Other factors included in this model (volumetric soil water content, VWC, and leaf-to-air vapor pressure deficit, VPD_L) and interactions with these factors are shown in Table S1. F -ratios are shown in Table S2.

Abbreviations: Rain, rainfall; Temp, temperature.

^aFor \hat{V}_{cmax} , effects including “Species” contained 3 instead of 4 df because the C_4 species, *A. gerardii*, was not included.

TABLE 1 Linear mixed-effects model probabilities ($p > F$) on leaf-level traits of five species

of the variance components because a negative variance component for the ring [CO₂] random effect did not allow the model to converge.

For all analyses, we controlled for multiplicity using the Benjamini–Hochberg false discovery rate controlling procedure with $q = 0.1$ (Benjamini & Hochberg, 1995). We show raw p values in tables but indicate in italics all factors for which we can reject the null hypothesis based on the Benjamini–Hochberg thresholds. Based on this procedure, only 10% of rejected null hypotheses in italics are potentially false discoveries.

Data were checked for assumptions of normality and homogeneity of variances; photosynthesis, stomatal conductance, iWUE, %N, VWC and SLA were log₁₀-transformed. For \hat{V}_{cmax} , we excluded one unrealistic value for *A. repens* from the related analysis and figures, along with all measurements of *A. gerardii* because the \hat{V}_{cmax} calculations do not apply to C₄ species, as described above. We also excluded three negative values of C_i, all belonging to *A. gerardii*, from the analysis of C_i and related figure, and we caution that measurements of C_i do not approximate C_c in C₄ species and this must be considered when interpreting the meaning of C_i responses in this species compared with C₃ species. All analyses were conducted with

statistical analysis software (JMP Pro 14.1.0, 2018, SAS Institute Inc., Cary, NC).

When we use the term “response” to discuss our results, it can be assumed that we are referring to both absolute and relative responses unless we specify otherwise (i.e. when different, we may refer to each individually). We define the “absolute response” as the absolute difference, that is, the ambient level subtracted from the non-ambient level. We define the “relative response” as the percent change, that is, the ambient level subtracted from the non-ambient level, then divided by the ambient level, then multiplied by 100.

3 | RESULTS

We first describe the main effects of each of the four global change treatments and any variation of these main effects among species, as we expected different responses depending on functional group identity in some cases. Then, we describe the key interactions related to our hypotheses (Figure 1).

TABLE 2 Linear mixed-effects model probabilities ($p > F$) on soil volumetric water content (VWC), foliar N concentration (%N) and specific leaf area

Effect	df	VWC		Foliar %N		Specific leaf area	
		F	<i>p > F</i>	F	<i>p > F</i>	F	<i>p > F</i>
Species	4	10.94	<.0001	163.18	<.0001	228.87	<.0001
CO ₂	1	0.34	.66	0.001	.99	0.01	.91
N	1	0.003	.96	14.77	.0002	11.04	.001
Rain	1	54.09	<.0001	17.37	.0002	9.20	.003
Temp	1	4.40	.04	6.22	.01	0.004	.95
Species × CO ₂	4	1.23	.30	1.03	.40	1.68	.15
Species × N	4	0.58	.68	3.12	.02	4.95	.0007
Species × rain	4	5.55	.0003	3.55	.009	3.50	.008
Species × temp	4	0.27	.90	1.99	.10	0.90	.47
CO ₂ × N	1	0.06	.81	0.11	.74	2.88	.09
CO ₂ × rain	1	1.08	.30	0.43	.52	0.40	.53
CO ₂ × temp	1	0.41	.53	0.001	.98	1.72	.19
N × rain	1	1.95	.16	0.21	.65	0.14	.71
N × temp	1	0.05	.83	0.05	.83	0.60	.44
Rain × temp	1	0.44	.51	1.41	.24	2.61	.11
Species × CO ₂ × N	4	1.05	.38	1.69	.16	3.14	.02
Species × CO ₂ × rain	4	0.88	.48	1.87	.12	0.68	.61
Species × CO ₂ × temp	4	0.33	.86	3.67	.009	1.24	.29
Species × N × rain	4	0.18	.95	0.80	.54	0.92	.45
Species × N × temp	4	0.23	.92	1.07	.38	3.03	.02
Species × rain × temp	4	0.42	.79	1.66	.17	4.64	.001

Note: $p < .1$ bolded to draw attention to the most important terms in the model. Italicized p values indicate values for which false discoveries are least likely; to control for multiplicity, we used the Benjamini–Hochberg false discovery rate controlling procedure and indicate in italics all factors for which we can reject the null hypothesis based on the B–H thresholds with $q = 0.1$. Raw p values are shown. Abbreviations: Rain = rainfall, Temp = temperature.

3.1 | Main effect of CO₂ (H1)

In accordance with H1, eCO₂ generally enhanced photosynthesis (Figure 2a,b) and did so for each species when averaged across all other factors; however, there is low confidence in a main effect of CO₂ ($p = .12$, Table 1) because responses to eCO₂ were influenced by other factors, described below. Average responses of C₃ species were greater than the C₄ species in all but one case. The greatest relative photosynthetic response to eCO₂, averaged across other factors, was observed in the C₃ grass *A. repens* (+38%), followed by the legumes *L. capitata* and *L. perennis* (+28%), then the C₄ grass, *A. gerardii* (+15%), with the lowest average response in the forb *S. rigida* (+6%). The general enhancement in photosynthesis under eCO₂ was accompanied by an increase in C_i that varied in

magnitude among species (species \times CO₂ for C_i: $p = .002$, Table 1, Figure 2i,j).

