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Water availability affects seasonal CO₂-induced photosynthetic enhancement in herbaceous species in a periodically dry woodland

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Abstract
Elevated atmospheric CO₂ (eCO₂) is expected to reduce the impacts of drought and increase photosynthetic rates via two key mechanisms: first, through decreased stomatal conductance (gₛ) and increased soil water content (Vₛ𝑤ᴄ) and second, through increased leaf internal CO₂ (Cᵢ) and decreased stomatal limitations (Sᵢₗₘ). It is unclear if such findings from temperate grassland studies similarly pertain to warmer ecosystems with periodic water deficits. We tested these mechanisms in three important C₃ herbaceous species in a periodically dry Eucalyptus woodland and investigated how eCO₂-induced photosynthetic enhancement varied with seasonal water availability, over a 3 year period. Leaf photosynthesis increased by 10%–50% with a 150 µmol mol⁻¹ increase in atmospheric CO₂ across seasons. This eCO₂-induced increase in photosynthesis was a function of seasonal water availability, given by recent precipitation and mean daily Vₛ𝑤ᴄ. The highest photosynthetic enhancement by eCO₂ (>30%) was observed during the most water-limited period, for example, with Vₛ𝑤ᴄ <0.07 in this sandy surface soil. Under eCO₂ there was neither a significant decrease in gₛ in the three herbaceous species, nor increases in Vₛ𝑤ᴄ, indicating no “water-savings effect” of eCO₂. Periods of low Vₛ𝑤ᴄ showed lower gₛ (less than ≈ 0.12 mol m⁻² s⁻¹), higher relative Sᵢₗₘ (>30%) and decreased Cᵢ under the ambient CO₂ concentration (aCO₂), with leaf photosynthesis strongly carboxylation-limited. The alleviation of Sᵢₗₘ by eCO₂ was facilitated by increasing Cᵢ, thus yielding a larger photosynthetic enhancement during dry periods. We demonstrated that water availability, but not eCO₂, controls gₛ and hence the magnitude of photosynthetic enhancement in the understory herbaceous plants. Thus, eCO₂ has the potential to alter vegetation functioning in a periodically dry woodland understory through changes in stomatal limitation to photosynthesis, not by the “water-savings effect” usually invoked in grasslands.

KEYWORDS
C₃ herbaceous species, elevated atmospheric CO₂, EucFACE, free-air CO₂ enrichment, net photosynthesis enhancement, stomatal limitations to photosynthesis, water limitation to productivity
INTRODUCTION

Grass–tree mixtures such as savannas and woodlands occupy extensive areas in tropical and subtropical regions and are characterized by strong seasonal variation in water availability (Baudena et al., 2015; Polley, Mayeux, Johnson, & Tischler, 1997). Due to the ongoing rise in atmospheric CO₂ these ecosystems are expected to undergo ecological changes via seedling establishment during dry periods (Bond & Midgley, 2000), changes in tree-grass interactions (Baudena et al., 2015), woody plant encroachment (Higgins & Scheiter, 2012), and altered fire regimes from the build-up of organic matter (Bond & Midgley, 2012). These changes may have profound effects on the structure and functioning of savannas and woodlands, with potentially large but unquantified implications for their capacity to sequester carbon and regulate water balances (Huxman et al., 2005; Prober et al., 2012). In spite of their importance for local and regional carbon and water cycles (Higgins & Scheiter, 2012; Snyder, Delire, & Foley, 2004), there is a significant knowledge gap in responses of savannas and woodlands to elevated atmospheric CO₂ (eCO₂) concentrations (Leakey, Bishop, & Ainsworth, 2012). Consequently, the expected impacts of eCO₂ on these warm ecosystems have been based on findings from cold temperate ecosystems (Leakey et al., 2012). Tropical and subtropical savannas and woodlands differ from cold temperate ones in important attributes like temperature, seasonal and total precipitation, maximal evapotranspiration and type of nutrient limitation (Cernusak et al., 2013), suggesting different and potentially larger responses to eCO₂ in these ecosystems on the basis of being warmer and drier than northern hemisphere temperate systems (Hickler et al., 2008). Both higher temperature and periodic low soil moisture have been hypothesized to increase the responsiveness to eCO₂ (Higgins & Scheiter, 2012; Morgan et al., 2011). Hence, there is a need for experiments addressing effects of eCO₂ on woodlands, in order to improve our ability to predict their vulnerabilities to climate change and improve their representations in Earth system models (Cernusak et al., 2013; Norby et al., 2016).

In general, eCO₂ increases CO₂ assimilation rates and plant biomass, decreases stomatal conductance and leaf nitrogen concentrations and increases water-use efficiency (Ainsworth & Rogers, 2007; Ellsworth et al., 2004; Morgan et al., 2011). However, the magnitude of these linked responses also depends on the availability of other resources such as soil nutrients and water (Rastetter & Shaver, 1992). Water availability is a primary factor limiting growth and productivity in many ecosystems including grasslands (Knapp et al., 2002), savannas, and woodlands (Baudena et al., 2015; Polley et al., 1997) so the response of these ecosystems to eCO₂ will in part depend upon water availability. One important way, through which eCO₂ is expected to ameliorate the negative impact of water limitation, is by stomatal closure resulting in decreased plant water use and increased soil water content (Morgan et al., 2011; Morgan et al., 2004). The increase in soil water content under eCO₂, also termed a “water-savings effect”, has led to the generalization that plant photosynthesis and productivity responses to eCO₂ will be strongest in dry conditions (Duursma & Medlyn, 2012; Ellsworth et al., 2012) although it is unclear if this best applies to short or long dry periods. Still, the generalization has been used to rationalize why the eCO₂-induced enhancement response of deserts will be large (Jordan et al., 1999), why arid and semi-arid zones have shown greening and shrub encroachment over the past 20 years (Ahlström et al., 2015; Donohue, Roderick, Mcvicar, & Farquhar, 2013) and why the eCO₂-induced enhancement of grasslands is larger in dry vs. wet years (Owensby, Ham, Knapp, & Auen, 1999). Hence, this particular phenomenon deserves closer investigation especially in water-limited ecosystems because even small increases in soil water content in dry climate zones can have significant effects on processes such as growing season length (Reyes-Fox et al., 2014), nutrient mineralization and organic matter decomposition (Morgan et al., 2004; Wullschleger, Tscharntek, & Norby, 2002), and survival of plants during dry periods (Bond & Midgley, 2012). Furthermore, earlier evidence from northern hemisphere temperate grasslands indicate that the extent, timing and duration of eCO₂-induced “water-savings effect” varies (Morgan et al., 2004) and may be determined by factors like species-specific water-use efficiencies (Blumenthal et al., 2013; Dijkstra, Blumenthal, Morgan, Lecain, & Follett, 2010), changes in leaf area index and canopy temperature (Gray et al., 2016; Kelly, Duursma, Atwell, Tissue, & Medlyn, 2016), and soil texture (Fay et al., 2012; Polley, Jin, & Fay, 2012). Although the eCO₂-induced increase in soil water content has been demonstrated for temperate grasslands (Blumenthal et al., 2013; Lecain, Morgan, Mosier, & Nelson, 2003; Morgan et al., 2011), it has not been substantiated for warm-climate savannas or woodlands. These occur in zones where potential evapotranspiration can exceed mean annual precipitation, so that the “water-savings effect” induced by eCO₂ may reduce such deficits.

