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Water availability affects seasonal CO\(_2\)-induced photosynthetic enhancement in herbaceous species in a periodically dry woodland

**Running head:** Soil water controls herb [CO\(_2\)] response

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**Type of Paper:** Primary Research Article
Abstract

Elevated atmospheric CO$_2$ (eCO$_2$) is expected to reduce the impacts of drought and increase photosynthetic rates via two key mechanisms: first, through decreased stomatal conductance ($g_s$) and increased soil water content ($V_{SWC}$) and second, through increased leaf internal CO$_2$ ($C_i$) and decreased stomatal limitations ($S_{lim}$). 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 C$_3$ herbaceous species in a periodically dry Eucalyptus woodland and investigated how eCO$_2$-induced photosynthetic enhancement varied with seasonal water availability, over a three-year period.

Leaf photosynthesis increased by 10-50% with a 150 µmol mol$^{-1}$ increase in atmospheric CO$_2$ across seasons. This eCO$_2$-induced increase in photosynthesis was a function of seasonal water availability, given by recent precipitation and mean daily $V_{SWC}$. The highest photosynthetic enhancement by eCO$_2$ (> 30%) was observed during the most water-limited period, e.g., with $V_{SWC} < 0.07$ in this sandy surface soil. Under eCO$_2$ there was neither a significant decrease in $g_s$ in the three herbaceous species, nor increases in $V_{SWC}$, indicating no ‘water-savings effect’ of eCO$_2$. Periods of low $V_{SWC}$ showed lower $g_s$ (less than $\approx 0.12$ mol m$^{-2}$ s$^{-1}$), higher relative $S_{lim}$ (> 30%) and decreased $C_i$ under the ambient CO$_2$ concentration (aCO$_2$), with leaf photosynthesis strongly carboxylation-limited. The alleviation of $S_{lim}$ by eCO$_2$ was facilitated by increasing $C_i$, thus yielding a larger photosynthetic enhancement during dry periods. We demonstrated that water availability, but not eCO$_2$, controls $g_s$ and hence the magnitude of photosynthetic enhancement in the understory herbaceous plants. Thus, eCO$_2$ 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.
Introduction

Grass-tree mixtures such as savannas and woodlands occupy extensive areas in tropical and sub-tropical regions and are characterised by strong seasonal variation in water availability (Baudena et al., 2015, Polley et al., 1997). Due to the ongoing rise in atmospheric CO$_2$ 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 et al., 2004), there is a significant knowledge gap in responses of savannas and woodlands to elevated atmospheric CO$_2$ (eCO$_2$) concentrations (Leakey et al., 2012). Consequently, the expected impacts of eCO$_2$ on these warm ecosystems have been based on findings from cold temperate ecosystems (Leakey et al., 2012). Tropical and sub-tropical 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 eCO$_2$ 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 hypothesised to increase the responsiveness to eCO$_2$ (Higgins & Scheiter, 2012, Morgan et al., 2011). Hence, there is a need for experiments addressing effects of eCO$_2$ 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, eCO$_2$ increases CO$_2$ 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 eCO$_2$ will in part depend upon water availability. One important way, through
which eCO$_2$ 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 eCO$_2$, also termed a
‘water-savings effect’, has led to the generalisation that plant photosynthesis and productivity
responses to eCO$_2$ will be strongest in dry conditions (Duursma & Medlyn, 2012, Ellsworth
et al., 2012) though it is unclear if this best applies to short or long dry periods. Still, the
generalisation has been used to rationalise why the eCO$_2$-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 et al., 2013)
and why the eCO$_2$-induced enhancement of grasslands is larger in dry vs. wet years
(Owensby et al., 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 mineralisation and organic matter decomposition (Morgan
et al., 2004, Wullschleger et al., 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 et al., 2010), changes in leaf area index and canopy temperature (Gray et al., 2016, Kelly et al., 2016), and soil texture (Fay et al., 2012, Polley et al., 2012). Though the eCO2-induced increase in soil water content has been demonstrated for temperate grasslands (Blumenthal et al., 2013, Lecain et al., 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.

Whilst 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 et al., 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 et al., 2004, Lecain et al., 2003, Morgan et al., 2011, Morgan et al., 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 et al., 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 et al., 2013).
In addition to a ‘water-savings effect’, another important mechanism through which C\textsubscript{3} plants might benefit from CO\textsubscript{2} fertilisation 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 CO\textsubscript{2} assimilation. This restriction of stomata to CO\textsubscript{2} supply, also termed as stomatal limitation, decreases leaf intercellular CO\textsubscript{2} concentrations (C\text{\textsubscript{i}}) 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 CO\textsubscript{2} response curve (Ellsworth et al., 2012). Under such conditions, CO\textsubscript{2} fertilisation can help alleviate the stomatal limitations by increasing C\text{\textsubscript{i}} and hence plants would experience larger photosynthetic enhancement (Kelly et al., 2016, Lawlor, 2002). The importance of such limitations in controlling eCO\textsubscript{2}-induced photosynthetic enhancement during dry periods has rarely been studied in the field (Galmès et al., 2007, Grassi & Magnani, 2005) and has not been investigated in eCO\textsubscript{2}.