In general, eCO₂ reduced stomatal conductance (Figure 2c,d), but as with photosynthesis, there is low confidence in a main effect of CO₂ (CO₂: $p = .56$, Table 1) due to interactions with other factors. The greatest relative reduction in stomatal conductance under eCO₂, averaged across other factors, was observed in the forb, *S. rigida* (−35%), followed by the C₄ grass, *A. gerardii* (−25%), then the C₄ grass, *A. repens* (−20%), with the lowest average responses in the legumes, *L. capitata* (−10%) and *L. perennis* (−1%). eCO₂ enhanced iWUE in all species, by 54% on average (CO₂: $p = .02$, Table 1, Figure 2e,f), because of average increases in photosynthesis and reductions in stomatal conductance. Despite enhanced iWUE, eCO₂ did not increase soil VWC (CO₂: $p = .66$, Table 2, Figure S1b).

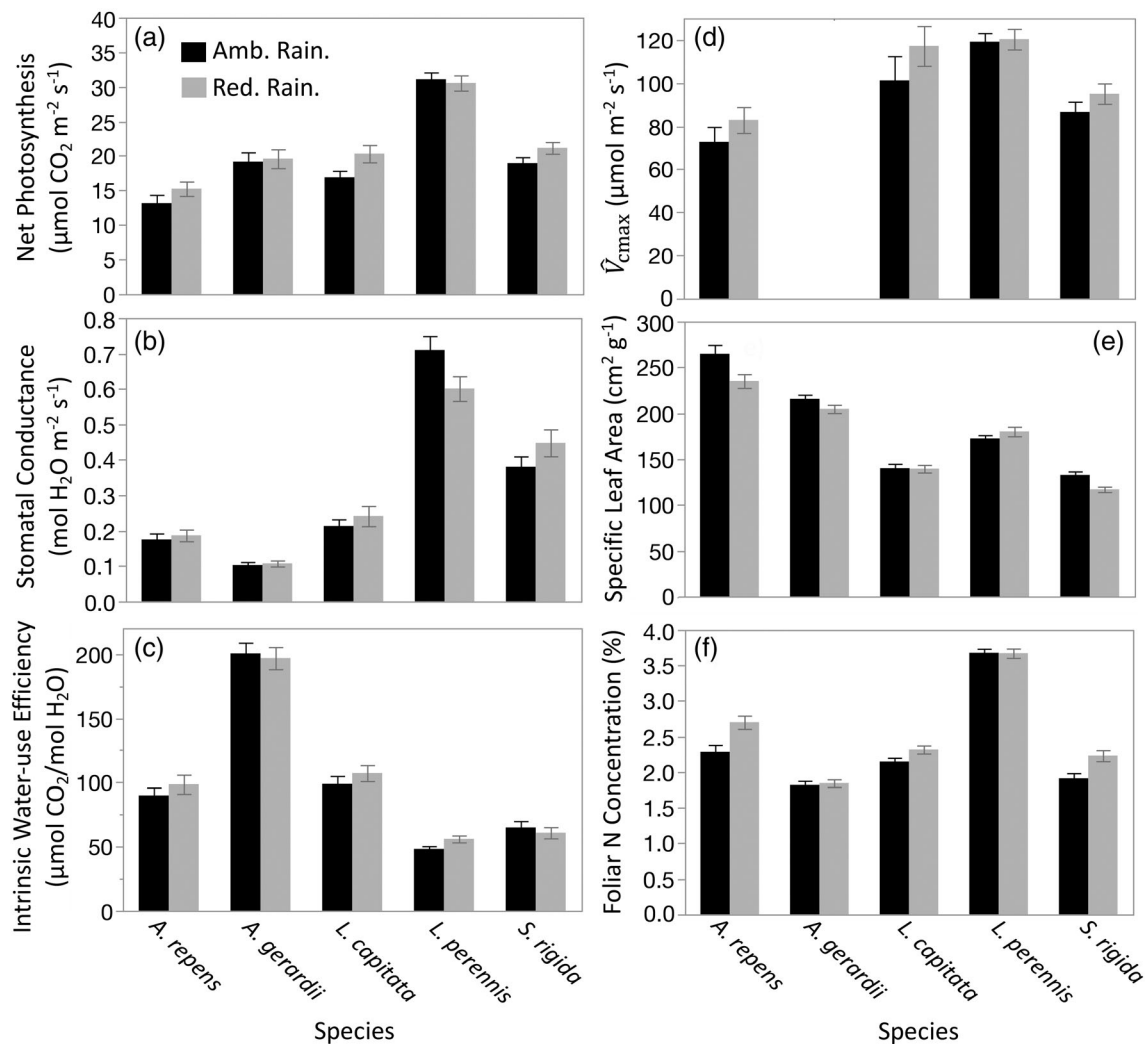


FIGURE 3 Leaf-level physiology under ambient (black) and reduced (grey) rainfall in five perennial grassland species: (a) leaf net photosynthesis; (b) stomatal conductance; (c) intrinsic water-use efficiency; (d) estimated maximum carboxylation capacity (\hat{V}_{cmax}); (e) specific leaf area; (f) foliar N concentration. \hat{V}_{cmax} calculations do not apply to C₄ species and thus no estimates are shown for *A. gerardii* in panel (d). $n = 480$, except for \hat{V}_{cmax} ($n = 383$) and foliar N concentration ($n = 320$). Error bars represent standard error. Results of statistical analyses are reported in Tables 1 and 2

3.2 | Main effect of N (H2)

The effect of N on photosynthesis depended on species such that H2 was only partly supported (species \times N: $p = .04$, Table 1, Figure 2a,b). As expected, N addition did not affect legume photosynthetic rates (Figure 2a,b). In contrast to expectations, N did not affect photosynthesis in *A. gerardii* and *S. rigida* (despite increases in foliar N concentration, species \times N: $p = .02$, Table 1, Figure 2k,l) and strongly reduced photosynthesis for *A. repens* (Figure 2a,b). However, the direction of responses to N for non-legume species was not always consistent among levels of CO₂, as described below (Table 1, Figure 2a,b).