While tests of the “water-savings effect” hypothesis largely emanate from a number of short-term glasshouse and controlled-environment studies (e.g., Dijkstra et al., 2010; Polley et al., 2012; Volk, Niklaus, & Körner, 2000), only a few field-based studies in grasslands support the corollary that photosynthesis and productivity responses to eCO₂ are strongest in dry seasons or years (Belote, Weltzin, & Norby, 2004; Lecain et al., 2003; Morgan et al., 2011, 2004; Niklaus & Körner, 2004). Some studies suggest that eCO₂ effect can be strongest in wet years (Morgan et al., 2004; Naumburg et al., 2003; Newingham et al., 2013; Smith et al., 2000; but see Norby & Zak, 2011). Water demand for herbaceous species varies seasonally (Knapp et al., 2002) suggesting that the benefit of eCO₂-induced water-savings should differ across seasons on the basis of their differences in water availability (Hovenden, Newton, & Wills, 2014). An understanding of the relationship between seasonal water availability and eCO₂ effect is essential since large changes in the timing of rainfall in seasonally dry regions are anticipated by climate models, even where total annual rainfall will remain unchanged (Berg et al., 2016; Sillmann, Kharin, Zhang, Zwiers, & Bronaugh, 2013).

In addition to a water-savings effect, another important mechanism through which C₃ plants might benefit from CO₂ fertilization during water-limited periods is via alleviation of diffusional
limitations (Lawlor, 2002). Stomatal closure, one of the first events to occur during water stress (Chaves et al., 2002), results in significant limitations on plant CO₂ assimilation. This restriction of stomata to CO₂ supply, also termed as stomatal limitation, decreases leaf intercellular CO₂ concentrations (Cᵢ) as well as photosynthetic rates (Grassi & Magnani, 2005; Lawlor, 2002). Thus, an important consequence of higher stomatal limitations in dry conditions is that plants operate on the steep linear phase of the photosynthetic CO₂ response curve (Ellsworth et al., 2012). Under such conditions, CO₂ fertilization can help alleviate the stomatal limitations by increasing Cᵢ and hence plants would experience larger photosynthetic enhancement (Kelly et al., 2016; Lawlor, 2002). The importance of such limitations in controlling eCO₂-induced photosynthetic enhancement during dry periods has rarely been studied in the field (Galmés, Medrano, & Flexas, 2007; Grassi & Magnani, 2005) and has not been investigated in eCO₂.

Building on knowledge from previous ecosystem studies (see Leakey et al., 2012), we examined eCO₂ responses of an herbaceous understory community in the Eucalyptus Free-Air CO₂ Enrichment Experiment (EucFACE). The EucFACE experiment is located in a mature, undisturbed Eucalyptus woodland in south eastern Australia which shows strong seasonal and interannual variability in precipitation (Gimeno et al., 2016). The 30 year mean potential evapotranspiration exceeded precipitation by 40%, evidence that water deficits are frequent (Duursma et al., 2016). These attributes provide a unique opportunity to test the mechanisms responsible for eCO₂ response in a periodically water-limited woodland ecosystem. We hypothesized that: H₁: Maximum photosynthetic enhancement by eCO₂ will be observed in dry seasons; H₂: This photosynthetic enhancement will be mediated by a decrease in stomatal conductance in eCO₂ and hence increases in soil water content; H₃: Elevated CO₂ will reduce stomatal limitations induced by stomatal closure during the dry periods thus resulting in increased photosynthetic rates.

To test the above hypotheses, we measured leaf CO₂ assimilation and stomatal conductance of a dominant C₃ grass across seasons over 3 years, as well as corroborating evidence from two sympatric C₃ forbs over 1½ years.

2 | MATERIALS AND METHODS

2.1 | Experimental design and site description

We conducted leaf-level gas exchange measurements on herbaceous understory in the first 3 years of the Eucalyptus Free-Air CO₂ Enrichment (EucFACE) experiment. EucFACE consists of six 25 m diameter circular plots or rings, with three of these maintained at ambient CO₂ (aCO₂) and three maintained at elevated CO₂ (ambient + 150 μmol mol⁻¹, eCO₂) since February 2013 (see Gimeno et al., 2016). CO₂ treatment was completely randomized among the six plots at the outset.