Building on knowledge from previous ecosystem studies (see Leakey et al., 2012), we examined eCO\textsubscript{2} responses of an herbaceous understory community in the Eucalyptus Free Air CO\textsubscript{2} Enrichment Experiment (EucFACE). The EucFACE experiment is located in a mature, undisturbed Eucalyptus woodland in south eastern Australia which shows strong seasonal and inter-annual 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 eCO\textsubscript{2} response in a periodically water-limited woodland ecosystem. We hypothesized that:

H1: Maximum photosynthetic enhancement by eCO\textsubscript{2} 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 three years, as well as corroborating evidence from two sympatric C₃ forbs over 1 ½ years.

Materials and Methods

Experimental design and site description

We conducted leaf level gas exchange measurements on herbaceous understory in the first three 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 randomised 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° 37' 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. Microlaena stipoides Labill., a native perennial C₃ grass, is the dominant herbaceous species at EucFACE (≈ 70% of total understorey biomass, Pathare unpubl. data) along with
the co-occurrence of $C_3$ forbs like *Lobelia purpurascens* R.Br., $C_4$ grasses like *Cymbopogon refractus* R.Br., and naturalised species such as *Senecio madagascariensis* Poir. We measured three common $C_3$ herbaceous understorey species in our study: the dominant $C_3$ grass (*M. stipoides*) and two prevalent $C_3$ 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-temperate with a mean annual temperature of 17°C, characterised 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) (Fig. 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 (Fig. 1b). The soil at the site is a well-drained, sandy loam with low organic carbon content (Gimeno *et al.*, 2016).

**Gas exchange measurements at EucFACE and model fitting**

For the purpose of 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 four time points per year, with each time point representing a season of the year. Measurements began, one week after initiation of full CO$_2$ 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 six 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 (Aₙₑₙ−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 non-overlapping leaves were placed across the Li-COR chamber and a minimum time of 15-min at light saturation was allowed for stabilisation of gas exchange before commencing measurements. After stabilisation, an initial measurement of net CO₂ assimilation rate (Aₙₑₙ; µmol m⁻² s⁻¹), stomatal conductance (gₛ; mol m⁻² s⁻¹), intercellular CO₂ (Cᵢ; µmol mol⁻¹) and the ratio of intercellular to growth CO₂ (Cᵢ/Cₐ) was conducted at growth CO₂ concentration, followed by the Aₙₑₙ−Cᵢ response curves. Aₙₑₙ−Cᵢ curves for the three species were done with a minimum of ten different steps of CO₂ 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 ($T_{\text{air}}$) during each measurement season (18, 22, 27 and 29 °C for winter, autumn, spring and summer respectively) (Fig. 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 three days. After each $A_{\text{net}}$-$C_i$ response curve, leaves were marked to assess the correct leaf area in the chamber, collected in self-sealing polythene bags, labelled 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.

$A_{\text{net}}$-$C_i$ curves were then fit using the biochemical model of Farquhar et al. (1980), in order to obtain kinetic coefficients associated with rates of maximum carboxylation ($V_{\text{cmax}}$; $\mu$mol m$^{-2}$ s$^{-1}$) and electron transport ($J_{\text{max}}$; $\mu$mol m$^{-2}$ s$^{-1}$) (see Crous et al., 2013, Duursma 2015). While estimating the rates of $V_{\text{cmax}}$ and $J_{\text{max}}$ we used a fixed mesophyll conductance value (0.2 mol m$^{-2}$ s$^{-1}$ for perennial herbaceous species; Flexas et al., 2008) to reflect the finite characteristics of this trait. The temperature responses of $V_{\text{cmax}}$ and $J_{\text{max}}$ are important to consider in model fitting (Medlyn et al., 2002), especially as seasonal temperatures varied. In order to do this, we carried out temperature response measurements on $M. \text{stipoides}$ 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 $V_{\text{cmax}}$ 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 et al., 1992 and Medlyn et al., 2002). The resulting kinetics derived by fitting the modified Arrhenius function for $V_{\text{cmax}}$ were used in the ‘fitacis’ function in the plantecophys package (Duursma, 2015) to obtain a temperature-normalised $V_{\text{cmax}}$ ($V_{\text{cmax,25}}$) from the $A_{\text{net}}$-$C_i$ response curves.
**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 \(A_{\text{net}}\) assuming a fractional increase in \(g_s\) and \(C_i\). Thus, relative stomatal limitations (\(S_{\text{lim}}\); 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). \(S_{\text{lim}}\) to photosynthesis were obtained by modelling the diffusional pathway and based on the \(A_{\text{net}}\) vs \(C_i\) response curves. For calculating \(S_{\text{lim}}\) to CO\(_2\) assimilation rates, we used the approach proposed by Grassi & Magnani (2005) which is similar to that defined in Jones (1985). We computed \(S_{\text{lim}}\) as follows:

\[
S_{\text{lim}} = \frac{\partial A_{\text{net}}/\partial C_i}{g_{sc}/g_s + \partial A_{\text{net}}/\partial C_i}
\]  