3.3 | Main effect of rainfall (H3)

In direct contradiction of H3, reduced rainfall modestly enhanced photosynthesis on average (rainfall: $p = .006$, Table 1, Figure 3a) despite strong reductions in soil VWC that showed that reduced rainfall treatments were realized on average (Table 2, Figure S1c). In the reduced rainfall treatment, plants experienced soil VWC that was 28.4% lower on average than those under ambient rainfall during leaf gas exchange measurements (mean reductions varied from 10 to 56% among species but shifted throughout the season, species \times rainfall: $p = .0003$, Table 2, Figure S1c and Figure 4). We note that this is a considerably larger difference in VWC than occurred on average at the plot level across rainfall treatments during the entire growing season (average 10.3% decrease in VWC due to reduced rainfall at the plot level). Positive responses of photosynthesis to reduced rainfall were primarily evident in three species from different functional groups, *A. repens*, *L. capitata* and *S. rigida*, and the magnitude of this positive effect varied modestly by CO₂ level (species \times CO₂ \times rainfall: $p = .04$, Table 1, Figures 3a and 6c,d). It occurred at both N and temperature levels except not at ambient temperature for *A. repens* (Table 1). Stomatal conductance did not decline in response to reduced rainfall for these three species and instead was enhanced, only at elevated temperature for

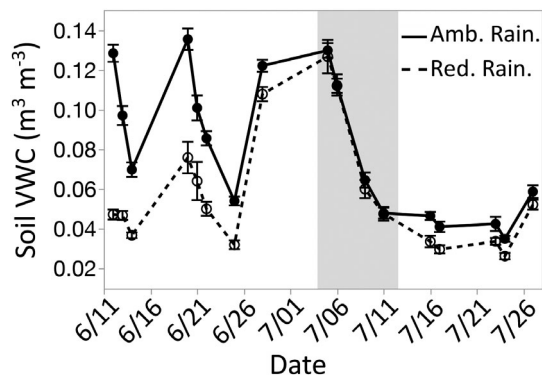


FIGURE 4 Average soil volumetric water content (VWC) at ambient (black line, closed circles) and reduced (dashed line, open circles) rainfall on each measurement date ($n = 480$). The shaded region includes dates for which VWC was very similar between rainfall treatment levels. Error bars represent standard error. Results of statistical analyses are reported in Table 2

A. repens and more strongly at ambient temperature for *L. capitata* and *S. rigida* (species \times temperature \times rainfall: $p = .03$, Table 1, Figure 3b). Observations of greater photosynthesis under reduced compared with ambient rainfall in only these three species primarily did not occur until the VWC differential between these two treatments disappeared (Figures 4 and 5a–e). Thus, the photosynthetic response to reduced rainfall for these species was generally most positive when the associated response in soil VWC was least negative (Figure S2).

Several leaf-level physiological adjustments to the reduced rainfall treatment were observed for the species that displayed enhanced photosynthesis (Table 3, Figure 3a–f). Reduced rainfall increased \hat{V}_{cmax} in these three species, indicating reduced biochemical limitations (rainfall: $p = .009$, Tables 1 and 3, Figure 3d). SLA was lower under reduced compared with ambient rainfall for *A. repens*, *S. rigida*, and to a lesser extent for *A. gerardii* (species \times rainfall: $p = .008$, Table 2, Figure 3e). Reduced rainfall increased foliar N concentration by 18, 17 and 8% on average for *A. repens*, *S. rigida* and *L. capitata*, respectively (species \times rainfall: $p = 0.009$ for %N, Table 2, Figure 3f).

3.4 | Main effect of temperature

We did not observe a main effect of temperature on photosynthesis or stomatal conductance (Table 1). However, elevated temperature reduced soil VWC by 10% on average (temperature: $p = 0.04$, Table 2, Figure S1a) and temperature was involved in an interaction with species and rainfall, described below.

3.5 | Interaction between CO₂ and N (H4)

N influenced the eCO₂ response of photosynthesis in all non-legume species (species \times CO₂ \times N: $p = .003$, Table 1, Figure 2a,b). For *A. gerardii* and *S. rigida*, eCO₂ only enhanced photosynthesis at the elevated N level, which is congruent with H4 (Figure 2a,b). This was associated with lower eCO₂-induced declines in stomatal conductance at the elevated compared with ambient N level in both species (species \times CO₂ \times N: $p = .04$, Table 1, Figure 2c,d) and, for *S. rigida*, less of an eCO₂-induced decline in \hat{V}_{cmax} at elevated compared with ambient N (species \times CO₂ \times N: $p = .02$, Table 1, Figure 2g,h). There was also a tendency for eCO₂ to modestly reduce foliar N concentration on average at ambient but not elevated N for *S. rigida* (Table 2, Figure 2k,l). In contrast, for *A. repens*, eCO₂ enhanced photosynthesis (and \hat{V}_{cmax}) at ambient but not elevated N, contrary to H4 (Figure 2a, b). Photosynthesis responded similarly to eCO₂ at both N levels in the two legume species, as expected (Figure 2a,b).