This experiment is located in a remnant patch of native Cumberland Plain Woodland (CPW) near Richmond, NSW Australia (33°57'S, 150°44.3'E) with substantial understory cover dominated by a C₃ grass, locally termed a grassy Eucalyptus woodland. The relatively high species diversity of this vegetation type (>60 species) is attributed to the herbaceous understory vegetation (Tozer, 2003) comprising a mixture of C₃ grasses, C₃ forbs, and C₄ grasses. *Microtis* *purpurascens* *Labill.*, a native perennial C₃ grass, is the dominant herbaceous species at EucFACE (~ 70% of total understory biomass, Pathare, 2017) along with the co-occurrence of C₃ forbs like *Lobelia purpurascens* R.Br., C₄ grasses like *Cymbopogon refractus* R.Br., and naturalized species such as *Senecio madagascariensis* Poir. We measured three common C₃ herbaceous understory species in our study: the dominant C₃ grass (*M. stipoides*) and two prevalent C₃ forbs (*L. purpurascens* and *S. madagascariensis*), denoted in figures by the genus initial and the first three letters of the species name.

The climate of the site is warm-tropical with a mean annual temperature of 17°C, characterized by a mean daily maximum temperature of 30.0°C during the warmest month (January) and 17.6°C during the coldest month (July) (http://www.bom.gov.au/climate/averages/tables/cw_067105.shtml) (Figure 1a). It is seasonally water-limited with a 20-year average annual precipitation of 800 mm and an estimated annual pan evapotranspiration of 1350 mm (Australian Bureau of Meteorology, station 067105, 8 km from the site; www.bom.gov.au). Precipitation timing is variable, with larger monthly rainfall amounts received mostly during summers (December through February in southern hemisphere). However, substantial amounts of rainfall occur periodically throughout the year thus resulting in multiple seasonal wet-dry cycles (Figure 1b). The soil at the site is a well-drained, sandy loam with low organic carbon content (Gimeno et al., 2016).

2.2 | Gas exchange measurements at EucFACE and model fitting

For measurements, the year was divided into four major seasons comprising summer (December to February), autumn (March to May), winter (June to August), and spring (September to November). Leaf-level gas exchange measurements were conducted at 4 time points per year, with each time point representing a season of the year. Measurements began, 1 week after initiation of full CO₂ fumigation, in February 2013, on *M. stipoides* as the dominant herbaceous species in the ecosystem, and two prevalent C₃ forb species (*L. purpurascens* and *S. madagascariensis*) were added starting from October 2014.

A set of portable infrared photosynthesis systems (Li-COR 6400XT; Li-COR Inc., Lincoln, NE, USA) with 6 cm² chambers were used for gas exchange measurements. In order to assess instantaneous and long-term effects of eCO₂ on the photosynthetic capacities of the species, photosynthetic CO₂ response curves (Aₙₐₑᵗ−Cᵢ curves) were measured, starting at the mean growth CO₂ concentration for each treatment (~ 400 μmol mol⁻¹ for aCO₂ and ~ 550 μmol mol⁻¹ for eCO₂). Average daytime CO₂ concentrations at the ground layer 20 cm above the soil were 582 ± 8.1 μmol mol⁻¹, measured at 8 points within each plot compared to the target of
ambient + 150 μmol mol$^{-1}$ (Craig McNamara, personal communication). Multiple nonoverlapping leaves were placed across the Li-COR chamber and a minimum time of 15 min at light saturation was allowed for stabilization of gas exchange before commencing measurements. After stabilization, an initial measurement of net CO$_2$ assimilation rate ($A_{net}$, μmol m$^{-2}$ s$^{-1}$), stomatal conductance ($g_s$, mol m$^{-2}$ s$^{-1}$), intercellular CO$_2$ ($C_i$, μmol mol$^{-1}$), and the ratio of intercellular to growth CO$_2$ ($C_i/C_1$) was conducted at growth CO$_2$ concentration, followed by the $A_{net}$--$C_i$ response curves. $A_{net}$--$C_i$ curves for the three species were done with a minimum of 10 different steps of CO$_2$ concentrations, ranging from 40 μmol mol$^{-1}$ to 1800 μmol mol$^{-1}$, while maintaining saturating light conditions (photon flux density of 1800 μmol m$^{-2}$ s$^{-1}$), 55%–65% relative humidity and prevailing leaf temperatures ($T_{leaf}$ °C). The canopy openings in this Eucalyptus woodland are relatively large with tree canopy leaf area index <2 (Duursma et al., 2016) and the high intensity sun flecks (>1000 μmol m$^{-2}$ s$^{-1}$) lasting about 30 min/day during summer and spring. Understory species rely on the sun flecks for achieving a majority of daily carbon gain (Chazdon & Pearcy, 1991). Hence, saturating light levels of 1800 μmol m$^{-2}$ s$^{-1}$ were used for gas exchange measurements to better reflect the rates during sun flecks. $T_{leaf}$ during the gas exchange measurement corresponded to the prevailing mean daily maximum air temperatures ($T_{air}$) during each measurement season (18, 22, 27, and 29°C for winter, autumn, spring, and summer, respectively) (Figure 1a). Measurements were taken during sunny days (09:30–14:30 local time) on fully expanded leaves exposed to sunlight. At least two measurements per CO$_2$ plot per species were undertaken at every time point and all measurements were completed over the course of 3 days. After each $A_{net}$--$C_i$ response curve, leaves were marked to assess the correct leaf area in the chamber, collected in self-sealing polythene bags, labeled and immediately placed on ice until further analyses. In the laboratory, the projected leaf area of the marked leaves in Li-COR 6400XT chamber was determined (Win Rhizo software, Regent Instruments Inc., Québec City, Canada) and gas exchange measurements were recalculated accordingly.

$A_{net}$--$C_i$ curves were then fit using the biochemical model of Farquhar, von Caemmerer, and Berry (1980), in order to obtain kinetic coefficients associated with rates of maximum carboxylation ($V_{cmax}$, μmol m$^{-2}$ s$^{-1}$) and electron transport ($J_{max}$, μmol m$^{-2}$ s$^{-1}$) (see Crous et al., 2013; Duursma 2015). While estimating the rates of $V_{cmax}$ and $J_{max}$ we used a fixed mesophyll conductance value (0.2 mol m$^{-2}$ s$^{-1}$ for perennial herbaceous species; Flexas, Ribas-Carbó, Díaz-Espejo, Galmés, & Medrano, 2008) to reflect the finite characteristics of this trait. The temperature responses of $V_{cmax}$ and $J_{max}$ are important to consider in model fitting (Medlyn, Loustau, & Delzon, 2002), especially as seasonal temperatures varied. In order to do this, we carried out temperature response measurements on M. stipoides following a procedure modified from Crous et al. (2013) (Supporting material; Supplementary methods for a description of the temperature response measurements). The temperature response of $V_{cmax}$ was fit in R (v3.2.2, R Foundation for Statistical Computing, Vienna, Austria) using the modified form of an Arrhenius function (peaked function; see Harley, Thomas, Reynolds, & Strain, 1992 and Medlyn et al., 2002). The resulting kinetics derived by fitting the modified Arrhenius function for $V_{cmax}$ were used in the “fitacis” function in the plantecophys package (Duursma, 2015) to obtain a temperature-normalized $V_{cmax}$ ($V_{cmax,25}$) from the $A_{net}$--$C_i$ response curves.

