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Grass and herb photosynthesis and productivity in a resource-limited *Eucalyptus* woodland under elevated atmospheric CO_2

by

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gururbrahma gururviṣṇu gururdevo maheśvaraḥ
guruḥ sākṣāt parabrahmā tasmai śrī gurave namaḥ

गुरुर्ब्रह्म गुरुर्विष्णु गुरुर्देवो महेश्वरः।
गुरुः साक्षात् परब्रह्मा तस्मै श्री गुरवे नमः॥

(From Guru Gita)

Guru is verily the representative of Brahma, Vishnu and Shiva. He creates, sustains knowledge and destroys the weeds of ignorance. I salute such a Guru.

This thesis is dedicated to all my 'gurus'- teachers, mother, father and husband for their endless support

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Statement of authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except where acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution

Varsha Shankar Pathare

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Table of contents

List of tables

List of supplementary tables

List of figures

List of supplementary figures

List of images

List of abbreviations

A_{max}	- Light and CO_2 saturated net CO_2 assimilation rate
A_{net}	- Light saturated net CO_2 assimilation rate
$A_{net\text{-}Ca}$	- Light saturated net CO_2 assimilation rate at common CO_2 concentration
A_{net}-C_i	- Net CO_2 assimilation rate versus intercellular CO_2 concentration
ANOVA	- Analysis of variance
aCO_2	- Ambient CO_2 concentrations
C	- Carbon
C_3	- C_3 photosynthetic pathway
C_4	- C_4 photosynthetic pathway
C_a	- Growth CO_2 concentration
C_i	- Intercellular CO_2 concentrations
C_i difference	- Difference between operating C_i and transition C_i
$[CO_2]$	- CO_2 concentration
CPW	- Cumberland Plain Woodland
ΔA_{net}	- Absolute enhancement of net CO_2 assimilation rates
df	- Degrees of freedom
eCO_2	- Elevated CO_2 concentration
EucFACE	- *Eucalyptus* free air CO_2 enrichment experiment
FACE	- Free air CO_2 enrichment experiment
$f_{N\text{-}Rubisco}$	- Fraction of N allocated to Rubisco enzyme
g_s	- Stomatal conductance
g_{sc}	- Stomatal conductance to CO_2
IPCC	- Intergovernmental Panel on Climate Change
J_{max}	- In situ maximum electron transport rate
$J_{max\text{-}25}$	- Temperature normalized maximum electron transport rate
k_{cat}	- Catalytic turnover number

LAR	- Leaf area ratio
lme	- Linear mixed effects model
n	- Sample size
N	- Nitrogen
NH_4^+	- Ammonium
NOAA	- National Oceanic and Atmospheric Administration
NO_3^-	- Nitrate
NPP	- Net primary productivity
P	- Phosphorus
Rubisco	- Ribulose-1, 5-bisphosphate carboxylase/oxygenase
RuBP	- Ribulose-1, 5-bisphosphate
SEM	- Structural equation modelling
S_{lim}	- Stomatal limitations
T_{air}	- Air temperature
T_{leaf}	- Leaf temperature
V_{cmax}	- *In situ* maximum carboxylation rate
$V_{cmax\text{-}25}$	- Temperature normalized maximum carboxylation rate
V_{SWC}	- Volumetric soil water content

Publications

Chapter 2 has been published as **Pathare VS**, Crous KY, Cooke J, Creek D, Ghannoum O, Ellsworth DS (2017) Water availability affects seasonal CO_2-induced photosynthetic enhancement in herbaceous species in a periodically dry woodland. *Global Change Biology*, **23**, 5164-5178. https://doi.org/10.1111/gcb.13778.

The thesis includes a revised version of above publication as Chapter 2.

Chapter 3 is anticipated to be submitted for publication in the journal of *Functional Ecology* as 'Photosynthetic acclimation and productivity responses of understory herbaceous species from a resource-limited *Eucalyptus* woodland'.

Chapter 4 is anticipated to be submitted for publication in the journal of *Oecologia* as 'Differential photosynthetic and biomass responses of C_3 grasses and C_3 forbs to elevated CO_2'.

Abstract

It has been suggested that plant species from the warmer ecosystems will show different and potentially larger photosynthesis and productivity responses to elevated CO_2 (eCO_2, ambient + 150 ppm) compared to those from the cold temperate ecosystems, on the basis of higher average annual temperature and greater water deficits in the former ecosystems. Based on these expectations, it has further been predicted that the warm water-limited ecosystems may have a greater potential to sequester the extra C that has been assimilated under eCO_2. However, empirical evidences testing these expectations are scarce. The overall aim of this thesis was to investigate the effects of eCO_2 on photosynthesis and productivity responses of the evergreen C_3 herbaceous species from the understory of a periodically water-limited warm-temperate *Eucalyptus* woodland. In a three-year field study conducted at the *Eucalyptus* free-air CO_2 enrichment experiment (EucFACE), I investigated how eCO_2-induced enhancement of photosynthetic rates (A_{net}) in herbaceous species varied with seasonal water availability. During the second and third year of CO_2 fertilisation at EucFACE, I measured the seasonal photosynthetic acclimation responses to eCO_2 in two dominant species- a C_3 forb and a C_3 grass, and measured responses of peak above-ground biomass to eCO_2 for total forbs and grasses. In a glasshouse experiment, I tested whether the species or functional groups growing under similar water inputs and nutrient availability differed in their photosynthetic or biomass allocation and growth responses to eCO_2 for two C_3 forbs and two C_3 grasses.

Findings from the field experiments demonstrate that eCO_2-induced A_{net} enhancement is a decreasing function of soil water availability, as the highest proportional increase in A_{net} under eCO_2 was evident during the driest periods. There was a lack of decrease in stomatal conductance (g_s) and increase in soil water content (V_{SWC}) under eCO_2, thus a 'water-savings effect' of eCO_2 was absent. The proportional enhancement of A_{net} under eCO_2 was not a consequence of a 'water-savings effect', but alleviation of drought-induced stomatal limitation via increase in intercellular [CO_2]. In spite of significant enhancement of A_{net} across the three years of the current study, there was

also evidence of photosynthetic acclimation under eCO_2 in the dominant C_3 herbaceous species, especially during the peak growing season of spring. Also, there was no proportional stimulation of peak above-ground biomass in the understory grasses and forbs, which may have been a result of lack of a 'water-savings effect' of eCO_2 and/or higher soil nutrient limitation. C_3 grasses and C_3 forbs differed in their photosynthetic and biomass allocation responses to eCO_2. Differences in leaf N content, N allocation and changes in above-ground biomass allocation likely affected the CO_2 responsiveness in these functional groups. In particular, there was an ability to maintain greater leaf area index, N allocation to photosynthesis and avoid down-regulation under eCO_2 by the grasses but not by the forbs. Findings from the current study suggest that interactions between seasonal water-availability eCO_2 will be critical in determining relative A_{net} enhancement response in herbaceous species from a water-limited ecosystem. However, the enhancement response may not be mediated via a 'water-savings effect' of eCO_2, which contrasts with the earlier findings from cold temperate ecosystems. Furthermore, evidence of photosynthetic capacity down-regulation in the dominant species and lack of relative increase in biomass under eCO_2, suggest a limited capacity of the understory herbaceous species from a grassy woodland to respond to eCO_2 and ultimately act as an aboveground C sink in future.

Chapter 1 : General Introduction

1.1 Rise in atmospheric [CO_2]

As a consequence of changes in land-use and increase in fossil fuel combustion, atmospheric [CO_2] has risen from the pre-industrial levels of about 280 ppm to the present high of 400 ppm in May 2013 (NOAA, 2013). Atmospheric [CO_2] is further expected to surpass the 550 ppm mark by the middle of this century (Keeling & Whorf, 2005, Prentice *et al.*, 2001). Sustained greenhouse gas emissions will continue to raise global average temperatures, with projected increases ranging from 0.3-4.8°C by the end of this century (IPCC, 2013). This increase in the global temperatures is expected to spatially and temporally alter the precipitation regimes, increase the intensity of droughts and heavy precipitation events (Luo *et al.*, 2008, Sillmann *et al.*, 2013). In addition to these effects on global climate, elevated CO_2 concentrations (eCO_2) are also expected to have considerable effects on the carbon and water balance of terrestrial ecosystems worldwide (IPCC, 2013), with some regional ecosystems responding more than others.

Photosynthesis and stomatal conductance are the most important processes by which higher plants and hence terrestrial ecosystems will respond to eCO_2. All other effects of eCO_2 on ecosystem processes will be meditated through these two fundamental processes (Ainsworth & Rogers, 2007). Consequently, how eCO_2 affects terrestrial ecosystem functioning via effects on photosynthesis and stomatal conductance, has been an important topic of research for several decades (Anderson *et al.*, 2001, Drake *et al.*, 1997, Mueller *et al.*, 2016, Owensby *et al.*, 1993, Sasek & Strain, 1988) and is fundamental to accurate prediction of future responses of both natural and agricultural ecosystems (Drake *et al.*, 1997). In the following sections, current understanding about effects of eCO_2 on the photosynthesis and productivity responses of terrestrial ecosystems will be reviewed and critical knowledge gaps will be highlighted. Fig.1.1 summarises the expected effects of eCO_2 on photosynthesis and stomatal conductance, leading to pools of biomass and soil water content respectively, in a model grass-dominated ecosystem.

1.2 Terrestrial ecosystem responses to eCO_2

Terrestrial feedbacks to climate could accelerate or mitigate the effects of climate change depending on whether they act as a source or sink under future high [CO_2] (Heimann & Reichstein, 2008). An unresolved question pertaining to climate change research is to what degree will terrestrial ecosystems mitigate the effects of rise in atmospheric [CO_2] by sequestering extra C (Arora *et al.*, 2013, Baig *et al.*, 2015, Friedlingstein *et al.*, 2014). To address this question, free-air CO_2 enrichment experiments have been conducted in a range of terrestrial ecosystems for last two decades (Ainsworth & Long, 2005, Hovenden *et al*., 2014, Morgan *et al*., 2011, Naumburg *et al.*, 2003, Norby & Zak, 2011, Nowak *et al.*, 2004). These previous studies have significantly improved our understanding about plant photosynthesis and productivity responses to climate change and provided valuable information for validating ecosystem models.

Despite the numerous studies cited above and elsewhere, our current understanding about photosynthesis and productivity responses to eCO_2 is strongly based on experimental data obtained from ecologically and economically important cold temperate ecosystems from the Northern hemisphere (Leakey *et al.*, 2012, Norby *et al.*, 2016). There is a lack of experimental data on photosynthesis and productivity responses to eCO_2 in many important biomes, but particularly for the warmer ecosystems from the warm-temperate, tropical and sub-tropical regions (Hickler *et al.*, 2008, Leakey *et al.*, 2012). Consequently, expected impacts of eCO_2 on these less-studied warm-temperate, tropical and sub-tropical ecosystems (henceforth referred as warmer ecosystems) have been modelled based on findings from the well-studied cold temperate ecosystems (Hickler *et al.*, 2008, Leakey *et al.*, 2012, Norby *et al.*, 2016, Rogers *et al.*, 2017).Warmer ecosystems differ from cold temperate ecosystems in important attributes like mean annual temperatures, amount and timing of precipitation, maximal evapotranspiration, type of nutrient limitation and vegetation type (Cernusak *et al.*, 2013), thus suggesting different responses to eCO_2 (Hickler *et al.*, 2008). Consequently, there is a need for experiments addressing the effects of eCO_2 on the less studied warmer ecosystems, in order to improve their representation in Earth

system models and accurately predict their capacity to mitigate or accelerate the impacts of climate change (Baig *et al.*, 2015, Cernusak *et al.*, 2013, Körner, 2004).

1.3 Plant photosynthetic responses to eCO_2

Since atmospheric CO_2 is an important substrate for photosynthesis, an increase in the availability of CO_2 can have profound impact on growth and physiology of plants (Fig.1.1). The well-documented effects of eCO_2 include increase in net photosynthetic rates (A_{net}) and intercellular [CO_2] (C_i), decrease in stomatal conductance (g_s) and increase in plant water-use efficiency (Ainsworth & Rogers, 2007, Long *et al.*, 2004). Since the C_3 photosynthetic pathway is CO_2-limited at current atmospheric [CO_2] and C_4 photosynthetic pathway is CO_2-saturated (Bowes, 1993), earlier predictions were that eCO_2 will enhance the A_{net} more in the C_3 plants compared to the C_4 plants (Bazzaz, 1990, Pearcy & Ehleringer, 1984, but see Ghannoum *et al.*, 2000). This short-term increase in A_{net} of C_3 species under eCO_2 may occur due to two basic reasons (Drake *et al.*, 1997, Long *et al.*, 2004). First, the Rubisco enzyme is substrate limited at current [CO_2] and hence increase in [CO_2] will increase C_i and carboxylation of Rubisco enzyme. Second, increase in [CO_2] will competitively inhibit oxygenation reaction of Rubisco enzyme and reduce photorespiratory carbon loss. Based on a biochemical theory for regulation of photosynthesis following Farquhar *et al*., (1980), a simulation model (Duursma, 2015) at 28°C and with some standard physiological parameters relevant for understory species (see Chapter 2; here modelled with a $V_{cmax-25}$ of 50 µmol m^{-2} s^{-1}, J_{max-25} of 80 µmol m^{-2} s^{-1} and $R_{light-25}$ of 0.78 µmol m^{-2} s^{-1}), a possible enhancement of photosynthesis can be modelled. Using these parameters, for an increase in atmospheric CO_2 concentration of 38%, A_{net} was increased by 48.6%. Also, the biochemical model of Farquhar *et al*., (1980), based on the kinetic properties of Rubisco, suggests that an increase in A_{net} with an increase in the [CO_2] will be greater at higher than lower leaf temperatures. This is because higher temperatures (like 35°C, for instance) favour the oxygenation reaction of Rubisco thus resulting in loss of carbon. An increase in [CO_2] competitively inhibits oxygenation thus causing increase in the A_{net} by repression of photorespiration at high temperatures (Farquhar *et al.*, 1980).

However, photosynthetic stimulation observed under eCO_2 does not always match the theoretical expectations (Ainsworth & Rogers, 2007, Nowak *et al.*, 2004). There are several factors that can interfere with and modify the responses of plants to eCO_2, particularly, under long-term exposure. Photosynthetic capacity down-regulation (Crous *et al.*, 2010, Inauen *et al.*, 2012), soil nutrient limitations (Ellsworth *et al.*, 2017, Reich *et al.*, 2006a), differences in seasonal growth conditions (Crous *et al.*, 2011, Lewis *et al.*, 1996, Onoda *et al.*, 2005), species and functional group differences in resource acquisition and allocation patterns (Ainsworth *et al.*, 2003a, Crous *et al.*, 2010, Ellsworth *et al.*, 2004) and water availability (Hovenden *et al.*, 2014, Morgan *et al.*, 2004, Perry *et al.*, 2012, Perry *et al.*, 2013) have all been shown to influence the magnitude of plant photosynthetic and productivity responses to eCO_2. In the following sections, I discuss the effects of water-limitation and photosynthetic capacity down-regulation on plant responses to eCO_2 and highlight the expected effects of these factors on the responses of warmer ecosystems.

1.4 Water-limitations and eCO_2

Based on the multiple resource-limitation hypothesis (Rastetter & Shaver, 1992), it was suggested that plant responses to eCO_2 will depend on the availability of soil resources like N, P and water (Rastetter *et al.*, 1997). Water is a primary environmental factor limiting growth and productivity in many terrestrial ecosystems like grasslands (Knapp *et al.*, 2002), deserts (Naumburg *et al.*, 2003), savannas and grassy woodlands (Polley *et al.*, 1997). Hence, water availability is expected to be crucial in determining the responses of these water-limited ecosystems to eCO_2 (Morgan *et al.*, 2004). Water-limited conditions are characterised by a decline in the soil water content, decrease in g_s and CO_2 uptake, down-regulation of light- and CO_2-saturated net photosynthesis (A_{max}), decrease in maximal rate of Rubisco carboxylation (V_{cmax}) and electron transport (J_{max}; Albert *et al.*, 2011, Craven *et al.*, 2011, Knapp *et al.*, 2001). Elevated CO_2 can mitigate the effects of drought mentioned above via two key mechanisms (Kelly *et al.*, 2016). First, through decrease in the stomatal conductance and increase in the soil water content and second, through direct stimulation of A_{net} because of increase in C_i (Fig.1.1). Consequently, the benefit of eCO_2 in terms of proportional

increase in photosynthesis and biomass is expected to be greater under water-limited conditions or in the water-limited ecosystems (Morgan *et al.*, 2004). In the following sections, I discuss the above mechanisms in detail and highlight the intervening factors.