3.6 | Interaction between CO₂ and temperature (H5)

We did not observe a strong influence of temperature on responses of photosynthesis to eCO₂ (CO₂ \times temperature: $p = .11$, Figure 6a,b).

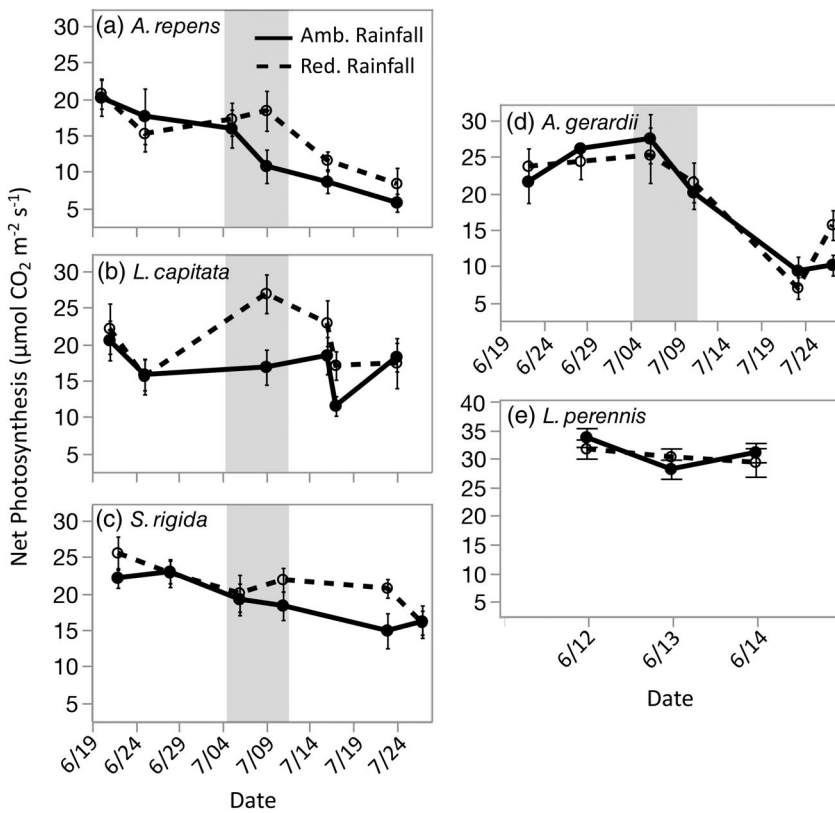


FIGURE 5 Leaf net photosynthesis throughout the growing season under ambient (solid line, closed circles) and reduced (dashed line, open circles) rainfall in each species. The three species for which photosynthesis responded positively to the reduced rainfall treatment on average include: (a) *A. repens*; (b) *L. capitata*; (c) *S. rigida*. The remaining two species include (d) *A. gerardii* and (e) *L. perennis*. $n = 480$. Note that both axes for *L. perennis* differ from those of the other species. The shaded region includes dates for which soil VWC was very similar between rainfall treatment levels (see Figure 4). Error bars represent standard error. Results of statistical analyses are reported in Table 1

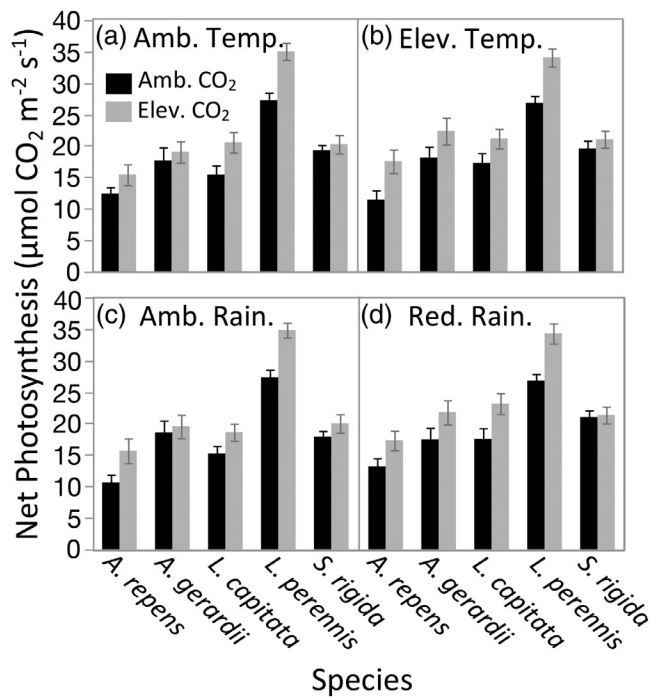


FIGURE 6 Leaf net photosynthesis under ambient (black) and elevated (grey) CO₂ at contrasting temperature (a: ambient; b: elevated) and rainfall (c: ambient; d: reduced) levels in five perennial grassland species. $n = 480$. Error bars represent standard error. Results of statistical analyses are reported in Table 1. There was an interaction of species \times CO₂ \times rainfall ($p = .04$)

However, providing some support for H5, both grasses showed modestly greater responses to eCO₂ at elevated compared with ambient temperature on average (25 vs. 53% response to eCO₂ for *A. repens* and 8 vs. 24% response to eCO₂ for *A. gerardii* at ambient vs. elevated temperature, respectively, Figure 6a,b). However, the other species showed similar responses on average at both temperature treatments (Figure 6a,b). Foliar N concentration was enhanced by elevated temperature at eCO₂ but not ambient CO₂ for *A. repens*, potentially contributing to the observed trend for this species, although elevated temperature increased foliar N concentration at both CO₂ levels for *A. gerardii* (species \times CO₂ \times temperature: $p = .009$, Table 2, not shown).