### 2.3 Relative stomatal limitations

Limitations to light-saturated CO$_2$ assimilation rates primarily occur through restrictions to the diffusion of CO$_2$ into intracellular leaf spaces, in liquid-phase to the chloroplast, or due to the biochemistry of CO$_2$ fixation at the chloroplast. Among these, the gas-phase diffusional limitations to CO$_2$, also termed as stomatal limitation, is controlled by stomata and requires computing the theoretical rates for $A_{net}$ assuming a fractional increase in $g_s$ and $C_i$. Thus, relative stomatal limitations ($S_{lim}$; fraction of total) can be defined as the ratio of change in CO$_2$ assimilation resulting from changes in $g_s$ to the total measured change in CO$_2$ assimilation resulting from the other processes (Wilson et al., 2000). $S_{lim}$ to photosynthesis were obtained by
modeling the diffusional pathway and based on the $A_{\text{net}}$–$C_i$ response curves. For calculating $S_{\text{lim}}$ to $C_0$ assimilation rates, we used the approach proposed by Grassi and Magnani (2005) which is similar to that defined in Jones (1985). We computed $S_{\text{lim}}$ as follows:

$$S_{\text{lim}} = \frac{\partial A_{\text{net}}/\partial C_i}{\partial A_{\text{net}}/\partial C_i}$$

(1)

where, $\partial A_{\text{net}}/\partial C_i$ is the partial derivative of net CO2 assimilation ($A_{\text{net}}$) for a relative change in leaf internal CO2 ($C_i$) and $\partial A_{\text{net}}/\partial C_i$ is the stomatal conductance to CO2 ($g_{\text{sc}}$). Our approach uses a static mesophyll conductance to CO2 ($g_{\text{mes}}$ of 0.2 mol m$^{-2}$ s$^{-1}$) as the study was focused on the whole-leaf scale, and the magnitude of $S_{\text{lim}}$ is not strongly affected by the inclusion of mesophyll conductance effects (Grassi & Magnani, 2005).

In addition to $S_{\text{lim}}$, we also derived $C_i$ difference using the $A_{\text{net}}$–$C_i$ responses curves. $C_i$ difference was calculated as the difference between the transition $C_i$ (or $C_i$ at the $V_{\text{cmax}}$–$J_{\text{max}}$ transition point) and operating $C_i$ (or $C_i$ under growth CO2 levels). It was thus an indicator of how high the operating $C_i$ is on the linear slope of the $A_{\text{net}}$–$C_i$ response curve.

2.4 Other field measurements

Values for mean daily $T_{\text{air}}$ were obtained from a temperature and humidity sensor (HMP 155 Vaisala, Vantaa, Finland) located at 2 m above ground in all six plots, while values for total precipitation (mm day$^{-1}$) were obtained from automated tipping buckets (Tipping Bucket Rain gauge TB4, Hydrological Services Pty Ltd, Liverpool, NSW, Australia) at the top of a tower in each of three plots. Data obtained from both sensor types were logged every 10 s and recorded every 15 min using CR3000 data loggers (Campbell Scientific, Logan, UT, USA). Volumetric soil water content ($V_{\text{SWC}}$; v/v) was measured up to a depth of 30 cm with permanently installed time-domain reflectometry probes inserted into the soil at a 45° angle (eight per plot; CS650-L; Campbell Scientific, Logan, UT, USA). $V_{\text{SWC}}$ content data were recorded at 15 min intervals by a data logger in each plot (C3000; Campbell Scientific, Logan, UT, USA). In our study, we report the daily averages for the plot-average $V_{\text{SWC}}$ measurements under aCO2 and eCO2 treatments. In addition to $V_{\text{SWC}}$, the field capacity for the top layer soil of the EucFACE facility was determined by using soil moisture release curves (Campbell & Norman, 2000) measured with pressure plates. Based on curve analysis, the field capacity and water potential of this sandy loam was determined to be 0.18 v/v.

2.5 Statistical analysis

Statistical analyses were performed using R (v3.2.2, R Foundation for Statistical Computing, Vienna, Austria). The EucFACE facility consists of three ambient and three elevated CO2 rings and hence the number of replicates was three for each of the two levels of CO2 treatment. The overall dataset was unbalanced with regard to the number of species measured and the measurement months. For $M$. stipoides, gas exchange measurements were carried out in at least two locations in each of the six rings across 13 measurement time points over 3 years. Similarly, for the other two $C_3$ species ($L$. purpurascens and S. madagascariensis), gas exchange measurements were carried out for 7 measurement time points (1.5-3 years). A mixed-model split-plot ANOVA with interactions was performed for the physiological and biochemical parameters $A_{\text{net}}$, $V_{\text{cmax}}$–$J_{\text{max}}$, $N$ content, $g_{\text{sc}}$, $C_i$, $S_{\text{lim}}$, and $C_i$ difference, with CO2 treatment as a whole-plot factor and measurement time point as a split-plot factor. Appropriate tests were conducted to check the data for normality and equal variances and wherever necessary, log or square root transformations were used to improve the homoscedasticity of data (Zar, 2007). Linear mixed effects models were fitted using the ‘lme’ function within the nlme package (Pinheiro, Bates, Debry, & Sarkar, 2016). Values of $p < 0.02$ were considered as statistically significant, because we used the Benjamini–Hochberg procedure for the number of tests we did to control the false discovery rate (Benjamini & Hochberg, 1995). In addition to the mixed level split-plot ANOVA, regression analyses were performed in order to examine the relationships between key variables of interest, particularly with regard to eCO2-induced $A_{\text{net}}$ enhancement. These key variables were chosen according to their causal hypothesized roles in regulating eCO2-induced photosynthetic enhancement (Ellsworth et al., 2012; see Supplemental Information for further details). We also employed Structural Equation Modeling approaches (Lamb, Shirltiffe, & May, 2011) to understand the processes underlying the relationships among variables describing photosynthetic enhancement by eCO2 using the lavaan package in R (Rosseel, 2012; see Supplemental Information). We used generalized additive models (mgcv package; Wood, 2006) to visualize the seasonal trends in $V_{\text{SWC}}$ and test the differences between the CO2 treatments during the 3 years of this experiment. Although both $C_i$ and $S_{\text{lim}}$ are recursive variables depending on both $A_{\text{net}}$ and $g_{\text{sc}}$ (Eq. 1), we included them in the structural equation models (Figure 7 and Figs. S6-S8) as they are key parts of the overall hypotheses we asked.