A general, though not universal, finding from previous studies is that, eCO_2 induces stomatal closure in most of the herbaceous species irrespective of the photosynthetic pathway (Ainsworth & Rogers, 2007, Wand *et al.*, 1999). Effects of eCO_2 on g_s and hence the pools of soil moisture are indicated in Fig.1.1. In particular, eCO_2-induced decrease in g_s can lead to a decrease in the transpiration and increase in the soil water content (Ainsworth & Rogers, 2007, Blumenthal *et al.*, 2013, Morgan *et al.*, 2004). This eCO_2-induced increase in the soil water content, also termed as a 'water-savings effect' of eCO_2, has been reported across a range of water-limited ecosystems (Blumenthal *et al.*, 2013, Fay *et al.*, 2012, Morgan *et al.*, 2004) and has led to significant effects on plant growth and ecosystem processes (Blumenthal *et al.*, 2013, Fay *et al.*, 2012, Morgan *et al.*, 2004). For instance, an increase in soil water content under eCO_2 has been found to delay drying and increase plant productivity during the dry periods (Morgan *et al.*, 2011, Morgan *et al.*, 2004), lengthen the growing season (Reyes-Fox *et al.*, 2014) and increase the nutrient mineralisation rates and organic matter decomposition (Dijkstra *et al.*, 2010, Wullschleger *et al.*, 2002). However, the extent, timing and duration of eCO_2-induced 'water-savings effect' has also been found to vary among different ecosystems and species thus leading to variation in the relative eCO_2-induced enhancement of A_{net} and plant biomass. This variation in the 'water-savings effect' of eCO_2 has been attributed to changes in leaf area index, canopy temperatures (Gray *et al.*, 2016, Kelly *et al.*, 2016) and differences in soil texture (Fay *et al.*, 2012, Polley *et al.*, 2012a). For instance, eCO_2-induced increase in leaf area index counteracted the reduction in transpiration resulting from reduced stomatal conductance under eCO_2 thus leading to lack of soil water savings at the SoyFACE facility (Gray *et al.*, 2016). Furthermore, the 'water-savings effect' of eCO_2 and the relative increase in above-ground productivity were strongest on the coarse-textured sandy loam soils compared to the fine-textured soils from a mesic grassland (Fay *et al.*, 2012). Also, concurrent rise in atmospheric temperatures and vapor pressure deficit

may offset the increase in soil water content under eCO_2 (Bernacchi and VanLoocke, 2015).

The second mechanism through which eCO_2 may mitigate the effects of drought is through alleviation of stomatal limitations (Lawlor, 2002). Drought-induced decrease in g_s often leads to higher stomatal limitations (Chaves *et al.*, 2002, Lawlor, 2002). Stomatal limitation (S_{lim}) is considered to decrease C_i and A_{net}, as a result of which leaves operate on the linear part or CO_2-responsive region of the A_{net}-C_i response curve. Elevated CO_2-induced increase in C_i may help overcome the stomatal limitations thus leading to increase in photosynthetic rates (Ellsworth *et al.*, 2012, Nowak *et al.*, 2004). However, this might be true only in case of mild to moderate drought where biochemical processes are not affected (Ghannoum *et al.*, 2003, Lawlor, 2002). Severe drought can result in biochemical limitations that decrease photosynthetic capacity (Grassi & Magnani, 2005) and hence any increase in the external CO_2 concentration may be unable to restore the photosynthetic rates (Gray *et al.*, 2016, Lawlor, 2002). For example, Gray *et al*., 2016 observed that stimulation of soybean yield by eCO_2 diminished to zero during severe drought, because decreases in g_s and depression of C_i were greater in eCO_2 compared to aCO_2. Overall, intensity of drought may also cause significant variation in the relative photosynthesis and biomass responses to eCO_2.

In addition to the above two mechanisms, interaction between eCO_2 and water availability can also have other effects on the physiology and morphology of plants (Wullschleger *et al.*, 2002). These effects include (i) impacts on the leaf water potential and osmotic adjustments; (ii) enhanced instantaneous and whole plant water use efficiencies; and (iii) higher allocation of carbon to root biomass improving the plant capacity for water exploitation. Taken together, above evidences suggest the importance of water availability in controlling plants and hence ecosystem responses to eCO_2. However, these conclusions largely emerge from studies conducted in the cold temperate ecosystems (Blumenthal *et al.*, 2013, Morgan *et al.*, 2001, Morgan *et al.*, 2004). Effects of water availability on plant responses to eCO_2 have been less studied in the warmer terrestrial ecosystems compared to the cold temperate

ecosystems (Blumenthal *et al.*, 2013, Morgan *et al.*, 2004). In particular, evaporative demand in the warmer regions often exceeds precipitation thus resulting in higher water deficits compared to the cold temperate ecosystems. Consequently, compared to the cold temperate ecosystems, warmer ecosystems may have larger, but unquantified, potential for eCO_2-induced photosynthetic and biomass enhancement mediated via soil water-savings and alleviation of drought-induced stomatal limitations.

Effects of the seasonal water-availability on plant responses to eCO_2 are also less studied (Hovenden *et al.*, 2014, Lecain *et al.*, 2003, Morgan *et al.*, 2004). Seasonal water limitation is a characteristic feature of warmer ecosystems like the savannas and grassy woodlands (Polley *et al.*, 1997). As previously suggested by Morgan *et al*., 2004, such ecosystems with multiple in season wet-dry cycles have the potential for more consistent and substantial responses to eCO_2, possibly due to moderate water-stress, compared to the ecosystems with prolonged and severe dry periods. For instance, eCO_2 can ameliorate the stomatal limitations imposed by moderate drought by increasing C_i and restoring photosynthetic rates (Lawlor, 2002). Furthermore, eCO_2-induced increase in soil water content during periodic droughts may facilitate the establishment of woody plants seedlings that would otherwise be excluded due to drought stress (Bond & Midgley, 2012). However, these expectations remain contentious because field studies addressing the interaction of effects of eCO_2 and seasonal water availability on photosynthesis, soil water savings and plant productivity in the seasonally water-limited ecosystems like the grassy woodlands and warm temperate/subtropical ecosystems are lacking (Leakey *et al*., 2012). An understanding of relationship between seasonal water availability and eCO_2 effect is critical since large changes in the timing of rainfall are anticipated by climate models, even where annual total is expected to remain unchanged (Berg *et al.*, 2016, Sillmann *et al.*, 2013).

1.5 Photosynthetic capacity down-regulation under eCO_2

Elevated atmospheric CO_2 is expected to increase the carboxylation of Rubisco enzyme and decrease the rates of photorespiration thus leading to enhanced photosynthesis and growth particularly in the C_3 species (Drake *et al.*, 1997, Long *et*

al., 2004). However, this short-term stimulation of photosynthetic rates may diminish under long-term (days to years) growth at eCO_2, a phenomenon termed as photosynthetic acclimation or photosynthetic capacity down-regulation (Drake *et al.*, 1997, Long *et al.*, 2004). Down-regulation of photosynthetic capacity is manifested as a reduction in the Rubisco carboxylation (V_{cmax}) and maximal electron transport rate under eCO_2 (J_{max}) (Ainsworth & Rogers, 2007, Ellsworth *et al.*, 2004, Stitt & Krapp, 1999). Photosynthetic capacity downregulation under eCO_2 has been reported in the C_3 species from temperate ecosystems and is mostly associated with plant N status and soil N availability (Ainsworth & Rogers, 2007, Ellsworth *et al.*, 2004, Lee *et al.*, 2011).

Low soil N availability, decrease in the leaf N due to increased carbohydrates (Inauen *et al.*, 2012, Lee *et al.*, 2011), increase in the plant N demand due to accelerated growth (Yin, 2002), decrease in the N acquisition capacities (Crous *et al.*, 2010, Feng *et al.*, 2015) and source-sink imbalance due to the inability of plants to use the excess photosynthate (Ainsworth & Rogers, 2007, Long *et al.*, 2004) have all been shown to result in down-regulation of photosynthetic capacity in plants exposed to eCO_2. Given the large amount of N that plants invest in Rubisco, the key carbon fixing enzyme, this type of acclimation under eCO_2 allows plant to optimize overall performance by providing N that can be re-allocated away from photosynthetic apparatus towards other plant functions like growth and nutrient foraging (Medlyn *et al.*, 1999, Sage, 1994). This re-distribution of N could help increase the N-use efficiency of plants growing at eCO_2 (Drake *et al.*, 1997, Ellsworth *et al.*, 2004). Taken together, above evidence suggests that photosynthetic capacity down-regulation in response to growth under eCO_2 will be greater in the low N conditions compared to the high N conditions (Isopp *et al.*, 2000, Moore *et al.*, 1999).

Despite the numerous studies, there is still debate on how frequently the reductions in photosynthetic capacity are realized (Ainsworth *et al.*, 2003a, Poorter & Evans, 1998). The cold temperate ecosystems are often considered to be N-limited (Schulze *et al.*, 1994). Whereas, evidences suggest that N availability generally tends to be higher in many warmer ecosystems relative to cold temperate ecosystems (Brookshire *et al.*, 2012, Hedin *et al.*, 2009). The macronutrient P, rather than N, could be a primary

nutrient limiting photosynthesis and productivity responses to eCO_2 in the warmer ecosystems with highly weathered soils (Ellsworth *et al.*, 2017, Lambers *et al.*, 2008, Vitousek *et al.*, 2010). Because photosynthetic capacity is strongly controlled by N, one could predict that photosynthetic capacity of plants from the warmer ecosystems will be maintained under eCO_2 due to high N availability (Isopp *et al.*, 2000, Stitt & Krapp, 1999). However, this might not always be the case since photosynthetic capacity down-regulation has been observed even under N sufficient/fertilized conditions (Crous *et al.*, 2010, Inauen *et al.*, 2012) and may be attributed to increase in microbial nutrient immobilisation (De Graaff *et al.*, 2006, Gill *et al.*, 2002), decrease in the plant N acquisition capacities (Feng *et al.*, 2015) and species specific differences in N acquisition, allocation and processing capacities (Crous *et al.*, 2010, Ruiz-Vera *et al.*, 2017). Understanding the extent of photosynthetic capacity down-regulation under eCO_2 in the warmer ecosystems is critical for assessing their capacity to sequester extra C under future climate change.

In addition to the soil N status, differences among plant species and functional groups (trees, grasses, forbs and legumes) have also been shown to affect the magnitude of eCO_2-induced down-regulation of photosynthetic capacity (Ainsworth & Rogers, 2007, Crous *et al.*, 2010, Ellsworth *et al.*, 2004). These differences have been largely attributed to the differences in resource acquisition, processing and allocation patterns (Crous *et al.*, 2010, Ellsworth *et al.*, 2004, Ruiz-Vera *et al.*, 2017). For instance, Crous *et al*., (2010) observed a decrease in V_{cmax} and J_{max} under eCO_2 in the C_3 forbs, but not in the C_3 grasses, even under high soil N availability. This down-regulation response was attributed to the lower root foraging capacities resulting in lower leaf N content in the forbs. Furthermore, in a study on two tobacco cultivars with contrasting ability to produce sink (leaves), Ruiz-Vera *et al*., (2017) reported that the greater ability to utilize photosynthate resulted in only 9% down-regulation of photosynthetic capacity under eCO_2 in cultivar with higher sink capacity compared to 25% down-regulation in cultivar with lower sink capacity. Legumes may also show a smaller tendency towards down-regulation of photosynthetic capacity under eCO_2, irrespective of the soil nutrient availability, because of their abilities to fix atmospheric N and the strong carbon demand (Ainsworth *et al.*, 2003b, Rogers *et al.*, 2009). Thus, plant species vary

in the magnitude of photosynthetic acclimation responses to eCO_2, which may be independent of nutrient supply (Inauen *et al.*, 2012, Lee *et al.*, 2011), and could be attributed to differences in traits and growth strategies. Such differential photosynthesis and productivity responses to eCO_2 among the plant species and functional groups can have significant impact on composition and functioning of the terrestrial ecosystems in future (Reich *et al.*, 2001, Zelikova *et al.*, 2014).

1.6 Plant productivity responses to eCO_2

Several previous studies suggest that CO_2 fertilisation increases A_{net} in the C_3 species (Ainsworth & Rogers, 2007, Long *et al.*, 2004). But does the increase photosynthesis under eCO_2 always lead to an increase in the plant biomass? Many studies report a positive CO_2 fertilisation effect on plant growth across a range of terrestrial ecosystems (Ainsworth & Long, 2005, McCarthy *et al.*, 2010, Talhelm *et al.*, 2014). In contrast, there are evidences showing a lack of eCO_2-induced increase in biomass in the trees and herbaceous species (Ellsworth *et al.*, 2017, Inauen *et al.*, 2012, Reich & Hobbie, 2013). Increase in the ecosystem productivity under eCO_2 has been expected to sequester more carbon in future thus resulting in a negative feedback on the climate change (Arora *et al.*, 2013, Baig *et al.*, 2015, Friedlingstein *et al.*, 2014). However, previous reports on variable stimulation of plant biomass under eCO_2 suggest that the capacities of different ecosystems to act as a carbon sink will differ depending on the plant species, type of nutrient limitation, water availability and climatic conditions (Ahlström *et al.*, 2015, Ahlström *et al.*, 2013). In principle, the C_3 species growing at higher average annual temperatures have greater potential to respond positively to eCO_2 in terms of photosynthesis and biomass, than the plants growing in regions with low average annual temperatures (Hickler *et al.*, 2008). This is because, high temperature decreases the CO_2 specificity of Rubisco thus increasing photorespiration (Farquhar *et al.*, 1980, Long, 1991). On the other hand, eCO_2 is expected to decrease photorespiration thus increasing the CO_2 assimilation rates and net primary productivity (Farquhar *et al.*, 1980, Long, 1991). Consequently, modelling studies based on the Rubisco kinetics predict a larger proportional stimulation of +35% in net primary productivity for the C_3 species growing in the warmer ecosystems, compared

to the +23% stimulation expected for the cooler higher latitude ecosystems (Hickler *et al.*, 2008). Furthermore, eCO_2-induced stimulation of biomass is also expected to be higher in the water-limited ecosystems because eCO_2 can ameliorate the negative effects of drought by increasing C_i and soil water content (Fatichi *et al.*, 2016, Morgan *et al.*, 2011, Morgan *et al.*, 2004). Because the warmer ecosystems also experience higher water-deficits compared to the cold temperate ecosystems, this may further support the expectation of larger stimulation of plant biomass in the former ecosystem. However, these estimates for eCO_2-induced increase in plant productivity for the warm, water-limited ecosystems, assume that changes in photosynthetic rates under eCO_2 drive changes in productivity, which is often not the case (Kirschbaum, 2011, Körner, 2004). Therefore, it is important to determine whether the prediction of higher eCO_2-induced productivity in warm, water-limited ecosystems is supported by experimental observations. Though high mean annual temperatures and water-limitation may stimulate biomass under eCO_2 in the warmer ecosystems, intervening factors like low P availability (Cernusak *et al.*, 2013), increased microbial nutrient immobilisation (Gill *et al.*, 2002) and decrease in N acquisition capacities (Feng *et al.*, 2015) may dampen this response (Fig.1.1). For instance, a recent study conducted in a P-limited sub-tropical grassy woodland ecosystem observed lack of eCO_2-induced increase in biomass in the mature trees (Ellsworth *et al.*, 2017). It would be interesting to investigate if a similar lack of growth response to eCO_2 is evident in the herbaceous species growing in P-limited soils.

1.7 Understory species and eCO_2

Over the past few decades, free-air CO_2 enrichment experiments have been conducted in different types of terrestrial ecosystems to assess their responses to eCO_2 in terms of photosynthesis, growth and carbon sequestration (Ellsworth *et al.*, 2012, Körner *et al.*, 2005, McCarthy *et al.*, 2010). However, these experiments largely focus on over story responses to eCO_2 (Ellsworth *et al.*, 2012, Liberloo *et al.*, 2009, Talhelm *et al.*, 2014) and responses of grasslands (Hovenden *et al.*, 2014, Lee *et al.*, 2011, Morgan *et al.*, 2011). Very few studies focus on the understory vegetation responses to eCO_2, but generally involve tree seedling and shrubs (Kim *et al.*, 2015, Naumburg & Ellsworth,

2000, Sefcik *et al.*, 2007, Springer & Thomas, 2007). Till date, less attention has been paid on the eCO_2 impacts on photosynthesis and productivity responses of the understory herbaceous vegetation (Bandeff *et al.*, 2006, Dawes *et al.*, 2015), in spite of its importance for overall ecosystem diversity and productivity (Misson *et al.*, 2007, Nilsson & Wardle, 2005, Oliver & Larson, 1996).