(Eq. 1)

where, \(\partial A_{\text{net}}/\partial C_i\) is the partial derivative of net CO\(_2\) assimilation (\(A_{\text{net}}\)) for a relative change in leaf internal CO\(_2\) (\(C_i\)) and \(g_{sc}\) is the stomatal conductance to CO\(_2\) (\(g_{sc} = g_s/1.6\)). Our approach uses a static mesophyll conductance to CO\(_2\) (\(g_{mes} = 0.2 \text{ mol m}^{-2} \text{ s}^{-1}\)) as the study was focussed at 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 \(A_{\text{net}}\) vs \(C_i\) responses curves. \(C_i\) difference was calculated as the difference between the transition \(C_i\) (or \(C_i\) at the \(V_{c_{\text{max}}-J_{\text{max}}}\) transition point) and operating \(C_i\) (or \(C_i\) under growth CO\(_2\) levels). It was thus an indicator of how high the operating \(C_i\) is on the linear slope of the \(A_{\text{net}}\) vs \(C_i\) response curve.
Other field measurements

Values for mean daily $T_{air}$ 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, Townsville, Australia). In each of the six EucFACE plots (referred to as rings), three photosynthetically active radiation (PAR) sensors (LI-190; Li-COR, Lincoln, NE, USA) were installed on metal posts at one-m height and data was recorded every minute. Volumetric soil water content ($V_{SWC}$; 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$^\circ$ angle (eight per plot; CS650-L; Campbell Scientific, Logan, UT, USA). $V_{SWC}$ content data was recorded at 15 min interval 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 $V_{SWC}$ measurements under aCO$_2$ and eCO$_2$ treatments. In addition to $V_{SWC}$, 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.

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 CO$_2$ rings and hence the number of replicates was three for each of the two levels of CO$_2$ treatment. The overall dataset was unbalanced with regard to 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 seven measurement time-points (~1.5 years). A mixed-model split-plot ANOVA with interactions was performed for the physiological and biochemical parameters $A_{\text{net}}$, $V_{\text{cmax},25}$, $J_{\text{max},25}$, $V_{\text{cmax}}$, $J_{\text{max}}$, N content, $g_s$, $C_i$, $S_{\text{lim}}$ and $C_i$ difference, with CO$_2$ 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 *et al.*, 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 eCO$_2$-induced $A_{\text{net}}$ enhancement. These key variables were chosen according to their causal hypothesised roles in regulating eCO$_2$-induced photosynthetic enhancement (Ellsworth *et al.*, 2012; see Supplemental Information for further details). We also employed Structural Equation Modelling approaches (Lamb *et al.*, 2011) to understand the processes underlying the relationships among variables describing photosynthetic enhancement by eCO$_2$ 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
$V_{SWC}$ and test the differences between the CO$_2$ treatments during three years of this
experiment. Although both $C_i$ and $S_{lim}$ are recursive variables depending on both $A_{net}$ and $g_s$
(Eq. 1), we included them in the structural equation models (Fig. 7 and Figs. S6-S8) as they
are key parts of the overall hypotheses we asked.

Results

Effect of CO$_2$ and measurement time on $A_{net}$ and $g_s$

*M. stipoides* was the dominant herbaceous species in the grassy woodland understorey, and
thus it was measured more intensively than the other species. CO$_2$ enrichment by 150 µmol
mol$^{-1}$ resulted in a significant increase in $A_{net}$ ($\approx 28\%$) across species measured for seven time
points from 1.5 to 3 years after the start of CO$_2$ enrichment ($P = 0.009$, Table 1, Fig. 2a-c).
Similarly, for the dominant *M. stipoides*, eCO$_2$ resulted in a significant increase in $A_{net}$ ($\approx
32\%$) across the 13 time points across three years ($P = 0.019$, Table S1, Fig. 2a). There was a
significant measurement time effect on $A_{net}$ across species ($P < 0.001$, Table 1 and S1, Fig.
2a-c) with average values ranging from $17 \pm 3.2$ µmol m$^2$ s$^{-1}$ during the warmer times
(Oct’15 and Feb ’16) to $11 \pm 2.4$ µmol m$^2$ s$^{-1}$ during the cooler time points (May’15 and
April’16). For *M stipoides*, maximum $A_{net}$ ($12 \pm 1.5$ µmol m$^2$ s$^{-1}$) occurred during the wet
and warmer times (Feb’13, Feb’14, Oct’14 and Feb’15), with minimum $A_{net}$ of $\sim 5 \pm 1.2$ µmol
m$^2$ s$^{-1}$ occurring in two dry periods, Oct’13 and Jul’14. We did not observe a significant CO$_2$
x measurement time effect on $A_{net}$ across the three species ($P > 0.02$, Table 1 and S1). Similar
to seasonal variation in $A_{net}$, the percent increase in photosynthetic rates due to eCO$_2$ also
varied among seasonal time points, with values ranging from 12-53%. The maximum
increase in photosynthetic rates due to CO$_2$ treatment across the species was observed during
Feb’16 (40%) and the minimum was observed in Feb’15 (13%). Similarly, for the dominant
*M. stipoides*, the maximum increase in \( A_{\text{net}} \) due to eCO\(_2\) was observed in Oct’13 (62%),
whereas minimum increase was reported in Feb’14 (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 (Fig. 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 (\( P > 0.02 \), Table 1, Fig. 2d-f).
However, there were highly significant measurement time effects on \( g_s \) in all species (\( P <
0.01 \), Table 1 and Table S1) with average values ranging from maximum of 0.27 ± 0.03 mol
m\(^{-2}\) s\(^{-1}\) in Oct’15 and Feb’16 to minimum of 0.18 ± 0.02 mol m\(^{-2}\) s\(^{-1}\) in May’15 and April’16.
For *M. stipoides*, maximum \( g_s \) (0.17 ± 0.02 mol m\(^{-2}\) s\(^{-1}\)) was observed during warmer time
points (Feb’13, Feb’14, Oct’14 and Feb’15), whereas, minimum \( g_s \) was observed in Oct’13
and Jul’14 as noted above for \( A_{\text{net}} \). Given that higher \( A_{\text{net}} \) values were observed during time
points with higher \( g_s \) (Fig. 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\(_2\) (\( r^2 = 0.64, P < 0.01 \),
Fig. S1a) and eCO\(_2\) (\( r^2 = 0.57, P < 0.01 \), Fig. S1b) concentrations.