3 RESULTS

3.1 Effect of CO2 and measurement time on $A_{\text{net}}$ and $g_{\text{sc}}$

$Microlaena$ stipoides was the dominant herbaceous species in the grassy woodland understory, and thus it was measured more intensively than the other species. CO2 enrichment by 150 μmol mol$^{-1}$ resulted in a significant increase in $A_{\text{net}}$ (≈ 28%) across species measured for 7 time points from 1.5 to 3 years after the start of CO2 enrichment ($p = .009$, Table 1, Figure 2a–c). Similarly, for the dominant $M$. stipoides, eCO2 resulted in a significant increase in $A_{\text{net}}$ (≈ 32%) across the 13 time points across 3 years ($p = .019$, Table S1,
photosynthetic enhancement. We will now further look into the sources of the variations in seasonal 

Figure 3a). There was a significant measurement time effect on $A_{\text{net}}$ across species ($p < .001$, Table 1 and S1, Figure 2a–c) with average values ranging from 17 ± 3.2 μmol m$^{-2}$ s$^{-1}$ during the warmer times (October 2015 and February 2016) to 11 ± 2.4 μmol m$^{-2}$ s$^{-1}$ during the cooler time points (May 2015 and April 2016). For $M$. stipoides, maximum $A_{\text{net}}$ (12 ± 1.5 μmol m$^{-2}$ s$^{-1}$) occurred during the wet and warmer times (February 2013, February 2014, October 2014, and February 2015), with minimum $A_{\text{net}}$ of -5 ± 1.2 μmol m$^{-2}$ s$^{-1}$ occurring in two dry periods, October 2013 and July 2014. We did not observe a significant CO$_2$ x measurement time effect on $A_{\text{net}}$ across the three species ($p > .02$, Table 1 and S1). Similar to seasonal variation in $A_{\text{net}}$, the percent increase in photosynthetic rates due to eCO$_2$ also varied along seasonal time points, with values ranging from 12% to 53%. The maximum increase in photosynthetic rates due to CO$_2$ treatment across the species was observed during February 2016 (40%) and the minimum was observed in February 2015 (13%). Similarly, for the dominant $M$. stipoides, the maximum increase in $A_{\text{net}}$ due to eCO$_2$ was observed in October 2013 (62%), whereas minimum increase was reported in February 2014 (13%). Overall, we observed a significant seasonal variation in the $A_{\text{net}}$ values and the magnitude of eCO$_2$-induced photosynthetic enhancement across all the species (Figure 2a–c). We will now further look into the sources of the variations in seasonal photosynthetic enhancement.

There was no CO$_2$ treatment effect on $g_s$ across the species ($p > .02$, Table 1, Figure 2d–f). However, there were highly significant measurement time effects on $g_s$ in all species ($p < .01$, Table 1 and Table S1) with average values ranging from maximum of 0.27 ± 0.03 mol m$^{-2}$ s$^{-1}$ in October 2015 and February 2016 to a minimum of 0.18 ± 0.02 mol m$^{-2}$ s$^{-1}$ in May 2015 and April 2016. For $M$. stipoides, maximum $g_s$ (0.17 ± 0.02 mol m$^{-2}$ s$^{-1}$) was observed during warmer time points (February 2013, February 2014, October 2014 and February 2015), whereas minimum $g_s$ was observed in October 2013 and July 2014 as noted above for $A_{\text{net}}$. Given that higher $A_{\text{net}}$ values were observed during time points with higher $g_s$ (Figure 2), the seasonal variation in $A_{\text{net}}$ could be partly ascribed to seasonal variation in the $g_s$. This dependence of $A_{\text{net}}$ on $g_s$ is evident from the positive correlation between $A_{\text{net}}$ and $g_s$ for the three species under both, aCO$_2$ ($r^2 = .64$, $p < .01$, Fig. S1a) and eCO$_2$ ($r^2 = .57$, $p < .01$, Fig. S1b) concentrations.

3.2 | Effect of water availability on $A_{\text{net}}$, $g_s$ and eCO$_2$-induced $A_{\text{net}}$ enhancement