An important driver of understory plant community is light availability (Chazdon & Pearcy, 1991, Springer & Thomas, 2007). Light availability in the understory is variable, with periods of low diffuse light alternating with periods of high light intensity called as sun flecks (Chazdon & Pearcy, 1991). Efficient utilisation of sun flecks determines the daily carbon gain of the understory species (Naumburg & Ellsworth, 2000). Is this low level of light likely to preclude growth stimulation by CO_2? In theory, eCO_2 should reduce photorespiration, increase quantum yield and, thus, decrease the light compensation point of photosynthesis (Long & Drake, 1991). Hence, the leaf carbon balance should be improved in low light under eCO_2, resulting in larger relative photosynthesis and growth enhancement responses in the understory species (Granados & Körner, 2002, Würth *et al.*, 1998). However, the few studies that have examined understory herbaceous species and woody seedling responses to eCO_2 show inconsistent results (Bandeff *et al.*, 2006, Kim *et al.*, 2015, Naumburg & Ellsworth, 2000, Souza *et al.*, 2010). For example, light-saturated net photosynthetic rates of the seedlings of four broadleaved species growing in the understory of loblolly pine forest increased under eCO_2 at the Duke FACE experiment (Naumburg & Ellsworth, 2000). Furthermore, the shade-tolerant species were found to be most responsive to eCO_2 in terms of increase in A_{net} whereas, the least shade tolerant species showed lower A_{net} enhancement under eCO_2 (Ellsworth *et al.*, 2012). Similar to photosynthetic responses, biomass responses of the understory vegetation to eCO_2 have also been found to be inconsistent with responses ranging from increased biomass (Souza *et al.*, 2010), lack of eCO_2-induced biomass enhancement (Bandeff *et al.*, 2006, Kim *et al.*, 2015) and decrease in biomass under eCO_2 (Awmack *et al.*, 2007). These inconsistent photosynthesis and biomass responses to eCO_2 in the understory vegetation could be attributed to variation in species shade-tolerance capacities, over story dynamics, water and nutrient availability (Belote *et al.*, 2004, Kim *et al.*, 2015,

Kubiske *et al.*, 2002, Sefcik *et al.*, 2007). Among these, over story dynamics has been found to be important in determining the understory photosynthesis and biomass responses to eCO_2 (Kim *et al.*, 2015, Sefcik *et al.*, 2007).

The responses of over story trees to eCO_2 can indirectly affect the understory responses to eCO_2, mostly by altering the understory environmental conditions like soil temperature, moisture and light availability (Bandeff *et al.*, 2006, Kim *et al.*, 2015). In particular, previous field studies report that eCO_2 increases the over story leaf production and increases leaf area index (Lewis *et al.*, 2010, Liberloo *et al.*, 2007, McCarthy *et al.*, 2010, Norby & Zak, 2011). Thereby, eCO_2 may decrease light availability in the understory. Such changes in understory light availability could offset or even reverse the positive effects of CO_2 fertilisation on the photosynthesis and biomass of the understory vegetation (Bandeff *et al.*, 2006, Kim *et al.*, 2015). For example, increases in over story leaf area index under eCO_2 led to a reduction in the understory light availability which nullified the growth enhancing effect of eCO_2 on the understory vegetation of a Pine forest (Kim *et al.*, 2015).

1.8 *Eucalyptus* woodlands *and Eucalyptus* free-air CO_2 enrichment experiment

Tree-grass ecosystems like savannas and grass-dominated woodlands cover more than 20% of the global terrestrial landscape, occupying extensive areas of tropical and sub-tropical regions in Africa, Asia, South America and Australia (Bond & Midgley, 2000). An important example of such tree-grass ecosystems are the grass-dominated *Eucalyptus* woodlands of Australia (Scheiter *et al*., 2015). Savannas and grass-dominated woodlands are characterized by the presence of tree-grass mixtures with seasonal water-limitation, grass-fire feedbacks and tree-grass competition as important mechanisms controlling the existence of these ecosystems (Baudena *et al*., 2015). Elevated CO_2 is expected to have profound effects on the composition and functioning of these ecosystems globally (Bond & Midgley, 2000, Scheiter *et al*., 2015), which may be mediated through increases in soil water content and plant productivity and altered tree-grass interactions (Bond & Midgley, 2000, Bond & Midgley, 2012, Polley *et al*., 1997). An important consequence of these eCO_2-induced changes could be

altered fire-regimes due to changes in soil water content and plant biomass (Bond & Midgley, 2012). Despite their importance for global productivity and biogeochemical cycles, the tree-grass ecosystems remain less studied in terms of response to eCO_2 (Leakey *et al*., 2012).

To address the knowledge gaps discussed in previous sections, I undertook a study of the herbaceous plant community from a *Eucalyptus* woodland near Richmond, NSW Australia (33° 37' S, 150° 44.3' E). Several distinct characteristics of this tree-grass ecosystem, called as Cumberland Plain Woodland (CPW), provide a unique opportunity to address the effects of eCO_2 on photosynthesis and productivity of herbaceous understory species growing in warm, water and nutrient limited ecosystem. CPW is characterised by a warm-temperate to sub-tropical climate with a mean annual temperature of 17^oC and a mean daily maximum temperature of 30^oC during the warmest month (January) and 17.6^oC during the coldest month (July). In addition to the year-round warm climate, the site also experiences water-limited conditions indicated by a 20-year average annual precipitation of 800 mm and an estimated annual pan evapotranspiration of 1350 mm (Duursma *et al.*, 2016). Precipitation occurs periodically and throughout the year thus resulting in multiple seasonal wet-dry cycles. This variability in seasonal water availability helps me address the effects of periodic droughts on eCO_2-induced photosynthetic enhancement and 'water-savings effects'. In addition to seasonal water availability, tree-grass interactions and fire have a profound effect on the existence of CPW (Watson, 2005). Furthermore, the site is characterised by presence of nutrient-limited soils, in particular low P availability (Crous *et al.*, 2015). This feature helps me test the effects of eCO_2 on photosynthetic capacity acclimation and biomass of plants growing under nutrient-limited, especially P-limited, conditions. Another significant feature of the site is the diverse vegetation type. The vegetation consists of an over story dominated by canopy forming trees like *Eucalyptus tereticornis* Sm. and *E. amplifolia* Naudin (Gimeno *et al.*, 2016). However, the relatively high species diversity of this vegetation type (≈ 70 species) is attributed to the herbaceous understory vegetation (Tozer, 2003) comprising a mixture of C_3 grasses, C_3 forbs and C_4 grasses. *Microlaena stipoides* Labill., a native C_3 grass, is the dominant herbaceous species at EucFACE (≈ 70% of total understorey biomass,

Chapter 3) along with the co-occurrence of native C_3 forbs like *Lobelia purpurascens* R.Br., and native C_4 grasses like *Cymbopogon refractus* R.Br. In addition, an invasive C_3 forb -*Senecio madagascariensis* Poir. and an invasive C_3 grass-*Nasella neesiana* (Trin. & Rupr.) Barkworth, have a significant presence in the CPW ecosystem (McNaught, 2006, Sands & Goolsby, 2011). Four species, that is, *M. stipoides, L. purpurascens, S. madagascariensis* and *N. neesiana* were the focus of current study. These four species are evergreen as they possess green leaves throughout the year, depending on water availability and show a progressive type of leaf senescence wherein the older leaves die but the young leaves are still active (Leopold, 1961). These species flower from spring season through to autumn season. *L. purpurascens* is a small, creeping forb growing about 30 cm in length (Image 1.2c). *S. madagascariensis* is an erect forb with numerous branches and grows up to 20-60 cm high (Image 1.2d).

Free-air CO_2 enrichment (FACE) experiments provide the most feasible method to study eCO_2 effects on the terrestrial ecosystems (Nowak *et al.*, 2004). These experiments typically involve the use of horizontal and vertical gas dispersal pipes around the experimental plots, forming a 10-30 m diameter rings (Image 1.1). These pipes emit regulated concentrations of CO_2 within the canopy thus exposing the plants to futuristic levels of CO_2. Since the experimental plots are not isolated from the surrounding natural environment, FACE experiments facilitate a direct field insight into effects of eCO_2 on the complex terrestrial ecosystem functioning. One such experiment is the *Eucalyptus* free-air CO_2 enrichment experiment (EucFACE, Image 1.1) located on an ancient alluvial flood plain in a remnant patch of native CPW near Richmond, NSW Australia (33° 37' S, 150° 44.3' E). EucFACE is one of three novel next generation FACE experiments (Norby *et al.*, 2016) and the very first forest FACE established in a mature forest growing on P-limited soils. Herein, this thesis presents results from the first three years of CO_2 fertilisation at EucFACE, addressing the effects of eCO_2 on seasonal photosynthesis, stomatal conductance, soil water savings (Chapter 2), photosynthetic acclimation and above-ground productivity (Chapter 3) of herbaceous understory. In addition, a glasshouse study was performed to investigate the effects of eCO_2 on photosynthesis, total biomass and biomass allocation for C_3 grasses and C_3 forbs growing under similar water and nutrient supply.

1.9 Thesis outline, objectives and hypotheses

I carried out a series of experiments at the EucFACE facility and in the glasshouse. The work is presented in this thesis as a series of three experimental papers, accepted or prepared for submission to peer-reviewed journals. There are total five chapters in this thesis which includes an introductory literature review (Chapter 1), three experimental chapters (Chapter 2, 3 and 4) and a final synthesis and general discussion (Chapter 5) that contextualises the research, discusses key findings and implications. In the following sections, I highlight the specific objectives and hypotheses of three experimental chapters.

Chapter 2: Water availability affects seasonal CO_2-induced photosynthetic enhancement in herbaceous species in a periodically dry woodland

(Published in the journal of *Global Change Biology* (Pathare *et al.*, 2017). The thesis includes a revised version of this publication as Chapter 2).

In Chapter 2, I investigated the relationship between seasonal water-availability and eCO_2-induced photosynthetic enhancement. I also investigated whether eCO_2 results in a 'water-savings effect' in a warm and seasonally water-limited grassy woodland. In addition to seasonal water-availability, I also tested whether seasonal variation in temperature affected the relative eCO_2-induced photosynthetic enhancement. Because water is an important factor limiting growth and productivity in this grassy woodland ecosystem, I hypothesized that,

(i) Maximum photosynthetic enhancement by eCO_2 will be observed in dry seasons;

(ii) This photosynthetic enhancement will be mediated by a decrease in stomatal conductance in eCO_2 and hence increases in soil water content;

(iii) Elevated CO_2 will alleviate stomatal limitations induced by stomatal closure during the dry periods thus resulting in increased photosynthetic rates.

Chapter 2 is based on a field experiment conducted at the EucFACE facility. In order to test above hypotheses, I conducted a series of seasonal photosynthetic gas exchange

measurements on the dominant understory C_3 herbaceous species at the EucFACE facility during the first three years of CO_2 fertilisation at this experiment.

Chapter 3: Photosynthetic acclimation and productivity responses of understory herbaceous species from a resource-limited Eucalyptus woodland

In Chapter 3, I investigated the effects of eCO_2 on seasonal photosynthetic acclimation responses of a dominant C_3 grass and C_3 forb growing in the understory of a warm, water and nutrient-limited, especially P-limited (Crous *et al.*, 2015), grassy woodland ecosystem. In addition to photosynthetic responses, I also investigated the aboveground biomass responses to eCO_2 for total grasses and total forbs. I hypothesized that,

(i) There would be a larger down-regulation of photosynthetic capacity under eCO_2 during autumn than spring and summer due to the lower growth sink capacity in that season;

(ii) There will be a significant increase in above-ground biomass of herbaceous species under eCO_2.

Chapter 3 is based on a field experiment conducted at the EucFACE facility. To test above hypotheses, I conducted a series of seasonal photosynthetic gas exchange, and N content measurements on the dominant C_3 grass and C_3 forb at the EucFACE facility during the second and third year of CO_2 fertilisation at this experiment. In addition, I measured the above-ground biomass of total grasses and total forbs for two peak growing seasons of summer during the second and third year of CO_2 fertilisation.

Chapter 4: Differential photosynthetic and biomass responses of C_3 grasses and C_3 forbs to elevated CO_2 under nutrient-limited conditions

The goal of Chapter 4 was to examine how co-existing C_3 grasses and C_3 forbs, provided with similar water inputs and nutrient supply from the soil for growth, may respond differently to eCO_2. In particular, I investigated the key photosynthetic and morphological traits responsible for differential species responses to eCO_2. Further, I also investigated the effects of eCO_2 on total biomass and biomass allocation for C_3 grasses and C_3 forbs. I hypothesized that,

(i) Photosynthetic capacity down-regulation will be manifested as a decrease in leaf N content and/or protein specific down-regulation.

(ii) Down-regulation of photosynthetic capacity would result in little or no enhancement of photosynthetic rates and biomass

Chapter 4 is based on a glasshouse experiment. This experiment was designed to simulate the nutrient-limited conditions at EucFACE and hence soil excavated from around the EucFACE facility was used for this experiment.

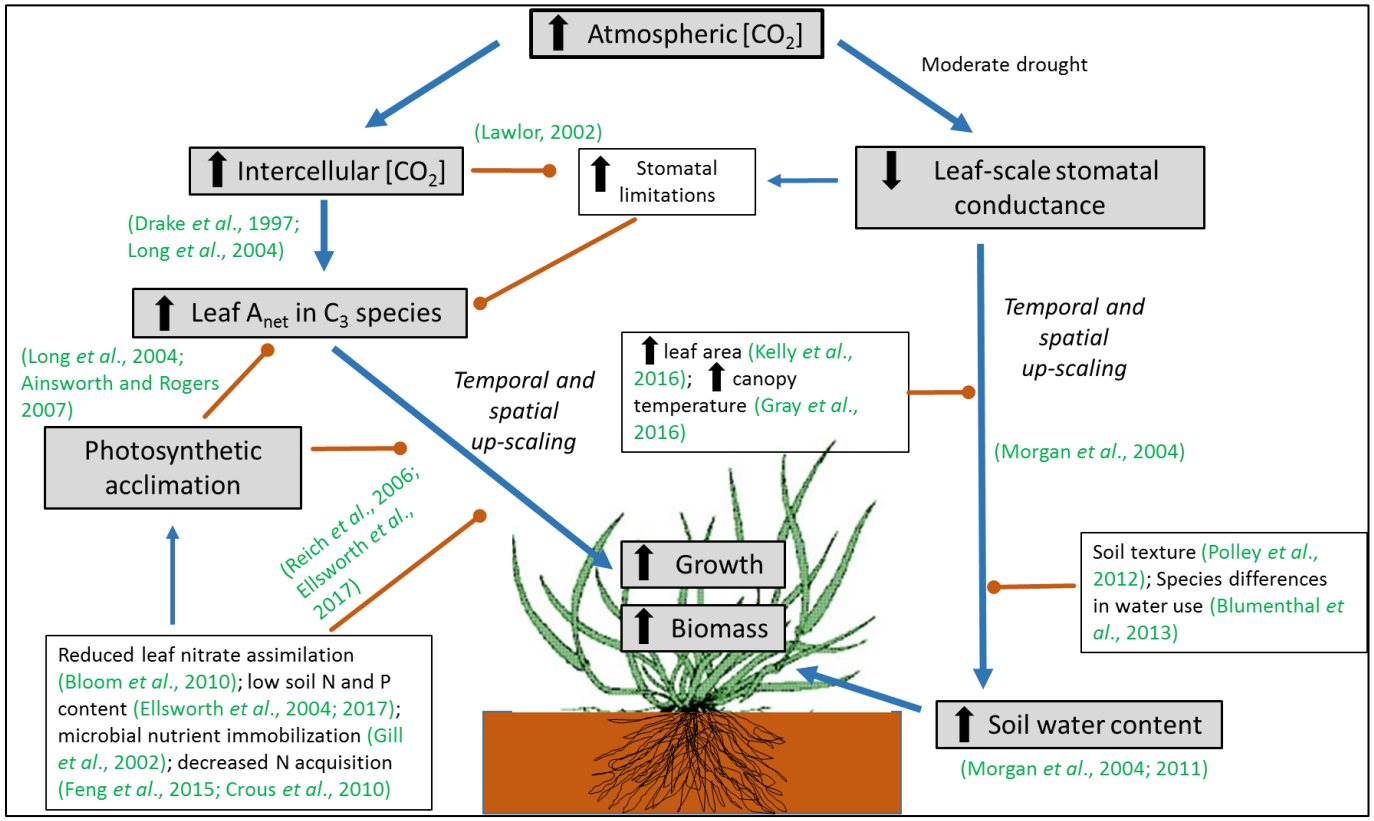

Fig. 1.1 Schematic representation of eCO_2 effects on the fundamental processes, that is, photosynthetic rates (A_{net}) and stomatal conductance leading to the pool of biomass and the pool of soil moisture, respectively.