3.7 | Interaction between CO₂ and rainfall (H6)

We found modest support for our hypothesis that responses of photosynthesis to eCO₂ would be greater at low rainfall. Two species displayed the hypothesized response, with a 5 versus 25% increase in photosynthesis under eCO₂ at ambient and reduced rainfall, respectively, for *A. gerardii* and a 22 versus 32% increase under eCO₂ at ambient and reduced rainfall, respectively, for *L. capitata* (species \times CO₂ \times rainfall: $p = .04$, Table 1, Figure 6c,d). We did not observe greater responses of photosynthesis to eCO₂ as soil VWC declined (species \times CO₂ \times VWC: $p = .31$, Table S1, not shown).

We observed a modest trend of greater responses of photosynthesis to eCO₂ as VPD_L increased, that is, as atmospheric water deficit

TABLE 3 Responses of leaf-level physiology to the reduced rainfall treatment

Leaf-level trait	<i>A. repens</i>	<i>L. capitata</i>	<i>S. rigida</i>	<i>A. gerardii</i>	<i>L. perennis</i>
Leaf net photosynthesis (A_{net})	↑ (eT)	↑ (aT)	↑	—	—
Stomatal conductance (g_s)	↑ (eT)	↑ (aT)	↑ (aT)	—	↓
Maximum carboxylation capacity (\hat{V}_{cmax})	↑ (eT)	↑ (aT)	↑	n/a ^a	—
Foliar %N	↑	↑	↑	—	—
Specific leaf area (SLA)	↓ (eT)	—	↓	↓	↑

Note: Statistics are reported in Tables 1 and 2. ↑ = increase in response to reduced rainfall, ↓ = decrease in response to reduced rainfall, — = no response to reduced rainfall, aT = occurred only (or more strongly) at ambient versus elevated temperature, eT = occurred only (or more strongly) at elevated versus ambient temperature.

Note: *A. repens*, *L. capitata* and *S. rigida* are the species for which photosynthesis responded positively to the reduced rainfall treatment on average, that is, those that showed evidence of acclimation to soil water-deficit under reduced rainfall.

^a \hat{V}_{cmax} could not be estimated for *C₄* species.

increased ($CO_2 \times VPD_L$: $p = .04$, Table S1, Figure 7a). This was observed, on average, for all species in terms of the relative response and for all *C₃* species except for *L. perennis* in terms of the absolute response (Figure S3a–e). Consistent with the typical coupling between photosynthesis and stomatal conductance, the negative response of stomatal conductance to eCO_2 decreased as VPD_L increased ($CO_2 \times VPD_L$: $p = .004$, Table S1, Figures 7b and S3f–j).

3.8 | Interaction between temperature and rainfall (H7)

We found modest species-dependent evidence consistent with our hypothesis that elevated temperature would lead to greater increases in photosynthesis at ambient compared with reduced rainfall. *L. capitata* is the only species that displayed the hypothesized response to the categorical treatments, with a modest positive response of photosynthesis (and greater positive response of \hat{V}_{cmax} , Table 1, Figure 8e,f) to warming only observed at ambient compared with reduced rainfall, which was associated with enhanced stomatal conductance (species \times rainfall \times temperature: $p = .002$ for photosynthesis and 0.03 for stomatal conductance, Table 1, Figure 8a–d). In contrast, for *A. repens*, the elevated temperature treatment increased photosynthesis (and \hat{V}_{cmax} , Table 1, Figure 8e,f) only with reduced rainfall (Figure 8a,b). Furthermore, when we examined the relationship of photosynthesis and soil VWC by temperature treatment, there was no evidence for greater temperature effects at higher VWC (temperature \times VWC: $p = .86$, Table S1, not shown).

4 | DISCUSSION

Overall, effects of four global change factors — CO_2 , N, rainfall and temperature — on leaf gas exchange differed among species, varied across levels of other treatments and/or depended on environmental conditions. Below, we first discuss the main effects of these factors, followed by interactions of interest based on our hypotheses.

4.1 | Main effects of CO_2 , N, rainfall and temperature

Though photosynthesis was higher and stomatal conductance was lower overall under eCO_2 , our analysis did not detect a main effect of CO_2 (H1) on either, as responses to eCO_2 depended on species, rainfall and N treatments, as well as on VPD_L . The average enhancement in photosynthesis under eCO_2 we observed was accompanied by increased C_i , indicating reduced stomatal limitation to CO_2 diffusion. eCO_2 also increased $iWUE$, as commonly observed in other studies and to a similar extent as shown in past work at BioCON (Ainsworth & Long, 2005; Drake et al., 1997; Pastore et al., 2019); however, we did not detect greater soil moisture under eCO_2 during leaf gas exchange measurements in this study, in contrast to previous work at BioCON (Adair et al., 2011).

The impact of soil N addition on photosynthesis depended on species. As expected, the two legume species did not respond to N addition (H2); however, photosynthesis in the *C₃* grass *A. repens* was lower under N addition, which may have manifested due to increased competition for light and other resources. Indeed, N addition increases biomass in this experiment (Reich & Hobbie, 2013), and rates of photosynthesis for *A. repens* tended to decrease with increasing plot-level biomass (not shown). Thus, the biotic environment may also play an important role in shaping photosynthetic responses. Photosynthesis was not notably enhanced by N addition in any species; thus we did not find evidence that N would enhance photosynthesis in non-legumes (H2), a response commonly observed in other studies, albeit often in young, small plants grown without competition (Reich et al., 2003). In a 21-year study in monoculture plots of 14 species exposed to factorial $CO_2 \times N$ in the BioCON experiment, N reduced photosynthesis the most in legumes and enhanced photosynthesis the most in *C₄* grasses, with variable effects on *C₃* grasses and forbs (Pastore et al., 2019). Average responses of the *C₄* grass and both legume species grown in mixtures, in this study, tended to follow a pattern similar to the monocultures, though perhaps it did not manifest as strongly because of interspecific interactions and/or factors that contribute to interannual variability in responses (e.g. weather).