Water supply and use is important to physiological activities of herbaceous species in other ecosystems (Knapp et al., 2002). Thus, in order to understand the effect of water availability on $A_{\text{net}}$, $g_s$, and eCO$_2$-induced $A_{\text{net}}$ enhancement in our study, these parameters were plotted as a function of seasonal water availability, determined as the recent week total precipitation and mean daily V$_{SWC}$ (Figure 3). The recent week for these measures was the 7 days prior to the initiation of gas exchange measurements at the EuCFACE. Figure 3a–d shows the responses of $A_{\text{net}}$ and $g_s$, respectively, for the dominant $M$. stipoides species, with respect to seasonal water availability. Lower values for $A_{\text{net}}$ (<9 μmol m$^{-2}$ s$^{-1}$; Figure 3a, b) and $g_s$ (<0.12 mol m$^{-2}$ s$^{-1}$; Figure 3c, d) were mostly observed during time points when recent week precipitation was <10 mm (Figure 3a, c) and mean daily V$_{SWC}$ was <0.10 v/v (Figure 3b, d). Figure 3e–h shows the effect of water availability on eCO$_2$-induced $A_{\text{net}}$ enhancement. For all the C$_3$ species considered together, eCO$_2$-induced $A_{\text{net}}$ enhancement was negatively correlated with both, total precipitation ($r^2 = .38$, $p < .01$, Figure 3e) and mean daily V$_{SWC}$ ($r^2 = .49$, $p < .01$, Figure 3f) of the preceding week. Similarly, for $M$. stipoides, eCO$_2$-induced $A_{\text{net}}$ enhancement was a decreasing function of total precipitation ($r^2 = .56$, $p < .01$, Figure 3g) and mean daily V$_{SWC}$ ($r^2 = .64$, $p < .01$, Figure 3h) of the preceding week. Overall, a photosynthetic enhancement of >20% under eCO$_2$ was observed during the relatively water-limited time points when the recent week total precipitation was <10 mm and mean daily V$_{SWC}$ was <0.10 v/v. Thus, there was evidence that water was an important regulator of $A_{\text{net}}$, $g_s$, and eCO$_2$-induced $A_{\text{net}}$ enhancement.

3.3 | Effect of CO$_2$ and measurement time on biochemical parameters

To understand the underlying biochemical regulation of $A_{\text{net}}$, we focused on V$_{\text{cmax}}$ and J$_{\text{max}}$, the parameters that are derived from the photosynthesis model of Farquhar et al. (Farquhar et al., 1980) and leaf N content. Although there was no significant CO$_2$ effect on the V$_{\text{cmax}}$ and J$_{\text{max}}$ values across the species ($p > .02$, Table S2 and S3, Fig. S3), we observed a highly significant measurement time effect on both the parameters ($p < .01$, Table S2 and S3). There was evidence of different species responses for these parameters (Fig. S3). Variation in V$_{\text{cmax}}$ and J$_{\text{max}}$ could be attributed to the variation in the measurement time weather conditions and the inherent temperature dependencies of these two biochemical parameters. Thus, V$_{\text{cmax}}$ and J$_{\text{max}}$ were normalized to a common standard temperature of 25°C using the activation energy and entropy parameters derived from instantaneous temperature responses of $M$. stipoides as indicated in supplementary methods (see Supporting Material). Although there was a significant measurement time effect on the normalized parameters (V$_{\text{cmax}}$-25 and J$_{\text{max}}$-25) across the species ($p < .01$, Table 1 and S1, Figure 4), they were less variable over measurement time compared to non-normalized V$_{\text{cmax}}$ and J$_{\text{max}}$ (Fig. S3). When averaged across the three species and CO$_2$ treatments, maximum values for V$_{\text{cmax}}$-25 and J$_{\text{max}}$-25 (80 ± 13.06 μmol m$^{-2}$ s$^{-1}$ and 129 ± 5.23 μmol m$^{-2}$ s$^{-1}$, respectively) were observed in October 2014 and October 2015.

We did not observe a significant CO$_2$ effect on V$_{\text{cmax}}$-25 and J$_{\text{max}}$-25 across the species ($p > .02$, Tables 1 and S1 and Figure 4). However, there was a nonsignificant CO$_2$ x measurement time interaction effect on V$_{\text{cmax}}$-25 and J$_{\text{max}}$-25 ($p < .1$, Tables 1 and S1 and Figure 4). In particular, there was a trend toward lower V$_{\text{cmax}}$-25 and J$_{\text{max}}$-25 under eCO$_2$ during October 2014 in $M$. stipoides and during October 2014 and October 2015 in L. purpureascens. Trends similar to V$_{\text{cmax}}$ and J$_{\text{max}}$ were also observed for leaf N content. There were no significant CO$_2$ or CO$_2$ x measurement time interaction effects on the
TABLE 1  Results of mixed-model split-plot ANOVA for net photosynthesis (Anet), temperature-normalized maximum carboxylation (Vcmax-25) and electron transport rates (Jmax-25), N content on area basis (Narea), stomatal conductance (gs), relative stomatal limitation (Slim), and Ci difference as the difference between the transition Ci and operating Ci, across the three C3 species measured for seven seasonal time points. Results shown are across M. stipoides, L. purpurascens, and S. madagascariensis. CO2 refers to the CO2 treatment and time refers to the seasonal time points during which measurements were carried out. p values for the split-plot ANOVA are shown in bold for significant effects when the false discovery rate is controlled using the Benjamini-Hochberg procedure. Three-way interactions were not statistically significant (p > .02) and hence are not shown in the table. The numerator degrees of freedom (df) are given for the statistical tests.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Variables</th>
<th>CO2</th>
<th>Time</th>
<th>Species</th>
<th>CO2 x Time</th>
<th>Species x CO2</th>
<th>Species x Time</th>
</tr>
</thead>
<tbody>
<tr>
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<td>df</td>
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<td>p value</td>
<td>df</td>
<td>F value</td>
<td>p value</td>
<td>df</td>
</tr>
<tr>
<td>Anet</td>
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<td>23.18</td>
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<td>6</td>
<td>13.85</td>
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</tr>
<tr>
<td>Vcmax-25</td>
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<td>0.815</td>
<td>6</td>
<td>4.95</td>
<td>0.002</td>
<td>2</td>
</tr>
<tr>
<td>Jmax-25</td>
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<td>0.32</td>
<td>0.602</td>
<td>6</td>
<td>8.69</td>
<td>&lt;0.001</td>
<td>2</td>
</tr>
<tr>
<td>Narea</td>
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<td>0.09</td>
<td>0.771</td>
<td>6</td>
<td>5.62</td>
<td>&lt;0.001</td>
<td>2</td>
</tr>
<tr>
<td>gs</td>
<td>1</td>
<td>2.35</td>
<td>0.200</td>
<td>6</td>
<td>5.94</td>
<td>0.002</td>
<td>2</td>
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<tr>
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<td>0.172</td>
<td>6</td>
<td>5.09</td>
<td>0.002</td>
<td>2</td>
</tr>
<tr>
<td>Ci difference</td>
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<td>0.002</td>
<td>6</td>
<td>2.99</td>
<td>0.025</td>
<td>2</td>
</tr>
</tbody>
</table>