Basic hypothesized effects of eCO_2 on the fundamental processes or pools are shown in grey boxes. Upward and downward pointing black arrows indicate increases and decreases, respectively, due to eCO_2. Blue arrows indicate promotion of the eCO_2 effect. Orange arrows indicate biotic and abiotic factors that may counteract/inhibit the eCO_2 effects. Key references are indicted in green

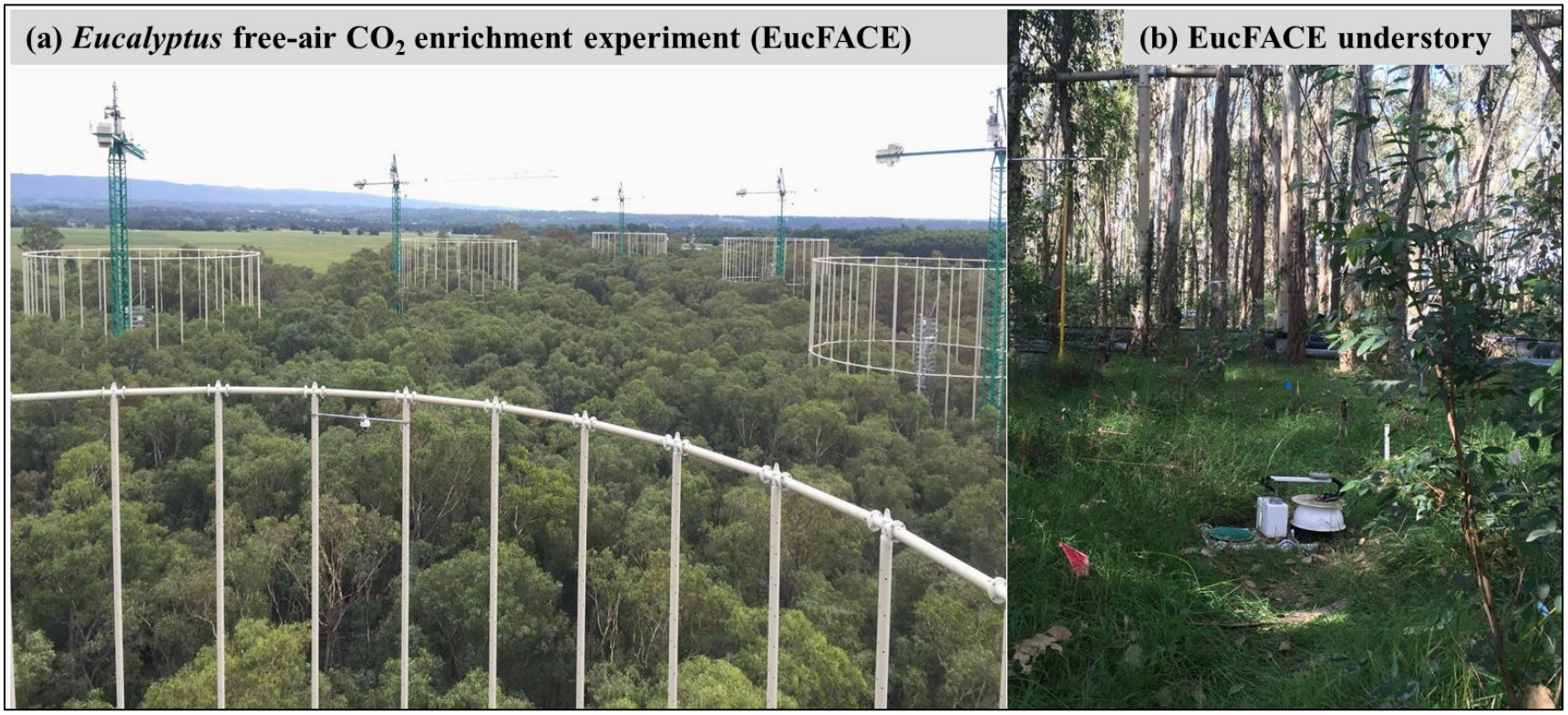

Image 1.1 *Eucalyptus* free-air CO_2 enrichment experiment (EucFACE).

(a) EucFACE is located in the native Cumberland Plain woodland ecosystem. The facility consists of three eCO_2 and three aCO_2 plots.
(b) Second image show the herbaceous understory of the EucFACE facility. Images were photographed by Ms. Varsha Pathare.

Image 1.2 Herbaceous plant species used in the current study.

(a) *Microlaena stipoides* Labill. - a native, evergreen C_3 grass growing naturally at the EucFACE. (b) *Nasella neesiana* (Trin. & Rupr.) Barkworth - an invasive, evergreen C_3 grass growing in a pot during the glasshouse experiment. (c) *Lobelia purpurascens* R.Br. - a native, evergreen C_3 forb growing naturally at the EucFACE. (d) *Senecio madagascariensis* Poir. - an invasive, evergreen C_3 forb growing naturally at the EucFACE. Images were photographed by Ms. Varsha Pathare.

Chapter 2 : Water availability affects seasonal CO_2-induced photosynthetic enhancement in herbaceous species in a periodically dry woodland

This Chapter has been published in the journal of Global Change Biology as:

Pathare VS, Crous KY, Cooke J, Creek D, Ghannoum O, Ellsworth DS (2017) Water availability affects seasonal CO_2-induced photosynthetic enhancement in herbaceous species in a periodically dry woodland. *Global Change Biology*, **23**, 5164-5178. https://doi.org/10.1111/gcb.13778.

This thesis includes a revised version of above publication.

2.1 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. I 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 10-62% 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}. A photosynthetic enhancement by eCO_2 of > 30% was observed during the most water-limited periods, 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 ≈ 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. I 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.

2.2 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 hypothesized 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, 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 rationalize 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 eCO_2-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.*, 2012a). Though the eCO_2-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 eCO_2 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 eCO_2 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 eCO_2 effect in terms of relative increases in photosynthesis and biomass 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), since water stress may limit plant response to eCO_2 during severe dry periods. Water demand for herbaceous species varies seasonally (Knapp *et al.*, 2002) suggesting that the benefit of eCO_2-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 eCO_2 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_3 plants might benefit from CO_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_2 assimilation. This restriction of stomata to CO_2 supply, also termed as stomatal limitation, decreases leaf intercellular CO_2 concentrations (C_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_2 response curve (Ellsworth *et al.*, 2012). Under such conditions, CO_2 fertilisation can help alleviate the stomatal limitations by increasing C_i and hence plants would experience larger photosynthetic enhancement (Kelly *et al.*, 2016, Lawlor, 2002). The importance of such limitations in controlling eCO_2-induced photosynthetic enhancement during dry periods has been less studied in the field conditions (Galmés *et al.*, 2007, Grassi & Magnani, 2005)..

Building on knowledge from previous ecosystem studies (see Leakey *et al.*, 2012), I examined eCO_2 responses of an herbaceous understory community in the *Eucalyptus* Free Air CO_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_2 response in a periodically water-limited woodland ecosystem. I hypothesized that:

H1: Maximum photosynthetic enhancement by eCO_2 will be observed in dry seasons;

H2: This eCO_2-induced photosynthetic enhancement will be mediated by a decrease in stomatal conductance in eCO_2 and hence increases in soil water content;

H3: Elevated CO_2 will reduce stomatal limitations induced by stomatal closure during the dry periods thus resulting in increased photosynthetic rates.

To test the above hypotheses, I measured leaf CO_2 assimilation and stomatal conductance of a dominant C_3 grass across seasons over three years, as well as corroborating evidence from two sympatric C_3 forbs over 1 ½ years.

2.3 Materials and Methods

2.3.1 Experimental design and site description

I conducted leaf level gas exchange measurements on herbaceous understory in the first three years of the *Eucalyptus* Free-Air CO_2 Enrichment (EucFACE) experiment (Image 2.1). EucFACE consists of six 25-m diameter circular plots or rings, with three of these maintained at ambient CO_2 (aCO_2) and three maintained at elevated CO_2 (ambient + 150 µmol mol^{-1}, eCO_2) since February 2013 (see Crous *et al*., 2015 and Gimeno *et al*., 2016). CO_2 treatment was completely randomised among the six plots at the outset. Ambient [CO_2] is constantly measured in the control rings. 150 ppm CO_2 is added to this measured value to get the set point for the eCO_2 rings. This is adjusted every second by the FACE Control Program (FCP). Also, CO_2 fumigation only occurs during daylight hours.

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 C_3 grass, locally termed a grassy *Eucalyptus* woodland. I measured three common C_3 herbaceous understorey species in current study (see Image 1.2 and section 1.8 of Chapter 1 for species details): 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^oC, characterised by a mean daily maximum temperature of 30.0^oC during the warmest month (January) and 17.6^oC during the coldest month (July) (http://www.bom.gov.au/climate/averages/tables/cw_067105.shtml) (Fig. 2.1a). 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. 2.1b). The soil at the site is a well-drained, sandy loam with low organic carbon content (Gimeno *et al*., 2016). The soil is nutrient poor,

particularly strongly P-limited (Crous *et al.*, 2015). (see Crous *et al*., 2015 and Hasegawa *et al*., 2016 for details of soil N and P content).

2.3.2 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 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 C_3 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 cm^2 chambers were used for gas exchange measurements. In order to assess instantaneous and long-term effects of eCO_2 on the photosynthetic capacities of the species, photosynthetic CO_2 response curves (A_{net}-C_i curves) were measured, starting at the mean growth CO_2 concentration for each treatment (≈ 400 µmol mol^{-1} for aCO_2 and ≈ 550 µmol mol^{-1} for eCO_2). Average daytime CO_2 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). Young active leaves were selected for measurement every season. 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 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_a) 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 ten 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}; ^oC). During the A_{net}-C_i measurements, [CO_2] in the cuvette was controlled as reference. 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 was controlled at the prevailing mean daily maximum air temperatures (T_{air}) during each measurement season (18, 22, 27 and 29 ^{o}C for winter, autumn, spring and summer respectively) (Fig. 2.1a). 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. Thus, 36 A_{net}-C_i responses curves were measured every season (two A_{net}-C_i responses curves per species per plot). All measurements were completed over the course of three days at the rate of 12 A_{net}-C_i response curves per day whilst measuring six A_{net}-C_i response curves from an aCO_2 plot and six from an eCO_2 plot. Three Li-COR 6400XT were used for gas exchange measurements during each time-point. All the three licors were user calibrated using a standard method mentioned in the licor manual. As a part daily warm-up tasks, licors were checked for flow meter zero, CO_2 and H_2O IRGA zeros and T_{leaf} zero. If required, IRGAs' were zeroed using fresh soda lime and drierite. To determine leaks, flow rate was set to 200 µmol s^{-1}. With the chamber closed and empty, air was exhaled around the chamber gaskets to look for any fluctuations in the sample cell [CO_2]. Increase in sample CO_2 values by less than 1 µmol mol^{-1} suggested absence of leaks. New chamber gaskets were used for each time-point measurement. 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, 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_{net}-Ci 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_{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} I used a fixed mesophyll conductance value of 0.2 mol m^{-2} s^{-1} for the evergreen herbaceous species (Flexas *et*

al., 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 *et al.*, 2002), especially as seasonal temperatures varied. In order to do this, I 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 *et al.*, 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-normalised V_{cmax} ($V_{cmax\text{-}25}$) from the A_{net}-C_i response curves.

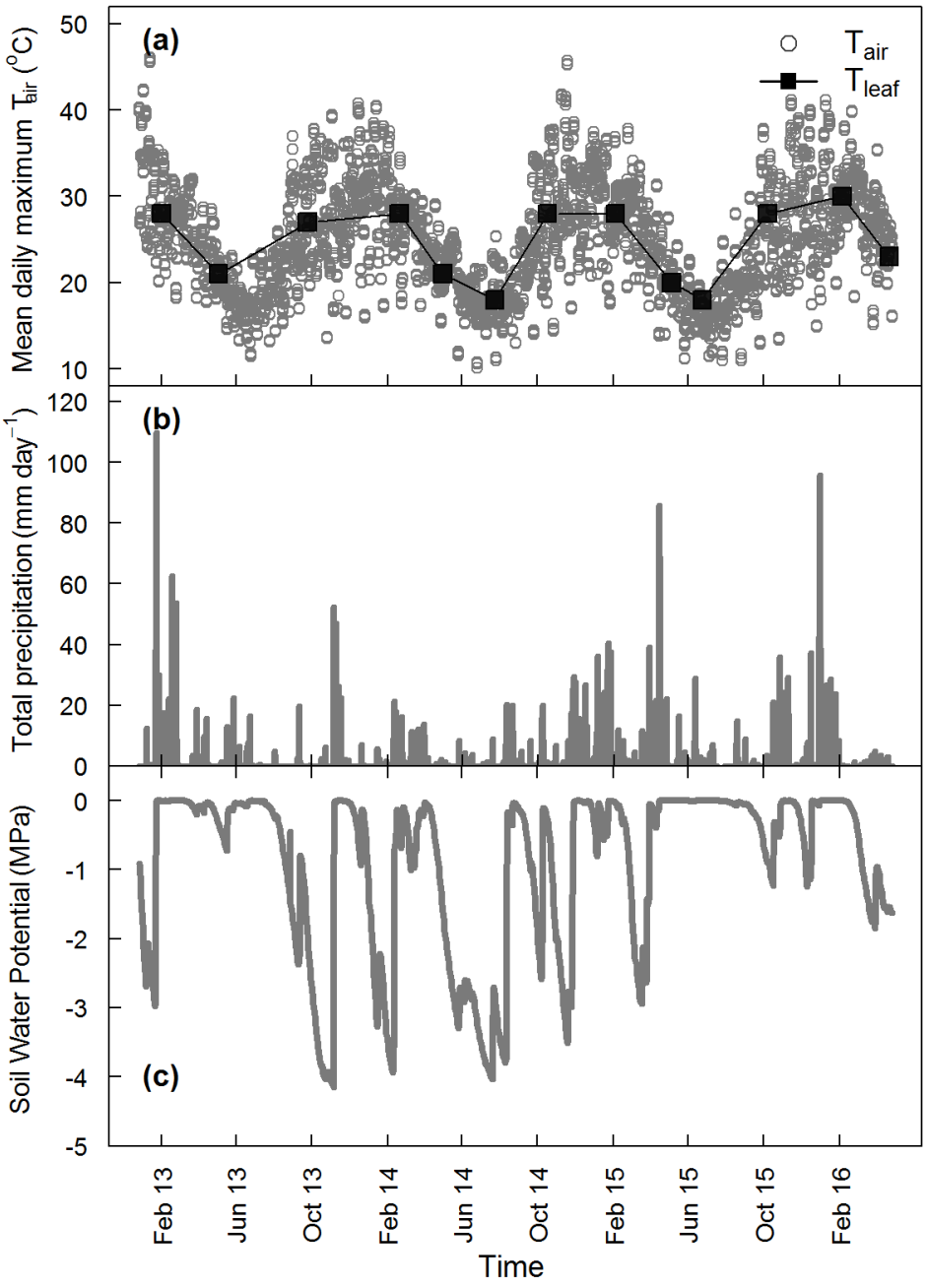

Fig. 2.1 Temperature, precipitation and soil water potential at EucFACE from February 2013 to May 2016.

Time course through the three measurement years for (a) daily maximum air temperature (T_{air} in ºC, open circles), and mean leaf temperature at the time of measurement (T_{leaf} in ºC, filled squares), (b) daily total precipitation received at the site, and (c) surface soil water potential (0-30cm depth). T_{leaf} is a mean of three understory species.

2.3.3 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 modelling the diffusional pathway and based on the A_{net}-C_i response curves. For calculating S_{lim} to CO_2 assimilation rates, I used the approach proposed by Grassi & Magnani (2005) which is similar to that defined in Jones, 1985. We computed S_{lim} as follows:

$$S_{lim} = \frac{\partial A_{net}/\partial C_i}{g_{sc} + \partial A_{net}/\partial C_i} \qquad \text{(Eq.2.1)}$$

where, $\partial A_{net}/\partial C_i$ is the partial derivative of net CO_2 assimilation (A_{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). My approach uses a static mesophyll conductance to CO_2 (g_{mes} of 0.2 mol m^{-2} s^{-1}) as the study was focused at the whole-leaf scale, and the magnitude of S_{lim} is not strongly affected by the inclusion of mesophyll conductance effects (Grassi & Magnani, 2005).