**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 \( V_{\text{SWC}} \) (Fig. 3). The recent week for these measures was the seven days prior to the
initiation of gas exchange measurements at the EucFACE. Fig. 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 $A_{\text{net}}$ ($< 9 \ \mu\text{mol m}^{-2} \text{s}^{-1}$; Fig. 3a, b) and $g_s$ ($< 0.12 \ \text{mol m}^{-2} \text{s}^{-1}$; Fig. 3c, d) were mostly observed during time points when recent week precipitation was $< 10 \ \text{mm}$ (Fig. 3a, c) and mean daily $V_{\text{SWC}}$ was $< 0.10 \ \text{v/v}$ (Fig. 3b, d). Fig. 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 ($r^2 = 0.38, P < 0.01$, Fig. 3e) and mean daily $V_{\text{SWC}}$ ($r^2 = 0.49, P < 0.01$, Fig. 3f) of the preceding week. Similarly, for $M. \ stipoides$, eCO$_2$-induced $A_{\text{net}}$ enhancement was a decreasing function of total precipitation ($r^2 = 0.56, P < 0.01$, Fig. 3g) and mean daily $V_{\text{SWC}}$ ($r^2 = 0.64, P < 0.01$, Fig. 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 $< 10 \ \text{mm}$ and mean daily $V_{\text{SWC}}$ was $< 0.10 \ \text{v/v}$. Thus, there was evidence that water was an important regulator of $A_{\text{net}}$, $g_s$ and eCO$_2$-induced $A_{\text{net}}$ enhancement.

**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. Though there was no significant CO$_2$ effect on the $V_{\text{cmax}}$ and $J_{\text{max}}$ values across the species ($P > 0.02$, Table S2 and S3, Fig. S3), we observed a highly significant measurement time effect on both the parameters ($P < 0.01$, 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, $V_{c,max}$ and $J_{max}$ were normalised 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). Though there was a significant measurement time effect on the normalised parameters ($V_{c,max25}$ and $J_{max25}$) across the species ($P < 0.01$, Table 1 and S1, Fig. 4), they were less variable over measurement time compared to non-normalised $V_{c,max}$ and $J_{max}$ (Fig. S3). When averaged across the three species and CO$_2$ treatments, maximum values for $V_{c,max25}$ and $J_{max25}$ (80 ± 13.06 µmol m$^{-2}$ s$^{-1}$ and 129 ± 5.23 µmol m$^{-2}$ s$^{-1}$ respectively) were observed in Oct’14 and Oct’15.

We did not observe a significant CO$_2$ effect on $V_{c,max25}$ and $J_{max25}$ across the species ($P > 0.02$, Tables 1 and S1 and Fig. 4). However, there was a non-significant CO$_2$ x measurement time interaction effect on $V_{c,max25}$ and $J_{max25}$ ($P < 0.1$, Tables 1 and S1 and Fig. 4). In particular, there was a trend towards lower $V_{c,max25}$ and $J_{max25}$ under eCO$_2$ during Oct’14 in *M. stipoides* and during Oct’14 and Oct’15 in *L. purpurascens*. Trends similar to $V_{c,max}$ and $J_{max}$ were also observed for leaf N content. There were no significant CO$_2$ or CO$_2$ x measurement time interaction effects on the leaf N content ($N_{area}$ and $N_{mass}$) across the three species ($P > 0.02$, Table 1 and S1, Fig. S4). However, we observed a significant measurement time effect of the leaf N content across the species and CO$_2$ treatments ($P < 0.01$, Table 1 and S1). Similarly, for *M. stipoides*, there were no statistically significant CO$_2$ and CO$_2$ x measurement time interaction effects on $N_{area}$ ($P > 0.02$, Table S1, Fig. S4a) and $N_{mass}$ ($P > 0.02$, Table S3, Fig. S4d). However, leaf N content of *M. stipoides* varied significantly with time across the CO$_2$ treatments ($P < 0.01$, Table S1 and S3). Overall, across the species we did not observed a significant decrease in any of the measured biochemical parameters under eCO$_2$, though individual species varied in this regard.
**Effect of CO\(_2\) and measurement time on \(V_{SWC}\)**