*All variables were transformed (square root or log transformation) to meet the normality assumptions for the mixed-model ANOVA.
significant measurement time effects on Ci difference of time interaction effect on Slim and Ci difference across the species ($p > .01$, Table 1 and S1). When there was a significant overall CO2 effect (Table 1), post hoc treatment differences were denoted by * ($p < .05$; t test) [Colour figure can be viewed at wileyonlinelibrary.com]
with season in *L. purpurascens* and *S. madagascariensis*. Overall, there were no statistically significant three-way interaction effects (CO2 × measurement time × species) on any of the measured physiological and biochemical parameters in our study (*p* > .02, Table 1 and S2).

4 | DISCUSSION

During 3 years of this study, photosynthetic rates under eCO2 were almost 30% higher on average (Figure 2), which we expect would have led to an increase in above- or below-ground production. However, the relative enhancement in photosynthetic rates by eCO2 across species varied substantially between seasons, with values ranging from 12% to 53%. We investigated the mechanisms underlying the seasonal variation in photosynthetic responses to eCO2 in three herbaceous C3 species from a periodically dry Eucalyptus woodland, with a focus on water availability and stomatal limitations, recognizing that this would be the driver for biomass accumulation responses. Our first hypothesis was supported, as we observed maximum photosynthetic enhancement by eCO2 during the dry periods (VSWC < 0.07). In contrast to our second hypothesis, we did not observe a significant increase in VSWC under eCO2 or decrease in stomatal conductance. The results indicate that eCO2-induced photosynthetic enhancement during dry periods was the result of alleviation of stomatal limitation by increasing Cₐ, thus supporting our third hypothesis.

4.1 | Maximum eCO2-induced Aₙe enhancement is observed during dry periods

The grassy Eucalyptus woodland in this study experienced frequent seasonal wet and dry periods (Figures 1b and 5a). Since herbaceous species respond quickly to events of water availability (Knapp et al., 2002), water was expected to be an important environmental factor controlling growth, productivity and probably the eCO2 response in the herbaceous species of this
ecosystem. The relationship between seasonal water availability (total precipitation and mean daily VSWC of preceding week) and eCO2-induced A\text{net} enhancement (Figure 3e–h) indicated that maximum eCO2-induced A\text{net} enhancement occurred during relatively dry periods, that is, when the total precipitation in the week preceding the measurements was <10 mm (Figure 3e, g) or the mean daily VSWC was <0.10 v/v (Figure 3f, h). Similar relationships have been observed between A\text{net} enhancement ratio and soil water content by Lecain et al. (2003) and between biomass enhancement and precipitation by Morgan et al. (2004), both for herbaceous species from temperate grasslands. The relationship between A\text{net} enhancement ratio and seasonal water availability in our study is in agreement with these previous reports, and support our first hypothesis.

How is seasonal water availability related to the eCO2-induced photosynthetic enhancement and its variability? We argue that this relationship emerges out of stomatal control of photosynthetic rates across a range of soil moistures. Previous studies addressing the interaction effects of eCO2 and drought (Kelly et al., 2016; Lecain et al., 2003; Morgan et al., 2004; Niklaus & Körner, 2004) indicate that eCO2 can mitigate the impact of water limitation via two key mechanisms; first, decreased g_s under eCO2 resulting in increased soil water content or “water-savings effect” and second, lower g_s and higher S\text{lim} during drought resulting in increased C\text{i} and hence A\text{net} under eCO2. We evaluated these two mechanisms and discuss them in the following sections.

### 4.2 Elevated CO2 does not increase soil water content

Previous studies in water-limited temperate ecosystems have reported improved photosynthetic rates and productivity under eCO2 during dry conditions, generally attributed to decreased g_s and the linked increase in soil water content (Blumenthal et al., 2013; Lecain et al., 2003; Morgan et al., 2011, 2004), called the “water-savings effect”. Although we observed the maximum CO2-induced photosynthetic enhancement in dry periods (Figure 3e–h), stomatal conductance (g_s) did not significantly decrease under eCO2 (Figure 2d–f) even during dry periods (Figure 3c, d). Stomatal...
Conductance showed significant variation across seasons, but was similar under both aCO2 and eCO2 conditions (Figure 2d–f), thus indicating that plants under both CO2 treatments were constrained by the same diffusional limitations. Also, there was no detectable increase in mean daily VSWC under eCO2 compared to aCO2 at any time point during 3 years of this study, not even during the dry periods when we expected a significant increase in VSWC (Figure 5). Unlike temperate ecosystems (Blumenthal et al., 2013; Lecain et al., 2003; Morgan et al., 2011, 2004), the “water-savings effect” of eCO2 was absent in the ground layer and upper soil of this subtropical grassy Eucalyptus woodland, rejecting our second hypothesis. Thus, we do not expect such an effect on plant biomass accumulation for the grassy understory, although this remains to be tested.

4.3 Higher stomatal limitations and A\text{net} enhancement by eCO2 during dry periods

Given that we did not find decreased stomatal conductance in eCO2 and hence no “water-savings effect”, we investigated the possibility of changed stomatal limitations in eCO2. S\text{lim} was a function of water availability, especially mean daily V\text{SWC} (Figure 5). Unlike temperate ecosystems (Blumenthal et al., 2013; Lecain et al., 2003; Morgan et al., 2011, 2004), the “water-savings effect” of eCO2 was absent in the ground layer and upper soil of this subtropical grassy Eucalyptus woodland, rejecting our second hypothesis. Thus, we do not expect such an effect on plant biomass accumulation for the grassy understory, although this remains to be tested.