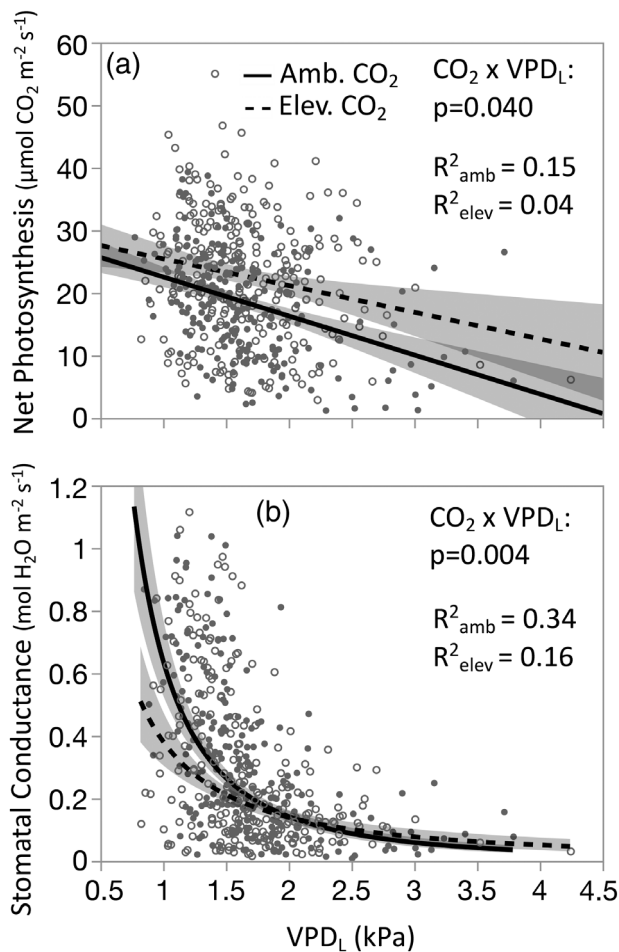


FIGURE 7 Leaf net photosynthesis (a) and stomatal conductance (b) under ambient (solid line, closed circles) and elevated (dashed line, open circles) CO_2 as a function of leaf-to-air vapour pressure deficit (VPD_L) across all five species. The best fits were achieved using linear fits for net photosynthesis and logarithmic fits for stomatal conductance. For (b), curves were fit with stomatal conductance \ln -transformed (shown here after conversion back to original scale). Shaded regions represent 95% confidence intervals. $n = 480$. Results of statistical analyses are reported in Table 1 and p values for $CO_2 \times VPD_L$ interactions are shown on the figure. R^2_{amb} corresponds to ambient CO_2 data and R^2_{elev} corresponds to elevated CO_2 data. Relationships by species are shown in Figure S3, although no species $\times CO_2 \times VPD_L$ interactions were detected

In contrast to H3, reduced rainfall increased photosynthesis on average, primarily in the C_3 grass, *A. repens*, the forb, *S. rigida* and the legume, *L. capitata*. On the surface, this appears contrary to the well-documented observation of photosynthetic impairment under low soil moisture due to reduced CO_2 diffusion across the leaf surface; however, our results are consistent with observations of acclimation to water deficits (Flexas, Bota, Galmes, Medrano, & Ribas-Carbó, 2006; Stewart, El Abidine, & Bernier, 1995). It is probable that physiological adjustments to the reduced rainfall treatment allowed these plants to circumvent stomatal closure, allowing for higher rates of gas exchange compared with plants grown in the ambient rainfall treatment.