There was no significant CO\(_2\) treatment effect on the mean daily \(V_{SWC}\) during the three years of this experiment, indicated by overlapping confidence intervals (Fig. 5b). Also, mean daily \(V_{SWC}\) during the weeks preceding gas exchange measurements was similar between aCO\(_2\) and eCO\(_2\) \((P > 0.02,\) Table S4). However, \(V_{SWC}\) varied substantially during the course of this study and there were several seasonal wet-dry periods (Fig. 5a). During a substantial amount of time (average 14 days per month or \(\approx 50\%\) of the time), \(V_{SWC}\) was \(< 0.10\) v/v (Fig. 5a).

Thus, the EucFACE facility experienced frequent dry periods during the duration of our measurements. Overall, there were no significant CO\(_2\) x measurement time interaction effects on mean daily \(V_{SWC}\) during the three years of measurement period indicated by overlapping confidence intervals in Fig. 5b as well as during the week preceding the gas exchange measurements across all the 13 measurement time points \((P > 0.02,\) Table S4).

**Effect of CO\(_2\) and measurement time on diffusional parameters**

Elevated CO\(_2\) resulted in a significant increase in \(C_i\) \((391 \pm 27 \mu\text{mol mol}^{-1})\) compared to aCO\(_2\) \((288 \pm 15 \mu\text{mol mol}^{-1})\) across the three species \((P < 0.01,\) Table S2 and S3, data not shown). However, this increase was not accompanied by a corresponding increase in the \(C_i/C_a\) ratio \((P > 0.02,\) Table S2 and S3). Both \(C_i\) and \(C_i/C_a\) varied significantly with measurement time across the species \((P < 0.001,\) Table S2 and S3). A result of increased atmospheric CO\(_2\) and hence increased \(C_i\), but no change in \(C_i/C_a\), should be a reduction in \(S_{\text{lim}}\) and \(C_i\) difference under eCO\(_2\), as leaves operate closer to the CO\(_2\) saturation for \(A_{\text{net}}\). We therefore examined the responses of \(S_{\text{lim}}\) and \(C_i\) difference across the species (Fig. 6). There was no significant CO\(_2\) effect on \(S_{\text{lim}}\) across the three species \((P > 0.02,\) Table 1 and S1, Fig. 6a-c). However, there was a highly significant measurement time effect on \(S_{\text{lim}}\) across the CO\(_2\) treatments and species \((P < 0.01,\) Table 1 and S1). Since there was a trend towards higher \(S_{\text{lim}}\) during the dry
time points (Fig. 6a-c) when values for $A_{\text{net}}$ (Fig. 2a) and $g_s$ (Fig. 2b) were lower, we plotted $S_{\text{lim}}$ as a function of water availability measured by total precipitation and mean daily $V_{\text{SWC}}$ of preceding week (Fig. S5). $S_{\text{lim}}$ was a decreasing function of $V_{\text{SWC}}$ across the species ($r^2 = 0.33, P = 0.016$, Fig. S5b) and for *M. stipoides* ($r^2 = 0.55, P = 0.02$, Fig. S5d). Thus, higher $S_{\text{lim}}$ were observed during periods of low water availability or when $V_{\text{SWC}}$ was < 0.10 v/v (Fig. S5b, d). Though the $S_{\text{lim}}$ were similar between aCO$_2$ and eCO$_2$ treatments (Fig. 6a-c), we observed a significant decrease in $C_i$ difference under eCO$_2$ across the species ($P < 0.01$, Table 1 and S1, Fig. 6d-f) indicating that plants in eCO$_2$ operated higher on the linear part of the $A_{\text{net}}$-$C_i$ curve. We did not observe a highly significant measurement time effect on $C_i$ difference across CO$_2$ treatments and three species ($P > 0.02$, Table 1). However, there were significant measurement time effects on $C_i$ difference of *M. stipoides* ($P < 0.01$, Table S1, Fig. 6d). Higher average $C_i$ difference was evident during the time points with higher relative $S_{\text{lim}}$ (Fig. 6). We expected that there would be a two-way interaction between CO$_2$ and time on $C_i$ difference, but overall there was no significant CO$_2$ x measurement time interaction effect on $S_{\text{lim}}$ and $C_i$ difference across the species ($P > 0.02$, Table 1 and S1). Taken together, higher relative $S_{\text{lim}}$ and $C_i$ difference were evident during water-limited time points (Fig. S5), suggesting that these diffusional factors may be responsible for seasonal variation in eCO$_2$-induced $A_{\text{net}}$ enhancement. Further evidence of this comes from a set of physiologically-based causal hypotheses laid out in a structural equation model (Fig. 7, see Supporting Material for details). Here, there was both a direct effect of the seasonal variation in $g_s$ affecting photosynthetic enhancement by eCO$_2$ as well as a strong effect mediated through $S_{\text{lim}}$.