In addition to S_{lim}, I also derived C_i difference using the A_{net}-C_i responses curves. C_i difference was calculated as the difference between the transition C_i (or C_i at the V_{cmax}-J_{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_{net}-C_i response curve.

2.3.4 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^{o} angle (CS650-L; Campbell Scientific, Logan, UT, USA). V_{SWC} data was recorded at 15 min interval by a data logger in each plot (C3000; Campbell Scientific, Logan, UT, USA). Eight CS650-L soil moisture probes were installed per CO_2 plot thus allowing accurate and highly replicated measurements of V_{SWC}. Campbell Scientific maintains that a soil-specific calibration is not required for the CS650-L soil moisture probes (https://www.campbellsci.com/cs650). Some important feature of CS650-L soil moisture probes are, lower error due to larger sample volume, measurement corrected for effects of soil texture and electrical conductivity and estimation of soil water content for a wide range of soil types. In the current study, I 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 and -0.006 MPa respectively.

2.3.5 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 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_{net}, $V_{cmax\text{-}25}$, $J_{max\text{-}25}$, V_{cmax}, J_{max}, N content, g_s, C_i, S_{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 I used the Benjamini-Hochberg procedure for the number of tests I 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_{net} enhancement. These key variables were chosen according to their causal hypothesized roles in regulating eCO_2-induced photosynthetic enhancement (Ellsworth *et al.*, 2012; see Supplemental information for further details). I also employed Structural Equation Modelling (SEM) 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). I 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), I included them in the structural equation models (Fig. 2.7 and Figs. S2.6 to S2.8) as they are key parts of the overall hypotheses asked.

Table 2.1 Results of mixed-model split-plot ANOVA for net photosynthesis (A_{net}), temperature normalised maximum carboxylation ($V_{cmax-25}$) and electron transport rates (J_{max-25}), N content on area basis (N_{area}), stomatal conductance (g_s), relative stomatal limitation (S_{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[1].

Results shown are across *M. stipoides*, *L. purpurascens* and *S. madagascariensis*. CO_2 refers to the 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.

	Source of variation													
	CO_2 (1, 4)		Season (2, 8)		Species (1, 12)		CO_2 x Season (2, 8)		CO_2 x Species (1, 12)		Season x Species (2, 12)		CO_2 x Season x Species (2, 12)	
Variables	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
A_{net}	11.330	**0.028**	31.440	**<0.001**	21.700	**0.001**	0.580	0.578	0.083	0.777	2.520	0.122	0.890	0.433
A_{net-Ca}	1.730	0.258	3.770	**<0.001**	9.530	**0.009**	2.207	0.172	0.140	0.714	1.161	0.346	0.979	0.403
$V_{cmax-25}$	1.569	0.278	12.050	**0.004**	36.726	**<0.001**	6.148	**0.024**	0.809	0.386	0.751	0.492	2.591	0.116
J_{max-25}	0.574	0.491	13.820	**0.003**	33.570	**<0.001**	4.875	**0.041**	0.000	0.996	0.326	0.728	0.837	0.456
N_{area}	1.460	0.290	8.650	**0.010**	9.670	**0.009**	0.207	0.817	1.020	0.333	1.160	0.346	1.470	0.268
N_{mass}	1.060	0.361	4.160	**0.057**	27.350	**<0.001**	2.430	0.149	0.064	0.804	1.758	0.214	1.153	0.348
$f_{N-Rubisco}$	0.037	0.857	2.770	0.121	24.930	**<0.001**	8.220	**0.011**	0.000	0.986	1.836	0.201	1.210	0.332
$J_{max-25}/V_{cmax-25}$	0.350	0.580	0.270	0.770	5.870	**0.032**	2.590	0.135	1.105	0.313	2.190	0.154	1.250	0.321
LMA	2.116	0.224	25.420	**<0.001**	2.659	0.129	1.257	0.335	0.434	0.522	0.760	0.491	3.480	**0.064**

[1]All variables were transformed (square root or log transformation) to meet the normality assumptions for the mixed-model ANOVA.

2.4 Results

2.4.1 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} (≈ 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. 2.2a-c). Similarly, for the dominant *M. stipoides*, eCO_2 resulted in a significant increase in A_{net} (≈ 32%) across the 13 time points across three years (P = 0.019, Table S2.1, Fig. 2.2a). There was a significant measurement time effect on A_{net} across species (P < 0.001, Table 2.1 and S2.1, Fig. 2.2a-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 April2016). For *M stipoides*, maximum A_{net} (12 ± 1.5 µmol m^{-2} s^{-1}) occurred during the wet and warmer times (February 2013, February 2014, October 2014 and February15), with minimum A_{net} of ~5 ± 1.2 µmol m^{-2} s^{-1} occurring in two dry periods, October13 and July14. I did not observe a significant CO_2 x measurement time effect on A_{net} across the three species (P > 0.02, Table 2.1 and S2.1). 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 February16 (40%) and the minimum was observed in February15 (13%). Similarly, for the dominant *M. stipoides*, the maximum increase in A_{net} due to eCO_2 was observed in October 2013 (62%), whereas minimum increase was reported in February 2014 (13%). Overall, I observed a significant seasonal variation in the A_{net} values and the magnitude of eCO_2-induced photosynthetic enhancement across all the species (Fig. 2.2a-c). I 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 2.1, Fig. 2.2d-f). However, there were highly significant measurement time effects on g_s in all species (P < 0.01, Table 2.1 and Table S2.1) with average values ranging from maximum of 0.27 ± 0.03 mol m^{-2} s^{-1} in October 2015 and February 2016 to 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_{net}. Given that higher A_{net} values were observed during time points with higher g_s (Fig. 2.2), the seasonal variation in A_{net} could be partly ascribed to seasonal variation in the g_s. This dependence of A_{net} on g_s is evident from the positive correlation between A_{net} and g_s for the three species under both, aCO_2 (r^2 = 0.64, P < 0.01, Fig. S2.1a) and eCO_2 (r^2 = 0.57, P < 0.01, Fig. S2.1b) concentrations.

2.4.2 Effect of water availability on A_{net}, g_s and eCO_2-induced A_{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_{net}, g_s and eCO_2-induced A_{net} enhancement in the current study, these parameters were plotted as a function of seasonal water availability, determined as the recent week total precipitation and mean daily V_{SWC} (Fig. 2.3). The recent week for these measures was the seven days prior to the initiation of gas exchange measurements at the EucFACE. Fig. 2.3a-d shows the responses of A_{net} and g_s respectively, for the dominant *M. stipoides* species, with respect to seasonal water availability. Lower values for A_{net} (< 9 µmol m^{-2} s^{-1}; Fig. 2.3a, b) and g_s (< 0.12 mol m^{-2} s^{-1}; Fig. 2.3c, d) were mostly observed during time points when recent week precipitation was < 10 mm (Fig. 2.3a, c) and mean daily V_{SWC} was < 0.10 v/v (Fig. 2.3b, d). Fig. 2.3e-h shows the effect of water availability on eCO_2-induced A_{net} enhancement. For all the C_3 species considered together, eCO_2-induced A_{net} enhancement was negatively correlated with both, total precipitation (r^2 = 0.38, P < 0.01, Fig. 2.3e) and mean daily V_{SWC} (r^2 = 0.49, P < 0.01, Fig. 2.3f) of the preceding week. Similarly, for *M. stipoides*, eCO_2-induced A_{net} enhancement was a decreasing function of total precipitation (r^2 = 0.56, P < 0.01, Fig. 2.3g) and mean daily V_{SWC} (r^2 = 0.64, P < 0.01, Fig. 2.3h) 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_{net}, g_s and eCO_2-induced A_{net} enhancement.

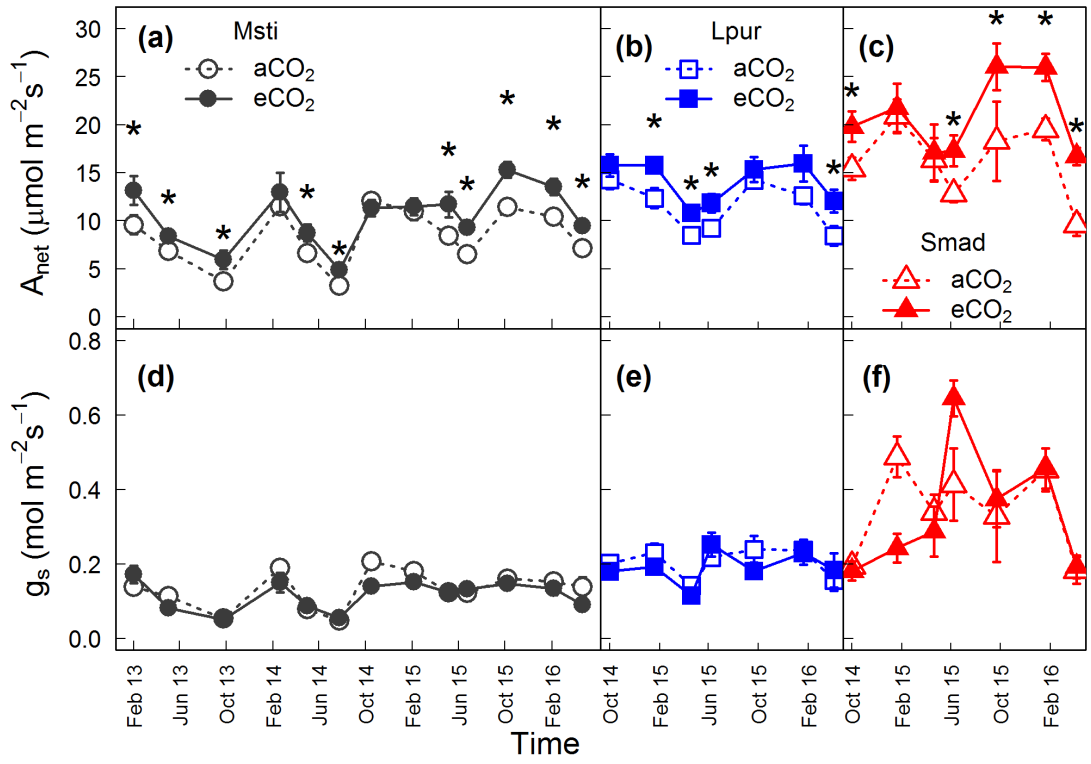

Fig. 2.2 Time course through the three measurement years for net CO_2 assimilation (A_{net}) and stomatal conductance (g_s) as a function of CO_2 treatment.

Time course is shown for (a) *M. stipoides* (Msti, black circles), (b) *L. purpurascens* (Lpur, blue squares) and (c) *S. madagascariensis* (Smad, red triangles). Open symbols indicate ambient CO_2 (aCO_2) and closed symbols indicate elevated CO_2 (eCO_2). The corresponding g_s is shown for (d) *M. stipoides*, (e) *L. purpurascens*, and (f) *S. madagascariensis*. When there was a significant overall CO_2 effect (Table 1.1), *post-hoc* treatment differences were denoted by * ($P < 0.05$; t-test).

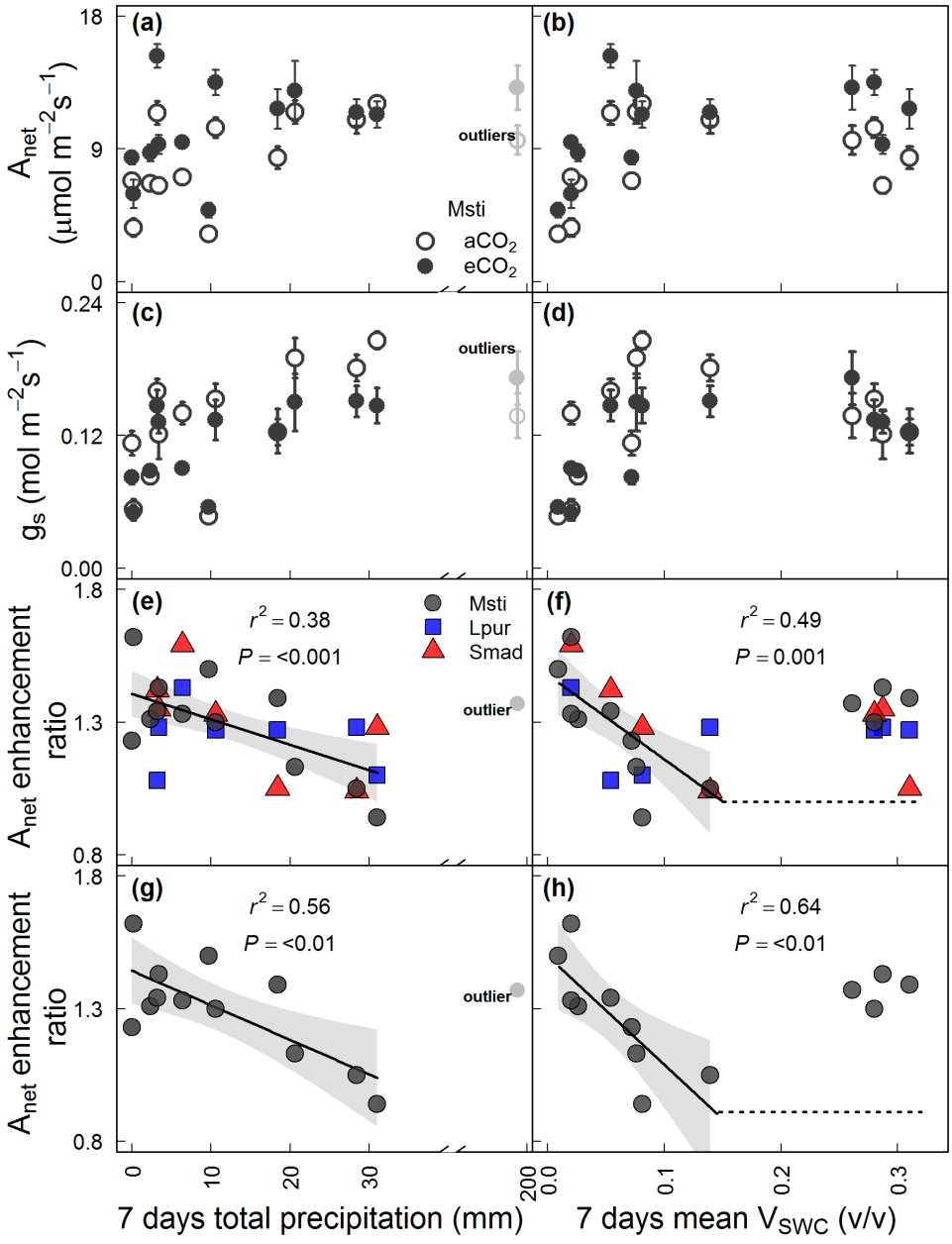

Fig. 2.3 Relationship of A_{net}, g_s and eCO_2-induced relative A_{net} enhancement with weekly precipitation and V_{SWC}.

(a, b) Seasonal A_{net} and (c, d) the corresponding seasonal g_s for *M.stipoides* along with (e, f) the A_{net} enhancement ratio for all three species, and (g, h) for *M. stipoides* only. A_{net}, g_s and A_{net} enhancement ratio are shown as a function of total precipitation (a, c, e and g) and mean daily volumetric soil water content (V_{SWC}; b, d, f and h) in the week preceding A_{net} 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). A_{net} enhancement ratio was calculated as mean A_{net} under eCO_2 divided by mean A_{net} under aCO_2. 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).

2.4.3 Effect of CO_2 and measurement time on biochemical parameters

To understand the underlying biochemical regulation of A_{net}, I focused on V_{cmax} and J_{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_{cmax} and J_{max} values across the species ($P > 0.02$, Table S2.2 and S2.3, Fig. S2.3), I observed a highly significant measurement time effect on both the parameters ($P < 0.01$, Table S2.2 and S2.3). There was evidence of different species responses for these parameters (Fig. S2.3). Variation in V_{cmax} and J_{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_{cmax} and J_{max} were normalized to a common standard temperature of 25 ^{o}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 normalized parameters ($V_{cmax-25}$ and J_{max-25}) across the species ($P < 0.01$, Table 2.1 and S2.1, Fig. 2.4), they were less variable over measurement time compared to non-normalized V_{cmax} and J_{max} (Fig. S2.3). When averaged across the three species and CO_2 treatments, maximum values for $V_{cmax-25}$ and J_{max-25} (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.