The “water-savings effect” of eCO2 has been expected to affect the structure and functioning of savannas and grassy woodlands through feedbacks on species composition, partly through the establishment of woody plant seedlings and tree-grass interactions (Bond & Midgley, 2012; Polley et al., 1997). For instance, the “water-savings effect” could favor the establishment of woody plant seedlings that were previously excluded due to low water availability (Polley et al., 1997) or could help lengthen the growing season, thus reducing the period when fires can occur (Bond & Midgley, 2012). An invasive grass, Microstegium, responded differently between years to eCO2 in a temperate plantation, which may be been due to interannual differences in soil moisture interacting with eCO2 (Belote et al., 2004). However, the above predictions might not be true in the case of warm-temperate grassy woodlands with periodic drought, as there was no evidence of eCO2-induced water savings in our study. We speculate that the dominance of C3 species in the ground layer at our site may have been a factor responsible for this finding, as suggested previously by Morgan et al. (2004).
such low $C_i$'s, CO$_2$ fertilization can facilitate the alleviation of $S_{\text{lim}}$ by increasing $C_i$, thus generating a larger photosynthetic enhancement during dry periods (Lawlor, 2002). In support to this prediction, we observed maximum increase in photosynthetic rates under eCO$_2$ when $S_{\text{lim}}$ were higher under aCO$_2$ concentrations (Figure 8a). A similar relationship was observed between eCO$_2$-induced An$_{\text{net}}$ enhancement and $C_i$ difference (Figure 8b). The $C_i$ difference is a measure of how high the operating point is, relative to a transition away from carboxylation limitation to photosynthesis. Larger $C_i$ difference indicates that plants have more capacity to increase carboxylation with increased atmospheric CO$_2$ concentrations. Thus, eCO$_2$ enables plants to overcome the higher $S_{\text{lim}}$ during water-limited periods resulting in increased $C_i$ and photosynthetic rates compared with plants grown in aCO$_2$. Examining the multivariate pathway to photosynthetic enhancement by eCO$_2$ in Figure 7, greater soil moisture in turn increased $g_s$ in ambient CO$_2$. There was both a direct pathway from $g_s$ to the enhancement in An$_{\text{net}}$ in eCO$_2$, as well as an indirect pathway through the change in relative stomatal limitation in aCO$_2$. This model clearly supports the mechanism of how higher stomatal limitations, caused by lower $g_s$ during dry periods, can be overcome by eCO$_2$ thus resulting in significant increase in photosynthetic rates. Taken together, the results indicate that seasonal variability in $S_{\text{lim}}$ was responsible for the variability in eCO$_2$-induced An$_{\text{net}}$ enhancement. The increased photosynthetic rates under eCO$_2$ suggest a potential for increased ecosystem C gain during dry periods. The phenology of different species would dictate if these responses could be translated to increased biomass accumulation, for which we currently have limited data. This is the first study to demonstrate the role of $S_{\text{lim}}$ in controlling eCO$_2$ response at field level and over multiple seasons in a periodically water-limited grassy woodland ecosystem.

Although eCO$_2$ overcomes $S_{\text{lim}}$, thus increasing A$_{\text{net}}$ during dry periods, this may not always be the case. The Eucalyptus woodland ecosystem in this study experienced frequent wet-dry periods resulting in moderate water stress (Figure 1b,c), likely enhanced by water extraction by nearby trees. Findings from this study might best apply in systems such as savannas and grasslands where frequent droughts are common, rather than the long and more intense dry periods observed in semi-arid to arid regions. In the latter case, metabolic limitations that decrease photosynthetic capacity become more important than stomatal limitations and any increase in external CO$_2$ is unable to increase photosynthetic rates (Ghannoum et al., 2003; Lawlor, 2002). For instance, eCO$_2$ was unable to increase photosynthetic rates in a desert shrub during severe drought as a consequence of reduced Rubisco content and low photosynthetic capacity (Naumburg et al., 2003). Similarly, Gray et al. (2016) observed that during severe droughts, decreases in $g_s$ and depression of $C_i$ were greater in eCO$_2$ than aCO$_2$. Consequently, there may be negative effects of severe restrictions on water availability that are manifest by nonstomatal effects that can override the stomatal ones under severe plant water deficits.

In summary, under field conditions and over 3 years of CO$_2$ fumigation, we investigated two key mechanisms that might be responsible for eCO$_2$-induced photosynthetic enhancement observed during periods of low water availability in C$_3$ herbaceous species of a grassy woodland. One of these, the “water-savings effect”, has been frequently assumed to be the main mechanism responsible for eCO$_2$ effect during dry conditions (Morgan et al., 2004) and has been used in global models (Ahlström, Smith, Lindström, Rummukainen, & Uvo, 2013; Zhu et al., 2016). Although we observed maximum eCO$_2$-induced photosynthetic enhancement during the dry periods, this enhancement was not mediated through the “effect”. Low water availability resulted in lower $g_s$, higher relative $S_{\text{lim}}$ and thus a greater increase in $C_i$ possible which led to a significant photosynthetic enhancement under eCO$_2$. The results demonstrate that water availability, but not eCO$_2$, controls $g_s$ and hence the

\[ A_{\text{net}} = \frac{1}{1 + \frac{S_{\text{lim}}}{S_{\text{lim}}}} \]

\[ C_i = \frac{1}{1 + \frac{S_{\text{lim}}}{S_{\text{lim}}}} \]

\[ A_{\text{et}} = \frac{1}{1 + \frac{S_{\text{lim}}}{S_{\text{lim}}}} \]

\[ p = 0.01 \]

\[ r^2 = 0.39 \]

\[ p = 0.01 \]

\[ r^2 = 0.44 \]

\[ p = 0.01 \]

\[ r^2 = 0.39 \]

\[ p = 0.01 \]
photosynthetic enhancement in the herbaceous understory of the dry grassy *Eucalyptus* woodland. Further, modeling photosynthetic enhancement should involve dynamic regulation of the set-point for gas exchange according to stomatal limitations across different times of year. Thus, eCO2 has the potential to alter the structure and functioning of warm and periodically dry grassy woodland ecosystems through alleviation of S$_{\text{sm}}$ and increase in photosynthetic CO2 assimilation, but not via a “water-savings effect” as is usually observed in temperate grasslands.

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