Relation between $S_{\text{lim}}$ and $A_{\text{net}}$ enhancement by eCO$_2$
To obtain a greater insight into the role of diffusional factors in controlling seasonal variation in eCO$_2$-induced $A_{\text{net}}$ enhancement we further plotted $A_{\text{net}}$ enhancement ratio as a function of $S_{\text{lim}}$ (Fig. 8a) and $C_i$ difference (Fig. 8b) under aCO$_2$ conditions. The eCO$_2$-induced $A_{\text{net}}$ enhancement was positively correlated with $S_{\text{lim}}$ at aCO$_2$ conditions across the species ($r^2 = 0.39, P < 0.01$, Fig. 8a) and for $M. stipoides$ ($r^2 = 0.63, P < 0.01$). Similar to $S_{\text{lim}}$, we observed a strong positive correlation between eCO$_2$-induced $A_{\text{net}}$ enhancement and $C_i$ difference at aCO$_2$ across the species ($r^2 = 0.44, P < 0.01$, Fig. 8b) and for $M. stipoides$ ($r^2 = 0.64, P < 0.01$). Overall, maximum enhancement in photosynthetic rates under eCO$_2$ were observed when $S_{\text{lim}}$ and $C_i$ difference were higher under aCO$_2$ conditions.

**Species effects and higher-order interactions**

The split-plot ANOVA (CO$_2$ x measurement time x species) for the seven time points, during which all three species were measured, indicated that species differed significantly in most of the measured physiological and biochemical parameters ($P < 0.01$, Table 1 and S2). When averaged across CO$_2$ treatments and seven measurement time points, we observed higher values for $A_{\text{net}}$ and $g_s$ (Fig. 2) in $S. madagascariensis$ ($18.5 \pm 4.4$ µmol m$^{-2}$ s$^{-1}$ and $0.34 \pm 0.13$ mol m$^{-2}$ s$^{-1}$, respectively) than the other species (average $A_{\text{net}}$ was $12 \pm 2.7$ µmol m$^{-2}$ s$^{-1}$ and $9.4 \pm 3.12$ µmol m$^{-2}$ s$^{-1}$ for $L. purpurascens$ and $M. stipoides$, respectively). A similar trend was observed for the biochemical parameters like $V_{\text{cmax-L25}}$ and $J_{\text{max-L25}}$ (Fig. 4), $V_{\text{cmax}}$ and $J_{\text{max}}$ (Fig. S3) and leaf N content (Fig. S4), with rates for the former ranking $S. madagascariensis > L. purpurascens > M. stipoides$. Species also differed significantly in all the diffusional parameters ($P < 0.01$, Table 1 and S2) except for $S_{\text{lim}}$ ($P > 0.02$, Table 1, Fig. 6a-c) which was similar across the three species ($\approx 33\%$) as expected given that it is a relative measure that already accounts for intrinsic physiological rates. We observed a significant species x CO$_2$ interaction effect only for two variables ($P < 0.01$, Table 1 and S2), as $S. madagascariensis$ had higher values for $J_{\text{max-L25}}$ (Fig. 4f) and $J_{\text{max}}$ (Fig. S3f) under eCO$_2$ than
for all other cases. Compared to *M. stipoides*, the biochemical ($J_{\text{max}}$, $V_{\text{cmax-25}}$, $J_{\text{max-25}}$) and diffusional ($g_s$, $C_i$, $C_i/C_a$, and $S_{\text{lim}}$) parameters varied substantially with season in *L. purpurascens* and *S. madagascariensis*. Overall, there were no statistically significant three-way interaction effects (CO$_2$ x measurement time x species) on any of the measured physiological and biochemical parameters in our study ($P > 0.02$, Table 1 and S2).
Discussion

During three years of this study, photosynthetic rates under eCO$_2$ were almost 30% higher on average (Fig. 2), which we expect would have led to an increase in above- or below-ground production. However, the relative enhancement in photosynthetic rates by eCO$_2$ across species varied substantially between seasons, with values ranging from 12-53%. We investigated the mechanisms underlying the seasonal variation in photosynthetic responses to eCO$_2$ in three herbaceous C$_3$ species from a periodically dry *Eucalyptus* woodland, with a focus on water availability and stomatal limitations, recognising that this would be the driver for biomass accumulation responses. Our first hypothesis was supported, as we observed maximum photosynthetic enhancement by eCO$_2$ during the dry periods (V$_{SWC}$ < 0.07). In contrast to our second hypothesis, we did not observe a significant increase in V$_{SWC}$ under eCO$_2$ or decrease in stomatal conductance. The results indicate that eCO$_2$ induced photosynthetic enhancement during dry periods was the result of alleviation of stomatal limitation by increasing C$_i$, thus supporting our third hypothesis.