I did not observe a significant CO_2 effect on $V_{cmax-25}$ and J_{max-25} across the species ($P > 0.02$, Tables 2.1 and S2.1 and Fig. 2.4). However, there was a non-significant CO_2 x measurement time interaction effect on $V_{cmax-25}$ and J_{max-25} ($P < 0.1$, Tables 2.1 and S2.1 and Fig. 2.4). In particular, there was a trend towards lower $V_{cmax-25}$ and J_{max-25} under eCO_2 during October 2014 in *M. stipoides* and during October 2014 and October2015 in *L. purpurascens*. Trends similar to V_{cmax} 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 2.1 and S2.1, Fig. S2.4). However, I observed a significant measurement time effect of the leaf N content across the species and CO_2 treatments ($P < 0.01$, Table 2.1 and S2.1). 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 S2.1, Fig. S2.4a) and N_{mass} ($P > 0.02$. Table S2.3, Fig. S2.4d). However, leaf N content of *M. stipoides* varied significantly with time across the CO_2 treatments ($P < 0.01$, Table

S2.1 and S2.3). Overall, across the species I did not observe a significant change in any of the measured biochemical parameters under eCO_2, though individual species varied in this regard.

2.4.4 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. 2.5b). Also, mean daily V_{SWC} during the weeks preceding gas exchange measurements was similar between aCO_2 and eCO_2 ($P > 0.02$, Table S2.4). However, V_{SWC} varied substantially during the course of this study and there were several seasonal wet-dry periods (Fig. 2.5a). During a substantial amount of time (average 14 days per month or ≈ 50% of the time), V_{SWC} was < 0.10 v/v (Fig. 2.5a). Thus, the EucFACE facility experienced frequent dry periods during the duration of 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. 2.5b as well as during the week preceding the gas exchange measurements across all the 13 measurement time points ($P > 0.02$, Table S2.4).

2.4.5 Effect of CO_2 and measurement time on diffusional parameters

Elevated CO_2 resulted in a significant increase in C_i (391 ± 27 µmol mol^{-1}) compared to aCO_2 (288 ± 15 µmol mol^{-1}) across the three species ($P < 0.01$, Table S2.2 and S2.3, data not shown). However, this increase was not accompanied by a corresponding increase in the C_i/C_a ratio ($P > 0.02$, Table S2.2 and S2.3). Both C_i and C_i/C_a varied significantly with measurement time across the species ($P < 0.001$, Table S2.2 and S2.3). 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_{lim} and C_i difference under eCO_2, as leaves operate closer to the CO_2 saturation for A_{net}. I therefore examined the responses of S_{lim} and C_i difference across the species (Fig. 2.6). There was no significant CO_2 effect on S_{lim} across the three species ($P > 0.02$, Table 2.1 and S2.1, Fig. 2.6a-c). However, there was a highly significant measurement time effect on S_{lim} across the CO_2 treatments and species ($P < 0.01$, Table 2.1 and S2.1). Since there was a trend towards higher S_{lim} during the dry time points (Fig. 2.6a-c) when values for A_{net} (Fig. 2.2a) and g_s (Fig. 2.2b) were lower, I plotted S_{lim} as a function of water availability measured by total

precipitation and mean daily V_{SWC} of preceding week (Fig. S2.5). S_{lim} was a decreasing function of V_{SWC} across the species (r^2 = 0.33, P = 0.016, Fig. S2.5b) and for *M. stipoides* (r^2 = 0.55, P = 0.02, Fig. S2.5d). Thus, higher S_{lim} were observed during periods of low water availability or when V_{SWC} was < 0.10 v/v (Fig. S2.5b, d). Though the S_{lim} were similar between aCO_2 and eCO_2 treatments (Fig. 2.6a-c), I observed a significant decrease in C_i difference under eCO_2 across the species (P < 0.01, Table 2.1 and S2.1, Fig. 2.6d-f) indicating that plants in eCO_2 operated higher on the linear part of the A_{net}-C_i curve. I did not observe a highly significant measurement time effect on C_i difference across CO_2 treatments and three species (P > 0.02, Table 2.1). However, there were significant measurement time effects on C_i difference of *M. stipoides* (P < 0.01, Table S2.1, Fig. 2.6d). Higher average C_i difference was evident during the time points with higher relative S_{lim} (Fig. 2.6). I 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_{lim} and C_i difference across the species (P > 0.02, Table 2.1 and S2.1). Taken together, higher relative S_{lim} and C_i difference were evident during water-limited time points (Fig. S2.5), suggesting that these diffusional factors may be responsible for seasonal variation in eCO_2-induced A_{net} enhancement. Further evidence of this comes from a set of physiologically-based causal hypotheses laid out in a structural equation model (Fig. 2.7, see Supporting Material for details). There was no significant CO_2 treatment effect on g_s (P > 0.02, Table 2.1). However, overall seasonal variation in g_s did affect the photosynthetic enhancement by eCO_2 which was mediated through the S_{lim}.

2.4.6 Relation between S_{lim} and A_{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_{net} enhancement I further plotted A_{net} enhancement ratio as a function of S_{lim} (Fig. 2.8a) and C_i difference (Fig. 2.8b) under aCO_2 conditions. The eCO_2-induced A_{net} enhancement was positively correlated with S_{lim} at aCO_2 conditions across the species (r^2 = 0.39, P < 0.01, Fig. 2.8a) and for *M. stipoides* (r^2 = 0.63, P < 0.01). Similar to S_{lim}, I observed a strong positive correlation between eCO_2-induced A_{net} enhancement and C_i difference at aCO_2 across the species (r^2 = 0.44, P < 0.01, Fig. 2.8b) and for *M. stipoides* (r^2 = 0.64, P < 0.01). Overall, maximum

enhancement in photosynthetic rates under eCO_2 were observed when S_{lim} and C_i difference were higher under aCO_2 conditions.

2.4.7 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 2.1 and S2.2). When averaged across CO_2 treatments and seven measurement time points, I observed higher values for A_{net} and g_s (Fig. 2.2) in *S. madagascariensis* (18.5 ± 4.4 µmol m^{-2} s^{-1} and 0.34 ± 0.13 mol m^{-2} s^{-1}, respectively) than the other species (average A_{net} was 12 ± 2.7 µmol m^{-2} s^{-1} and 9.4 ± 3.12 µmol m^{-2} s^{-1} for *L. purpurascens* and *M. stipoides*, respectively). A similar trend was observed for the biochemical parameters like $V_{cmax-25}$ and J_{max-25} (Fig. 2.4), V_{cmax} and J_{max} (Fig. S2.3) and leaf N content (Fig. S2.4), 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 2.1 and S2.2) except for S_{lim} ($P > 0.02$, Table 2.1, Fig. 2.6a-c) which was similar across the three species (≈ 33%) as expected given that it is a relative measure that already accounts for intrinsic physiological rates. I observed a significant species x CO_2 interaction effect only for two variables ($P < 0.01$, Table 2.1 and S2.2), as *S. madagascariensis* had higher values for J_{max-25} (Fig. 2.4f) and J_{max} (Fig. S2.3f) under eCO_2 than for all other cases. Compared to *M. stipoides*, the biochemical (J_{max}, $V_{cmax-25}$, J_{max-25}) and diffusional (g_s, C_i, C_i/C_a, and S_{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 the current study ($P > 0.02$, Table 2.1 and S2.2).

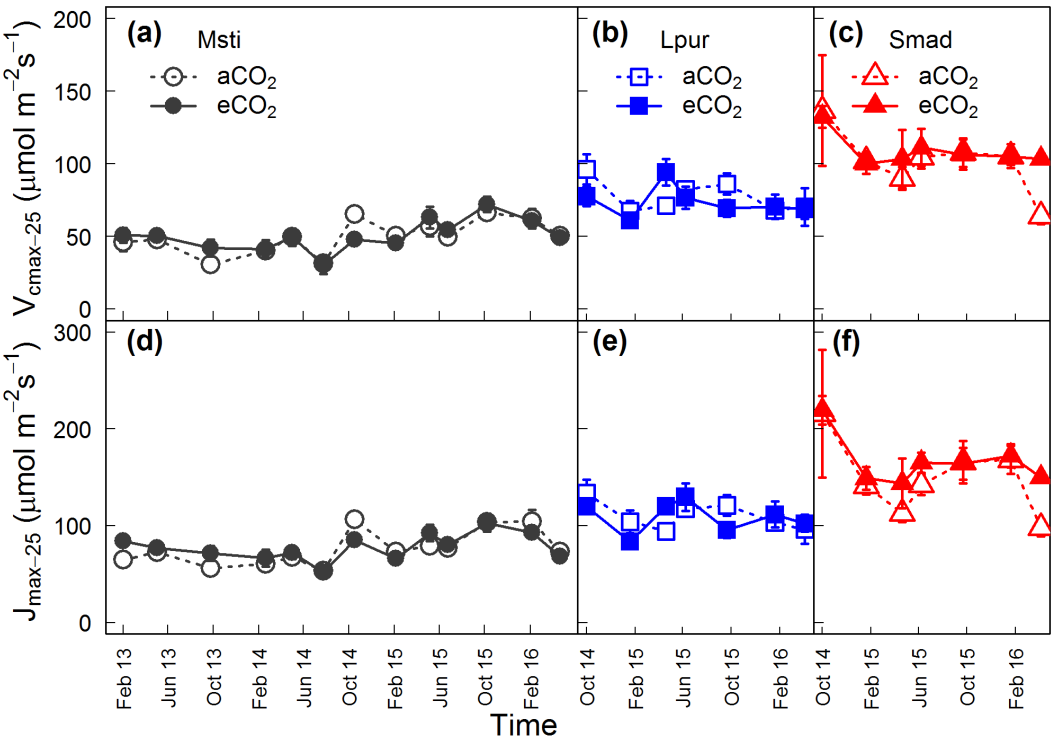

Fig. 2.4 Time course through the three measurement years for maximum carboxylation (V_{cmax}) and electron transport (J_{max}) as a function of CO_2 treatment.

The rates have been normalised to a standard leaf temperature of 25 ºC, indicated by (a, b and c) $V_{cmax-25}$ and (d, e and f) J_{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).

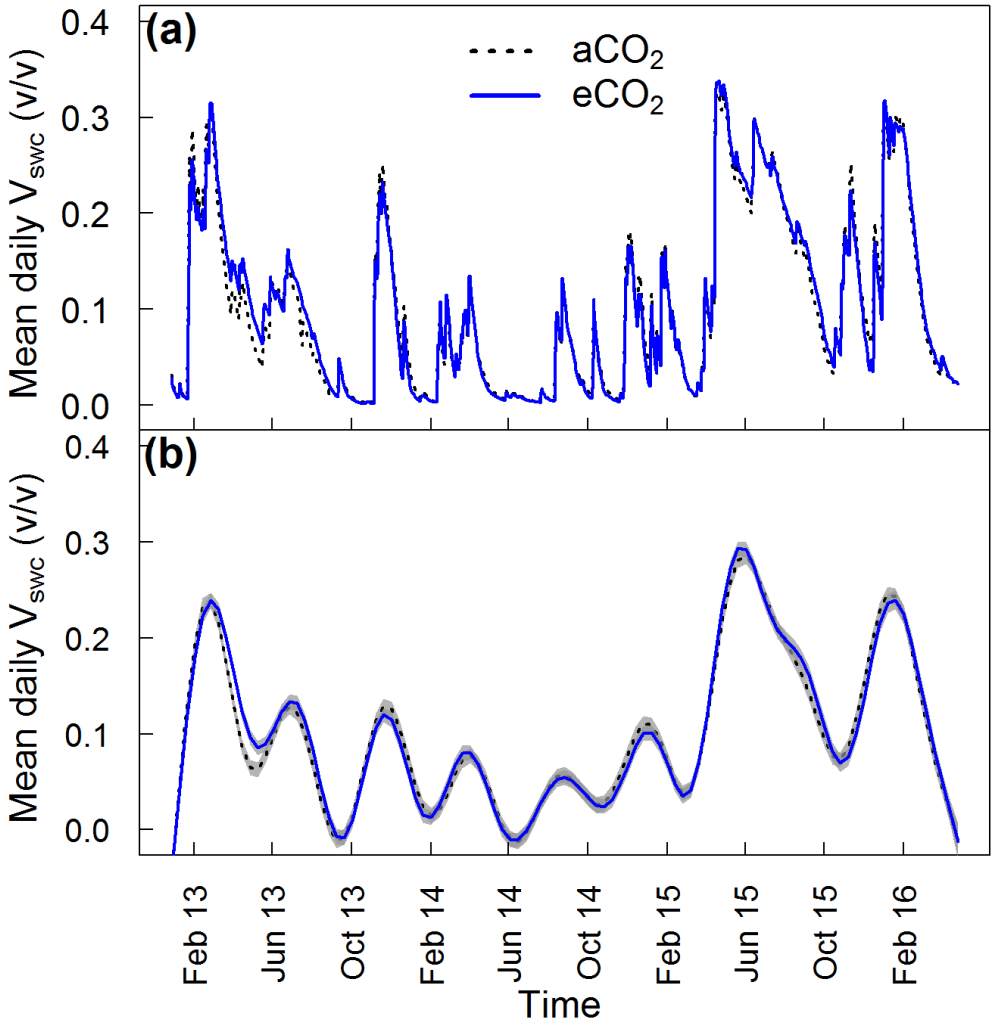

Fig. 2.5 Time course through the three measurement years for mean daily V_{SWC} content as a function of CO_2 treatment.

Panel (a) indicates V_{SWC} under aCO_2 (black dashed line) and eCO_2 (blue solid line) and (b) indicates smoothed regressions with 95% confidence intervals (gray areas) around the smooth terms for V_{SWC} under aCO_2 and eCO_2.

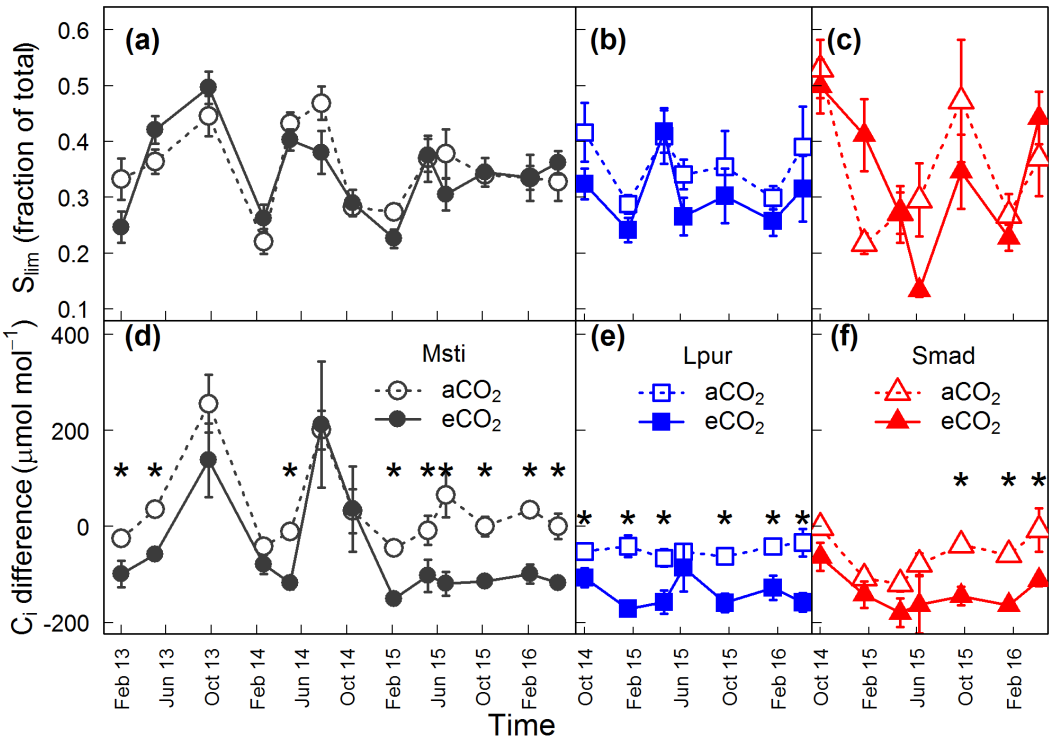

Fig. 2.6 Time course of relative stomatal limitations (S_{lim}) and the difference between operating C_i and transition C_i (C_i difference) as a function of CO_2 treatments.

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 CO_2 effect (Table 1), *post-hoc* treatment differences were denoted by * ($P < 0.05$; t-test).