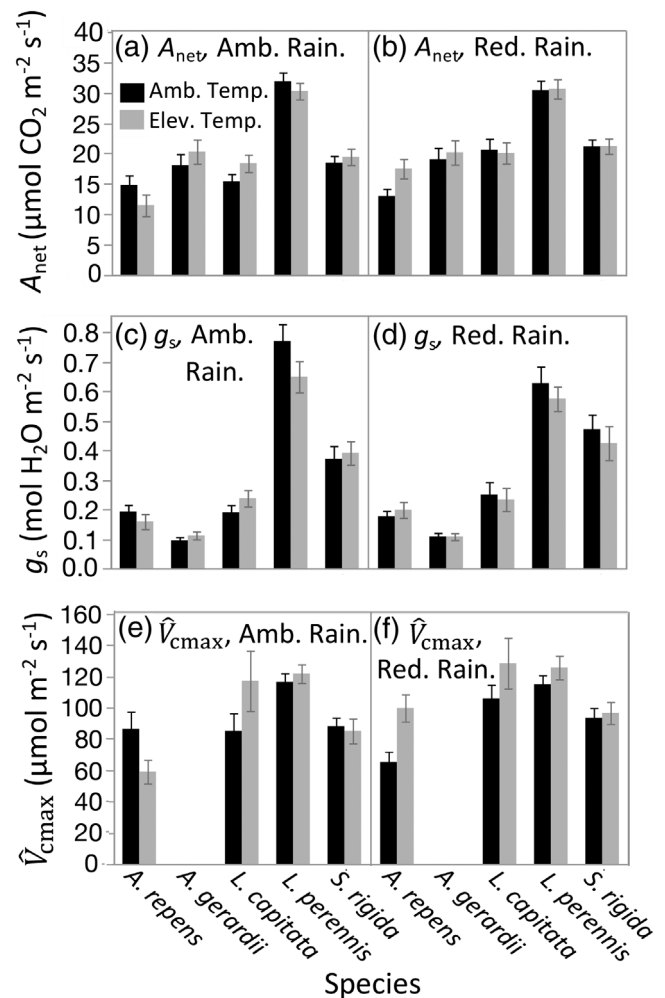


FIGURE 8 Leaf net photosynthesis (A_{net} : a and b), stomatal conductance (g_s : c and d), and estimated maximum carboxylation capacity (\hat{V}_{cmax} : e and f) under ambient (black) and elevated (grey) temperature at contrasting rainfall levels in five perennial grassland species: (a, c, e) ambient rainfall; (b, d, f) reduced rainfall. $n = 480$, except for \hat{V}_{cmax} ($n = 383$). \hat{V}_{cmax} calculations do not apply to C_4 species and thus no estimates are shown for *A. gerardii* in panels (e) and (f). Error bars represent standard error. Results of statistical analyses are reported in Table 1. There were interactions of species \times rainfall \times temperature for net photosynthesis ($p = .002$), stomatal conductance ($p = .03$) and \hat{V}_{cmax} ($p = .03$)

Specifically, we surmise that these species, although functionally disparate, acclimated to avoid tissue dehydration through physiological adjustments like osmotic adjustment, increasing leaf thickness, investing more in photosynthetic capacity and increasing the biomass of deep rooting systems rather than by merely closing stomata (Chaves et al., 2003; Reddy et al., 2004). We did observe decreased SLA under reduced rainfall in two of these species, which could indicate thicker leaves with a higher density of chlorophyll and proteins per unit area and hence greater photosynthetic capacity (Niinemets, 1999). Plot-level fine root mass fraction from 20 to 100 cm under reduced rainfall also increased (Reich et al., unpublished data), which could allow for greater access to water and nutrients.

Moreover, we observed enhanced foliar N concentration in these species when grown under reduced rainfall; this is a typical trait adjustment in low rainfall (Wright et al., 2005; Wright, Reich, & Westoby, 2003), but it could also be related to lower leaching as soil nitrate tends to be greater under reduced compared with ambient rainfall in this experiment (Reich et al., unpublished data). Overall, these three physiologically distinct species (a C₃ grass, a forb and a legume) maintained high rates of photosynthesis when soil VWC was low and photosynthetically outperformed their conspecifics in the ambient rainfall treatment when measured at similar VWC, resulting in greater rates of photosynthesis under reduced compared with ambient rainfall on average. Of the two species that showed similar rates of photosynthesis under ambient and reduced rainfall, one is a C₄ species characterized by high WUE and is well adapted to cope with water deficits (Knapp, 1984; Kocacinar & Sage, 2003), and the other is a legume that achieved peak growth and set seed earlier in the growing season, thereby likely escaping water deficits.

We did not observe a main effect of temperature, although temperature did interact with species and rainfall. As we measured plants grown at ambient and elevated temperature treatments at a common temperature, these results suggest that the $\approx 2.5^{\circ}\text{C}$ higher growth temperature has not altered photosynthetic capacity. To conclusively determine whether photosynthesis in these species has thermally adjusted to the warming treatment or not, it would be useful to perform temperature-response curves.

4.2 | CO₂, N, rainfall and temperature interact in complex ways to affect leaf gas exchange and related parameters

Although eCO₂ did not consistently reduce foliar N concentration in this study, N strongly affected photosynthetic responses to eCO₂ in non-legume species (H4). This is in contrast to monocultures at BioCON, for which N did not influence responses of photosynthesis to eCO₂ (Lee, Barrott, & Reich, 2011; Pastore et al., 2019). Biomass is greater and soil nitrate lower in 9-species mixtures compared with monocultures at BioCON (Craine et al., 2003; Mueller, Hobbie, Tilman, & Reich, 2013; Reich et al., 2001), and thus N fertilization may have affected responses of photosynthesis to eCO₂ more in mixtures because of greater competition for soil nitrate. Depending on species and N level, eCO₂ altered photosynthesis by -8 to $+65\%$. eCO₂ only enhanced photosynthesis for *A. gerardii* and *S. rigida* at elevated compared with ambient N, in accordance with H4. In contrast to expectations, however, the average response of photosynthesis to eCO₂ in the C₄ species, *A. gerardii*, at elevated N was similar to responses of C₃ species, adding to a growing body of evidence showing that C₄ photosynthesis can respond to eCO₂ (Pastore et al., 2019; Wand et al., 1999). In contrast to H4, eCO₂ only enhanced photosynthesis of *A. repens* at ambient N, perhaps because of increased competition under N addition, as described above. For the two legume species that eCO₂ responses did not depend on N is not surprising, as legumes are less likely to experience N constraints (Rogers, Ainsworth, & Leakey, 2009).