**Maximum eCO$_2$-induced $A_{net}$ enhancement is observed during dry periods**

The grassy *Eucalyptus* woodland in this study experienced frequent seasonal wet and dry periods (Fig. 1b and Fig. 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 eCO$_2$ response in the herbaceous species of this ecosystem. The relationship between seasonal water availability (total precipitation and mean daily V$_{SWC}$ of preceding week) and eCO$_2$-induced $A_{net}$ enhancement (Fig. 3e-h) indicated that maximum eCO$_2$-induced $A_{net}$ enhancement occurred during relatively dry periods, that is, when the total precipitation in the week preceding the measurements was <
10 mm (Fig. 3e, g) or the mean daily $V_{SWC}$ was < 0.10 v/v (Fig. 3f, h). Similar relationships have been observed between $A_{net}$ 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 $A_{net}$ 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 eCO$_2$-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 eCO$_2$ and drought (Kelly et al., 2016, Lecain et al., 2003, Morgan et al., 2004, Niklaus & Körner, 2004) indicate that eCO$_2$ can mitigate the impact of water-limitation via two key mechanisms; first, decreased $g_s$ under eCO$_2$ resulting in increased soil water content or ‘water-savings effect’ and second, lower $g_s$ and higher $S_{lim}$ during drought resulting in increased $C_i$ and hence $A_{net}$ under eCO$_2$. We evaluated these two mechanisms and discuss them in the following sections.

**Elevated CO$_2$ does not increase soil water content**

Previous studies in water-limited temperate ecosystems have reported improved photosynthetic rates and productivity under eCO$_2$ 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, Morgan et al., 2004), called the ‘water-savings effect’. Although we observed the maximum CO$_2$-induced photosynthetic enhancement in dry periods (Fig. 3e-h), stomatal conductance ($g_s$) did not significantly decrease under eCO$_2$ (Fig. 2d-f) even during dry periods (Fig. 3c, d). Stomatal conductance showed significant variation across seasons, but was similar under both aCO$_2$ and eCO$_2$ conditions (Fig. 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 Vₜₜₑₜₜ under eCO₂ compared to aCO₂ at any time point during three years of this study, not even during the dry periods when we expected a significant increase in Vₜₜₑₜₜ (Fig. 5). Unlike temperate ecosystems (Blumenthal et al., 2013, Lecain et al., 2003, Morgan et al., 2011, Morgan et al., 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, though 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 favour 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).

**Higher stomatal limitations and Aₜₜₑₜₜ enhancement by eCO₂ during dry periods**
Given that we did not find decreased stomatal conductance in eCO$_2$ and hence no ‘water-savings effect’, we investigated the possibility of changed stomatal limitations in eCO$_2$. $S_{\text{lim}}$ was a function of water availability, especially mean daily V$_{\text{SWC}}$ (Fig. S5b,d). As a result, lower $g_s$ (Fig. 3d) and consequently higher $S_{\text{lim}}$ (Fig. S5b,d) were observed during the water-limited periods than during wet periods. From this we infer that water availability controlled the variability in $S_{\text{lim}}$ to photosynthesis as depicted in the path analysis in Figure 7. A similar relationship was previously observed between soil water content and diffusional limitation by Grassi & Magnani (2005). A consequence of lower $g_s$ and higher $S_{\text{lim}}$ observed during water-limitation is a decrease in $C_i$ and $A_{\text{net}}$ with plants operating deeper in the carboxylation-limited zone, and so more responsive to eCO$_2$. At such low $C_i$’s, CO$_2$ fertilisation 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 (Fig. 8a). A similar relationship was observed between eCO$_2$-induced $A_{\text{net}}$ enhancement and $C_i$ difference (Fig. 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 to 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 $A_{\text{net}}$ 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 $S_{lim}$ was responsible for the variability in eCO$_2$-induced $A_{net}$ 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 $S_{lim}$ in controlling eCO$_2$ response at field level and over multiple seasons in a periodically water-limited grassy woodland ecosystem.

Though eCO$_2$ overcomes $S_{lim}$ thus increasing $A_{net}$ 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 (Fig. 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 non-stomatal effects that can override the stomatal ones under severe plant water deficits.

In summary, under field conditions and over three 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 eCO₂ effect during dry conditions (Morgan et al., 2004) and has been used in global models (Ahlström et al., 2013, Zhu et al., 2016). Though we observed maximum eCO₂-induced photosynthetic enhancement during the dry periods, this enhancement was not mediated through the ‘water-savings effect’. Low water availability resulted in lower gs, higher relative S_lim and thus a greater increase in C_i possible which led to a significant photosynthetic enhancement under eCO₂. The results demonstrate that water availability, but not eCO₂, controls gs and hence the photosynthetic enhancement in the herbaceous understorey of the dry grassy *Eucalyptus* woodland. Further, modelling 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_lim and increase in photosynthetic CO₂ assimilation, but not via a ‘water-savings effect’ as is usually observed in temperate grasslands.