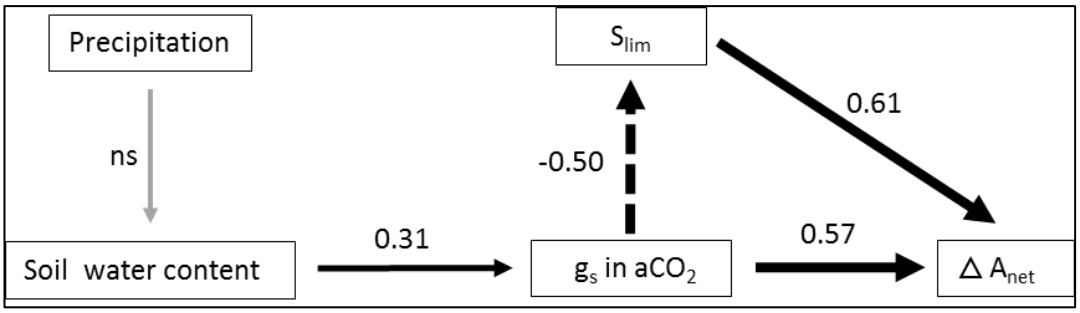

Fig. 2.7 The fitted structural equation model (SEM) depicting causal hypotheses underlying the photosynthetic enhancement by eCO_2 for herbaceous species measured at discrete points in the EucFACE experiment.

Significant standardized path coefficients ($P < 0.05$) are shown near each arrow, with the width of the line proportional to the size of the standardized coefficients. The dashed line denotes a negative relationship, and non-significant pathways are indicated in grey. ΔA_{net} denotes the absolute enhancement of A_{net} by eCO_2 with similar outcomes for the same model using the relative enhancement of A_{net}.

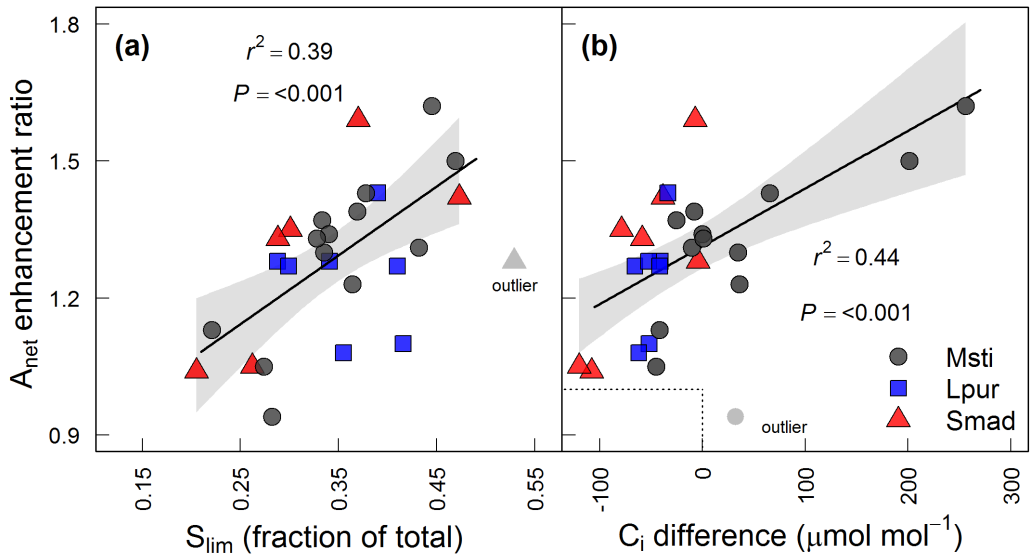

Fig. 2.8 The relative A_{net} enhancement ratio as a function of (a) S_{lim} (fraction of total limitations), and (b) C_i difference for all three species.

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 A_{net} enhancement in eCO_2. Gray shaded portions in panels (a) and (b) indicate 95% confidence intervals for the mean values, and the same outlier as shown in Fig. 2.3 is denoted.

2.5 Discussion

During three years of this study, photosynthetic rates under eCO_2 were almost 30% higher on average (Fig. 2.2). However, the relative enhancement in photosynthetic rates by eCO_2 across species varied substantially between seasons, with values ranging from 12-53%. I 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, recognizing that this would be the driver for biomass accumulation responses that will be tested later in this thesis (Chapter 3). My first hypothesis was supported, as I observed maximum photosynthetic enhancement by eCO_2 during the dry periods ($V_{SWC} < 0.07$). In contrast to the second hypothesis, I 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 the third hypothesis.

2.5.1 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. 2.1b and Fig. 2.5a). 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. 2.3e-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. 2.3e, g) or the mean daily V_{SWC} was < 0.10 v/v (Fig. 2.3f, 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 the current study is in agreement with these previous reports, and support my first hypothesis.

How is seasonal water availability related to the eCO_2-induced photosynthetic enhancement and its variability? I 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. I evaluated these two mechanisms and discuss them in the following sections.

2.5.2 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 I 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. 2.2d-f) even during dry periods (Fig. 2.3c, d). Stomatal conductance showed significant variation across seasons, but was similar under both aCO_2 and eCO_2 conditions (Fig. 2.2d-f), thus indicating that plants under both CO_2 treatments were constrained by the same diffusional limitations. Also, there was no detectable increase in mean daily V_{SWC} under eCO_2 compared to aCO_2 at any time point during three years of this study, not even during the dry periods when I expected a significant increase in V_{SWC} (Fig. 2.5). Unlike temperate ecosystems (Blumenthal *et al.*, 2013, Lecain *et al.*, 2003, Morgan *et al.*, 2011, Morgan *et al.*, 2004), the 'water-savings effect' of eCO_2 was absent in the understory and upper soil layer of this sub-tropical grassy *Eucalyptus* woodland, rejecting our second hypothesis. Thus, I do not expect such an effect on plant biomass accumulation for the grassy understory, though this remains to be tested later in this thesis (Chapter 3). The *Eucalyptus* woodland ecosystem in the current experiences an average annual precipitation of 800 mm. Thus, this ecosystem is not particularly dry compared to the previous studies from arid environments (annual precipitation < 400 mm) reporting

increases in soil water content (Blumenthal *et al.*, 2013, Lecain *et al.*, 2003, Morgan *et al.*, 2011). This could be a contributing factor in the non-significant effects of eCO_2 on soil water content reported in the current study.

The 'water-savings effect' of eCO_2 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 eCO_2 in a temperate plantation, which may have been due to interannual differences in soil moisture interacting with eCO_2 (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 eCO_2-induced water savings in the current study. Previous studies addressing the effects of eCO_2 on plant-water relations suggest that the C_4 species will benefit more from decreased stomatal conductance and increased soil water content, whereas C_3 species would benefit from a direct stimulation of photosynthetic rates due to increase in C_i (Morgan *et al*., 2011, Morgan *et al*., 2004). I speculate that the dominance of C_3 species in the understory at our site may have been a factor responsible for the lack of soil water-savings, as suggested previously by Morgan *et al.* (2004).

2.5.3 Higher stomatal limitations and A_{net} enhancement by eCO_2 during dry periods

Given that I did not find decreased stomatal conductance in eCO_2 and hence no 'water-savings effect', I investigated the possibility of changed stomatal limitations in eCO_2. S_{lim} reflects a complex function of both net CO_2 assimilation rates and stomatal conductance provides clarity about how these two processes balance with regard to the absolute photosynthetic enhancement in eCO_2.Lower g_s (Fig. 2.3d) and consequently higher S_{lim} (Fig. S2.5b, d) were observed during the water-limited periods than during wet periods. From this I infer that water availability controlled the variability in S_{lim} to photosynthesis as depicted in the path analysis in Figure 2.7. A similar relationship was previously observed between soil water content and diffusional limitation by

Grassi & Magnani (2005). A consequence of higher S_{lim} observed during water-limitation is a decrease in C_i and A_{net} with plants operating deeper in the carboxylation-limited zone. At such low C_i's, CO_2 fertilisation can facilitate the alleviation of S_{lim} by increasing C_i, thus generating a larger photosynthetic enhancement during dry periods (Lawlor, 2002). In support to this prediction, I observed maximum increase in photosynthetic rates under eCO_2 when S_{lim} were higher under aCO_2 concentrations (Fig. 2.8a). A similar relationship was observed between eCO_2-induced A_{net} enhancement and C_i difference (Fig. 2.8b). 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_{lim} during water-limited periods resulting in increased C_i and photosynthetic rates compared to plants grown in aCO_2. The multivariate pathway analysis shown in Figure 2.7 clearly supports the mechanism of how higher stomatal limitations during the dry periods can be overcome by eCO_2 thus resulting in a significant increase in the photosynthetic rates. The increased photosynthetic rates under eCO_2 suggest a potential for increased ecosystem C gain during dry periods. However, the phenology of different species would dictate if these responses could be translated to increased biomass accumulation.

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. 2.1b, 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 because 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.

2.5.4 Conclusions

In summary, under field conditions and over three years of CO_2 fumigation, I 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 *et al.*, 2013, Zhu *et al.*, 2016). Though I observed maximum eCO_2-induced photosynthetic enhancement during the dry periods, this enhancement was not mediated through the 'water-savings effect'. Low water availability resulted in lower g_s, higher relative S_{lim} and thus a greater increase in C_i possible which led to a significant photosynthetic enhancement under eCO_2. The results demonstrate that interaction between water availability andeCO_2, controls g_s and hence the photosynthetic enhancement in the herbaceous understory 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, eCO_2 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 CO_2 assimilation, but not via a 'water-savings effect' as is usually observed in temperate grasslands.

2.6 Supporting information

2.6.1 Supplementary tables

Table S 2.1 Results of the mixed-model split-plot ANOVA similar to Table 1.1, but for *M. stipoides* measured for 13 seasonal time points[1].

Shown are effects for net photosynthesis (A_{net}), temperature normalised maximum carboxylation ($V_{cmax-25}$) and electron transport rates (J_{max-25}), N content on area basis (N_{area}), stomatal conductance (g_s), relative stomatal limitation (S_{lim}) and C_i difference as the difference between the transition C_i and operating C_i for *M. stipoides*. CO_2 refers to the 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. df indicates the numerator degrees of freedom for the statistical tests.

	Source of variation								
	CO_2			Time			CO_2 x Time		
Variables	df	*F*-value	*P*-value	df	*F*-value	*P*-value	df	*F*-value	*P*-value
A_{net}	1	14.53	**0.019**	12	27.01	**<0.001**	12	0.80	0.645
$V_{cmax-25}$	1	0.09	0.770	12	6.48	**<0.001**	12	0.89	0.560
J_{max-25}	1	0.25	0.640	12	6.99	**<0.001**	12	1.12	0.370
N_{area}	1	0.13	0.734	12	8.38	**<0.001**	12	2.27	0.031
g_s	1	4.47	0.101	12	20.04	**<0.001**	12	1.05	0.418
S_{lim}	1	0.71	0.450	12	11.12	**<0.001**	12	1.59	0.130
C_i difference	1	21.93	**0.009**	12	10.05	**<0.001**	12	1.33	0.358

[1]All variables were transformed (square root or log transformation) to meet the normality assumptions for the mixed-model ANOVA.

Table S 2.2 Results of mixed-model split-plot ANOVA for *in situ* maximum carboxylation (V_{cmax}) and electron transport rates (J_{max}), N content on mass basis (N_{mass}), intercellular CO_2 concentration (C_i), ratio of intercellular to growth CO_2 concentration (C_i/C_a) and C_i transition as the CO_2 level for the transition between V_{cmax}-limited and J_{max} -limited A_{net}, across the three C_3 species measured for seven seasonal time points[1].

Results shown are across *M. stipoides*, *L. purpurascens* and *S. madagascariensis*. CO_2 refers to the 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 not shown in the table. df indicates the numerator degrees of freedom for the statistical tests.

	Source of variation																
	CO_2			Time			Species			CO_2 x Time			Species x CO_2			Species x Time		
Variables	df	*F* -value	*P* -value	df	*F* -value	*P* -value	df	*F* -value	*P* -value	df	*F* -value	*P* -value	df	*F* -value	*P* -value	df	*F* -value	*P* -value
V_{cmax}	1	4.33	0.106	6	128.18	**<0.001**	2	151.18	**<0.001**	6	1.85	0.132	2	2.96	0.062	12	1.78	0.080
J_{max}	1	1.74	0.258	6	47.53	**<0.001**	2	149.52	**<0.001**	6	2.34	0.064	2	5.68	**0.006**	12	1.93	0.054
N_{mass}	1	1.30	0.317	6	11.81	**<0.001**	2	63.77	**<0.001**	6	1.70	0.163	2	0.21	0.808	12	2.80	**0.005**
C_i	1	330.42	**<0.001**	6	9.24	**<0.001**	2	4.78	**0.012**	6	1.26	0.311	2	0.28	0.754	12	3.47	**0.001**
C_i/C_a	1	0.56	0.494	6	10.08	**<0.001**	2	5.49	**0.007**	6	1.34	0.277	2	0.28	0.755	12	3.59	**0.001**
C_i transition	1	0.43	0.549	6	4.16	**0.005**	2	9.42	**<0.001**	6	1.43	0.243	2	1.92	0.156	12	1.38	0.204

[1]All variables were transformed (square root or log transformation) to meet the normality assumptions for the mixed-model ANOVA.

Table S 2.3 Results of mixed-model split-plot ANOVA for *in situ* maximum carboxylation (V_{cmax}) and electron transport rates (J_{max}), N content on mass basis (N_{mass}), intercellular CO_2 concentration (C_i), ratio of intercellular to growth CO_2 concentration (C_i/C_a) and C_i transition as the CO_2 level for the transition between V_{cmax}-limited and J_{max} -limited A_{net}, for *M. stipoides* measured for 13 seasonal time points[1].

CO_2 refers to the 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. df indicates the numerator degrees of freedom for the statistical tests.

	Source of variation								
	CO_2			Time			CO_2 x Time		
Variables	df	*F* -value	*P* -value	df	*F* -value	*P* -value	df	*F* -value	*P* -value
V_{cmax}	1	1.85	0.240	12	21.27	**<0.001**	12	0.34	0.974
J_{max}	1	0.63	0.470	12	18.18	**<0.001**	12	1.36	0.230
N_{mass}	1	0.05	0.829	12	9.03	**<0.001**	12	0.73	0.712
C_i	1	440.00	**<0.001**	12	4.67	**<0.001**	12	1.34	0.230
C_i/C_a	1	0.97	0.379	12	3.22	**0.002**	12	1.25	0.281
C_i transition	1	0.00	0.970	12	7.29	**<0.001**	12	0.72	0.730

[1]All variables were transformed (square root or log transformation) to meet the normality assumptions for the mixed-model ANOVA.

Table S 2.4 Result of mixed-model split plot ANOVA for mean volumetric soil water content (V_{SWC}) measured in the week prior to the initiation of gas exchange measurements for 13 seasonal time points.

CO_2 refers to the 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 when significant ($P < 0.05$). df indicates the numerator degrees of freedom for the statistical tests.

	Source of variation								
	CO_2			Time			CO_2 x Time		
Variable	df	*F* -value	*P* -value	df	*F* -value	*P* -value	df	*F* -value	*P* -value
V_{SWC}	1	0.16	0.706	12	87.01	**<0.001**	12	0.56	0.855

[1]All variables were transformed (square root or log transformation) to meet the normality assumptions for the mixed-model ANOVA.

2.6.2 Supplementary figures

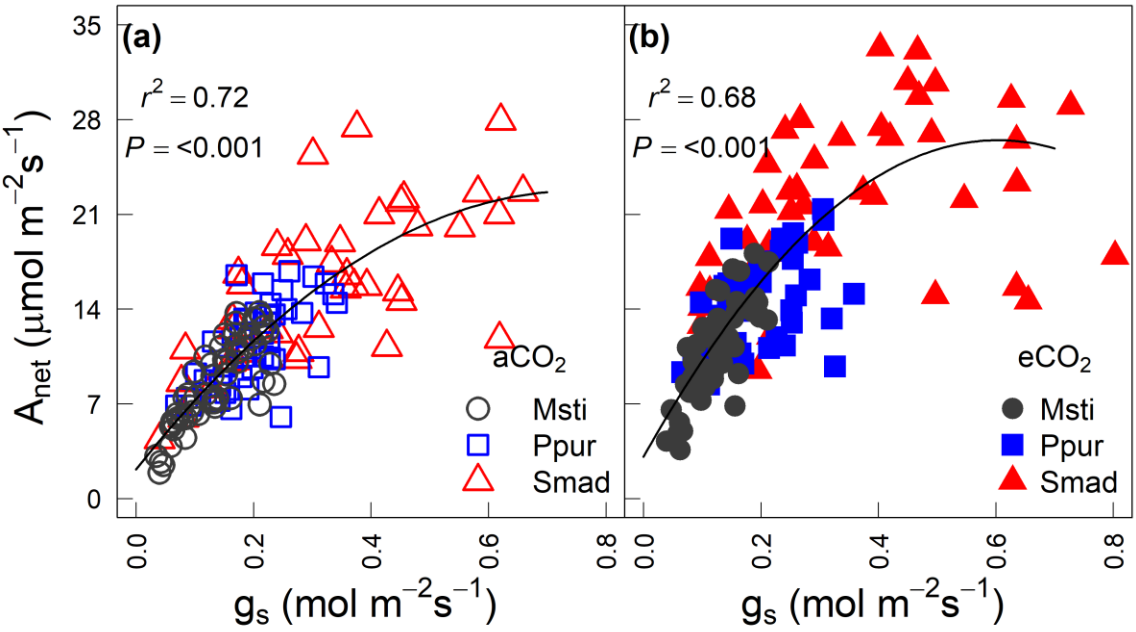

Fig. S 2.1 Relationship of A_{net} and g_s.