We did not observe a strong influence of temperature on responses of photosynthesis to eCO₂ (and vice versa). However, both the C₃ and C₄ grass species tended to respond more strongly to eCO₂ at the elevated compared with ambient temperature treatment, providing some support for H5. A greater thermal optimum under eCO₂ may have contributed, particularly for the C₃ species (*A. repens*) (Long, 1991), but cannot be evaluated without performing temperature response curves. For *A. repens*, this trend may also have been related to alleviation of N limitation, as foliar N concentration was enhanced by elevated temperature in this species at the eCO₂ concentration, although this was not true of *A. gerardii*. Soil moisture was reduced at elevated temperature because of greater evaporation from plants and soils (10% average decrease in soil VWC due to warming). However, during leaf gas exchange measurements, there was no detectable effect of eCO₂ on soil moisture. Thus, the lack of a strong CO₂ \times temperature interaction is perhaps unsurprising given that eCO₂ did not increase soil moisture to offset the reduction in soil moisture caused by higher temperature. This is in contrast to other studies that have observed CO₂ \times temperature interactions due to eCO₂-induced soil moisture savings. For instance, the C₃ forb *Linaria dalmatica* in a mixed-grass prairie FACE experiment showed more positive effects of eCO₂ on photosynthesis at elevated compared with ambient temperature because of indirect CO₂ effects on soil moisture (Blumenthal et al., 2013). In contrast, another study examining CO₂ \times temperature effects on leaf gas exchange in grasses found that photosynthesis was enhanced less by eCO₂ in plants grown under $+4^{\circ}\text{C}$, when all plants were measured at 25°C , due to long-term acclimation to warming (Morgan, Hunt, Monz, & LeCain, 1994). Studies including temperature response curves and separating short- and long-term effects of warming on responses of photosynthesis to eCO₂ may help to resolve the varied responses observed in the literature and reveal the mechanisms involved.

Responses of photosynthesis to eCO₂ were modestly influenced by the rainfall treatment (and vice versa). As described above, eCO₂ did not increase soil moisture, whereas the reduced rainfall treatment strongly reduced soil moisture (28% average decrease in soil VWC due to reduced rainfall). As such, we only found modest evidence for our hypothesis that responses of photosynthesis to eCO₂ would be more positive under reduced compared with ambient rainfall (H6) despite enhanced iWUE under eCO₂ in all species. The hypothesized trend only occurred in the C₄ grass, *A. gerardii* and legume, *L. capitata* and was modest. A growth chamber study using the C₃ grass, *Leymus chinensis* did not observe strong increases in soil moisture under eCO₂ despite enhanced iWUE, similar to our study, but still observed greater increases in photosynthesis with eCO₂ when simulated rainfall was low compared with normal or high (Li et al., 2014). Perhaps physiological or growth adjustments associated with acclimation to low soil moisture, which were evident in some species in our experiment, reduced the effects of any plant water conservation under eCO₂ on leaf gas exchange in our study.

VPD is another type of water deficit (though in the atmosphere rather than soils) that may interact with eCO₂ to affect leaf gas exchange. Indeed, responses of photosynthesis to eCO₂ tended to

increase as VPD_L increased. This may be explained by the tight coupling between photosynthesis and stomatal conductance. Stomatal conductance declined with rising VPD_L as stomates closed, but less steeply under eCO_2 ; eCO_2 thereby partially alleviated increases in stomatal limitation of CO_2 diffusion across the leaf surface with rising VPD_L . Thus, rates of photosynthesis declined less steeply with rising VPD_L under eCO_2 compared with ambient CO_2 . In a study of sweetgum trees in the Oak Ridge National Laboratory FACE experiment, the absolute positive response of photosynthesis to eCO_2 was similar across all levels of VPD_L , which ranged from ≈ 1.5 to 3.2 kPa (compared with 0.8–4.2 kPa in our study) and relative responses were variable at high VPD_L , that is, when stomatal conductance was low (Gunderson et al., 2002). Thus, while our results indicate that the benefits of reduced stomatal limitation to CO_2 diffusion under eCO_2 may be greater when atmospheric water deficit is high, further studies are needed to determine whether this is true of other systems.

Elevated temperature had positive, neutral or negative effects on photosynthesis depending on species and rainfall level. We expected that responses to elevated temperature would be less positive (or more negative) under reduced compared with ambient rainfall (H7), as low soil moisture can reduce or reverse positive responses to warming (Reich et al., 2018). However, the most positive response to elevated temperature occurred in *A. repens* under reduced rainfall, contrary to H7, and was accompanied by enhanced \hat{V}_{cmax} . Only the response of the legume *L. capitata* supported H7, with a greater positive response of photosynthesis (and \hat{V}_{cmax}) to elevated temperature at ambient compared with reduced rainfall for this species. The lack of consistently greater responses of photosynthesis to elevated temperature at the higher level of rainfall may be explained by apparent acclimation to soil water deficits in some species.

5 | CONCLUSIONS

Our study is, to our knowledge, the first to report responses of leaf gas exchange to independent and combined manipulations of CO_2 , N, rainfall and temperature in a field study. This lack of prior evidence highlights the enormous knowledge gap that remains for multiple global change effects on plant function (Reich et al., 2014). Overall, our results show that interactions among environmental factors that are changing globally may complicate simple predictions based on theoretical expectations of the main effects of each factor. For instance, responses of photosynthesis to eCO_2 may be greater at increased soil N supply for some non-leguminous species when grown in mixtures and under reduced rainfall or high VPD for certain species from various herbaceous functional groups. Responses to warming may be positive, neutral or negative depending on rainfall, species and perhaps acclimation to low soil moisture. Though responses were often species-dependent in our study, we have little basis to predict for which species particular global change factors or combinations of factors will most influence photosynthesis; further work is needed to elucidate the patterns most likely to predominate within and among ecosystems. Thus, these highly context-dependent leaf-level

physiological responses may not be useful for predicting the net impact of multiple, interactive global changes on plant growth and ecosystem C balance.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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