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Table 1 Results of mixed-model split-plot ANOVA for net photosynthesis ($A_{\text{net}}$), temperature normalised maximum carboxylation ($V_{c_{\text{max}-25}}$) and electron transport rates ($J_{\text{max-25}}$), N content on area basis ($N_{\text{area}}$), stomatal conductance ($g_s$), relative stomatal limitation ($S_{\text{lim}}$) and $C_i$ difference as the difference between the transition $C_i$ and operating $C_i$, across the three $C_3$ species measured for seven seasonal time points\textsuperscript{1}. Results shown are across $M. \text{stipoides}$, $L. \text{purpurascens}$ and $S. \text{madagascariensis}$. $\text{CO}_2$ refers to the $\text{CO}_2$ 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 ($P > 0.02$) and hence are not shown in the table. The numerator degrees of freedom (df) are given for the statistical tests.
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All variables were transformed (square root or log transformation) to meet the normality assumptions for the mixed-model ANOVA.
Fig. 1 Time course through the three measurement years for (a) daily maximum air temperature (Tair in °C, open circles), and mean leaf temperature at the time of measurement (Tleaf in °C, filled squares), (b) daily total precipitation received at the site, and (c) surface soil water potential (0-30cm depth). Tleaf is a mean of three ground layer species.
Fig. 2 Time course through the three measurement years for (a) seasonal net CO2 assimilation (Anet) as a function of CO2 treatment for M. stipoides (Msti, black circles), (b) L. purpurascens (Lpur, blue squares) and (c) S. madagascariensis (Smad, red triangles). Open symbols indicate ambient CO2 (aCO2) and closed symbols indicate elevated CO2 (eCO2). The corresponding stomatal conductance is shown for (d) M. stipoides, (e) L. purpurascens, and (f) S. madagascariensis. When there was a significant overall CO2 effect (Table 1), post-hoc treatment differences were denoted by * (P < 0.05; t-test).
Fig. 3 (a, b) Seasonal Anet and (c, d) the corresponding seasonal gs for M. stipoides along with (e, f) the Anet enhancement ratio for all three species, and (g, h) for M. stipoides only. Anet, gs and Anet enhancement ratio are shown as a function of total precipitation (a, c, e and g) and mean daily volumetric soil water content (VSWC; b, d, f and h) in the week preceding Anet measurements. In the legends, the three species are indicated as M. stipoides (Msti, black circles), L. purpurascens (Lpur, blue squares and S. madagascariensis (Smad, red triangles). Anet enhancement ratio was calculated as mean Anet under eCO2 divided by mean Anet under aCO2. Gray shaded portions indicate 95% confidence intervals for the mean values. In panels f and h, a broken stick function is shown, with fit to the linear part below the field capacity for this soil (0.18 v/v).
Fig. 4 Time course of rates of maximum carboxylation ($V_{\text{cmax}}$) and electron transport ($J_{\text{max}}$) as a function of CO2 treatments. The rates have been normalised to a standard leaf temperature of 25 °C, indicated by (a, b and c) $V_{\text{cmax}-25}$ and (d, e and f) $J_{\text{max}-25}$, respectively. These parameters are shown for M. stipoides (Msti; a,d; black circles), L. purpurascens (Lpur; b, e; blue squares) and S. madagascariensis (Smad; c, f; red triangles).
Fig. 5 Time course through the three measurement years for (a) mean daily VSWC under aCO2 (black dashed line) and eCO2 (red solid line) and (b) smoothed regressions with 95% confidence intervals (gray areas) around the smooth terms for VSWC under aCO2 and eCO2.
Fig. 6 Time course of (a, b and c) relative stomatal limitations (Slim) and (d, e and f) the difference between operating Ci and transition Ci (Ci difference) as a function of CO2 treatments. These parameters are shown for M. stipoides (Msti; a, d; black circles), L. purpurascens (Lpur; b, e; blue squares) and S. madagascariensis (Smad; c, f; red triangles). When there was a significant overall CO2 effect (Table 1), post-hoc treatment differences were denoted by * (P < 0.05; t-test).
Fig. 7 The fitted structural equation model depicting causal hypotheses underlying the photosynthetic enhancement by eCO2 for herbaceous species measured at discrete points in the EucFACE experiment (see Fig. 2). Significant standardised path coefficients (P < 0.05) are shown near each arrow, with the width of the line proportional to the size of the standardised coefficients. The dashed line denotes a negative relationship, and non-significant pathways are indicated in grey. ∆Anet denotes the absolute enhancement of Anet by eCO2 with similar outcomes for the same model using the relative enhancement of Anet.
Fig. 8 The relative Anet enhancement ratio as a function of (a) Slim (fraction of total limitations), and (b) Ci difference for all three species. The species are M. stipoides (black circles), L. purpurascens (blue squares) and S. madagascariensis (red triangles). In (b), the dashed box in the lower left-hand corner of the panels denotes the null hypothesis of no Anet enhancement in eCO2. Gray shaded portions in panels (a) and (b) indicate 95% confidence intervals for the mean values, and the same outlier as shown in Fig. 3 is denoted.