Growing season A_{net} as a function of growing season g_s under (a) aCO_2 (open symbols) and (b) eCO_2 (filled symbols) for the three species - *M. stipoides* (Msti, black circles), *L. purpurascens* (Lpur, blue squares) and *S. madagascariensis* (Smad, red triangles). Slopes of the regression lines between aCO_2 and eCO_2 are not significantly different ($P = 0.057$).

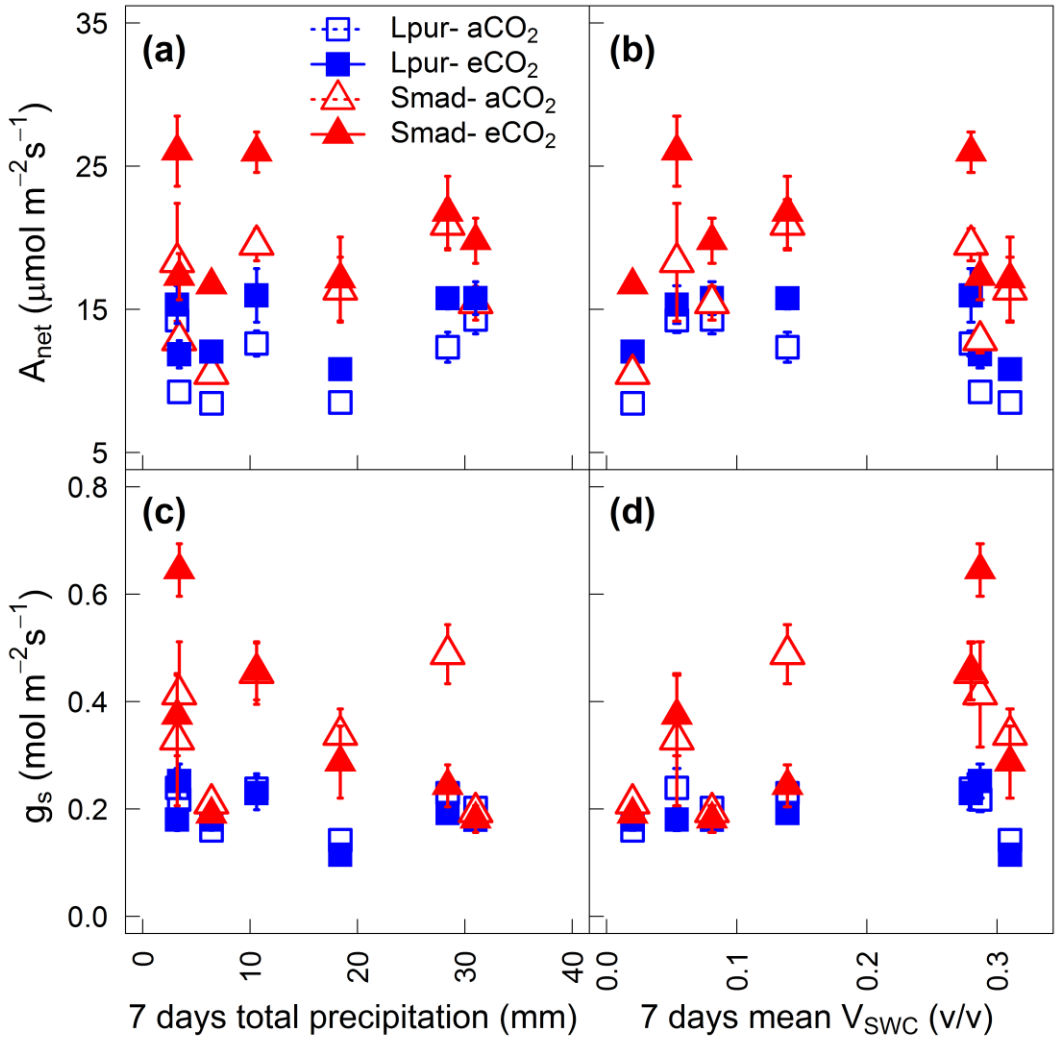

Fig. S 2.2 Relationship of A_{net} and g_s with weekly precipitation and V_{SWC}.

(a,b) Mean growing season A_{net} and (c, d) mean growing season g_s as a function of (a, c) total precipitation and (b, d) mean daily V_{SWC} over the week preceding A_{net} measurements for *L.purpurascens* (Lpur, blue squares) and *S. madagascariensis* (Smad, red triangles). Open circles indicate aCO_2 and filled circles indicate eCO_2.

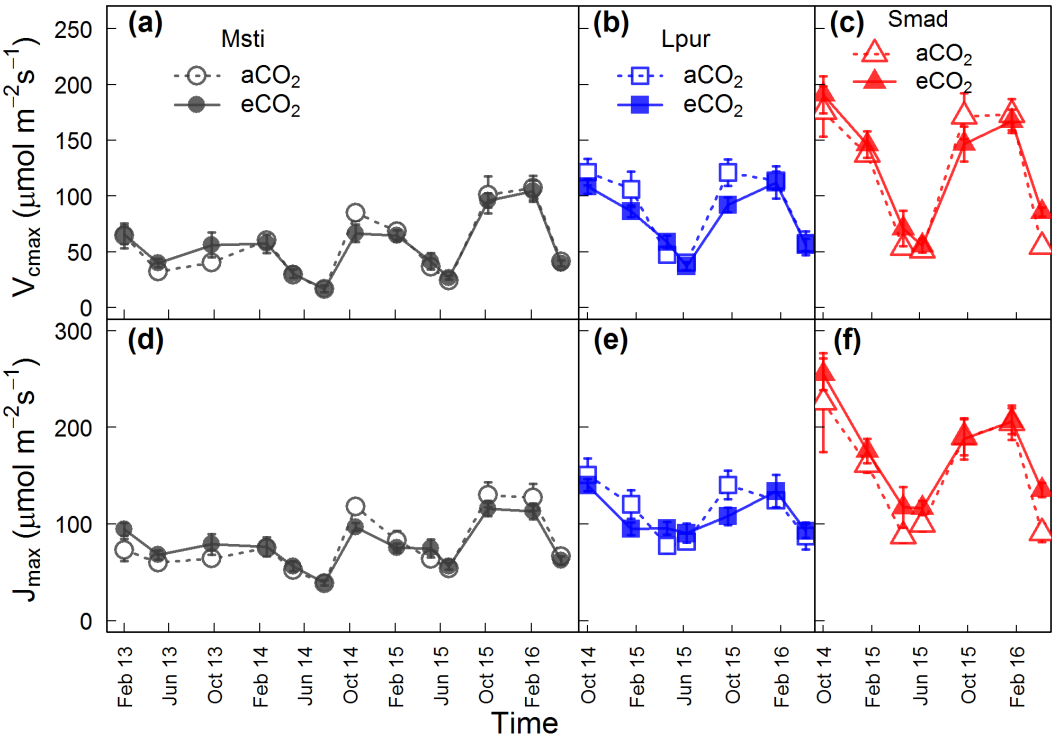

Fig. S 2.3 Time course for *in situ* maximum carboxylation (V_{cmax}) and electron transport (J_{max}) as a function of CO_2 treatments.

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).

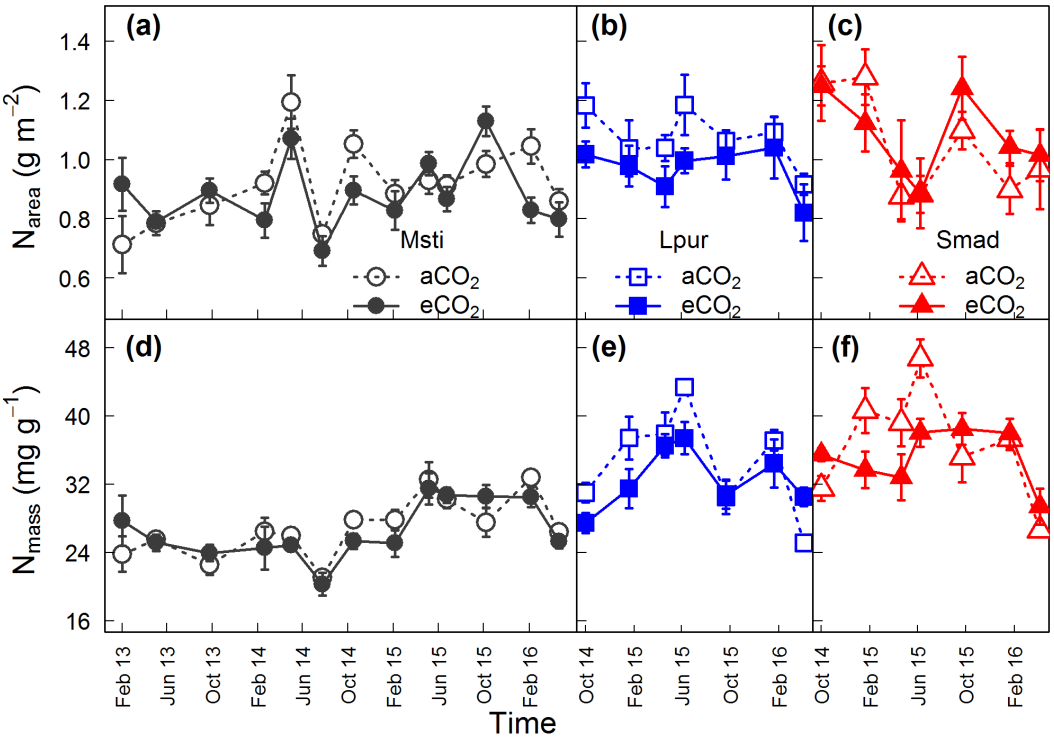

Fig. S 2.4 Time course for N content as a function of CO_2 treatments.

Mean growing season N content is expressed on (a, b and c) area basis (N_{area}), and (d, e and f) mass basis (N_{mass}). 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).

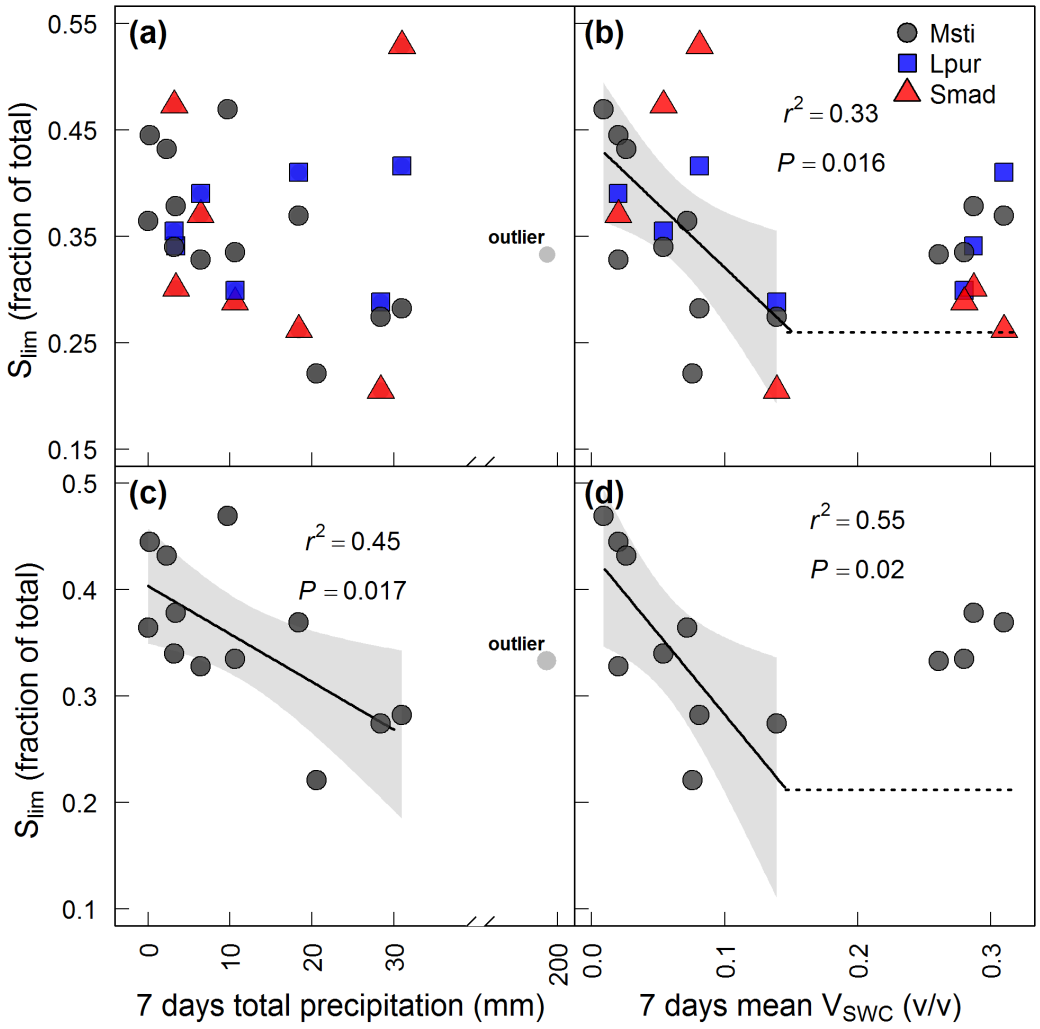

Fig. S 2.5 Relationship of S_{lim} with weekly precipitation and V_{SWC}.

Seasonal S_{lim} under aCO_2 as a function of (a, c) total precipitation and (b, d) mean daily V_{SWC} over the week preceding A_{net} measurements (a, b) for all three species - *M. stipoides* (Msti, black circles), *L. purpurascens* (Lpur, blue squares) and *S. madagascariensis* (Smad, red triangles) and (c, d) for *M. stipoides* only. Gray shaded portions indicate 95% confidence intervals for the mean values. In panels b and d, a broken stick function is shown, with fit to the linear part below the field capacity for this soil (0.18 v/v).

2.6.3 Description of the Structural Equation Modelling (SEM) approach and figures

I employed structural equation modelling in R as an approach for multivariate statistical modelling to investigate the networks of connections among components that contribute to leaf photosynthetic enhancement in elevated CO_2 in these herbaceous plants. The approach helped us examine complex cause-effect hypotheses about the mechanisms driving this photosynthetic enhancement. Photosynthetic enhancement in eCO_2 was examined both as an enhancement ratio and as an absolute difference, with very similar results between these two focal variables.

I specified a formal model that included two environmental inputs (any pair of precipitation, temperature and soil water content) and several physiological variables associated with the regulation of gas exchange of leaves. Following conventional understanding of how stomatal conductance and photosynthesis are regulated (Farquhar & Sharkey, 1982) and first-order theory of how elevated CO_2 would affect photosynthesis (Pearcy & Björkman, 1983), I formulated an initial path diagram hypothesizing the causal relationships among these variables (Fig. S2.6). Following the structural equation modelling approach (Grace, 2006, Lamb *et al.*, 2011) we set up a set of linear equations that establish an expected pattern to the variance-covariance matrix in the actual data. Using the Lavaan package in R (Rosseel, 2012), I applied the maximum likelihood approach to then minimize deviations between the observed data and the covariances appropriate for our initial model. Standardized path coefficients were expressed in terms of standard deviations so they could be compared. I then used the Chi-square test to determine whether the covariances implied by the model adequately fit the actual covariance structures of the data. I also formulated variations on the basic model in Fig. S2.6 to examine whether new variables (difference in C_i, or C_i/C_a ratio rather than S_{lim}) were more relevant than the ones chosen for the initial, basic model.

In the simple core model depicted in Fig. S2.6 and Fig. 2.7, precipitation provides soil moisture, which in turn affects g_s in ambient CO_2. There is a direct pathway from g_s to the absolute enhancement in A_{net} in eCO_2. Also there is an indirect pathway from g_s in ambient CO_2 to the absolute A_{net} enhancement in eCO_2, which is mediated by relative stomatal limitation in aCO_2, C_i/C_a ratio in aCO_2 or increase in C_i in eCO_2.Other variables could be included, but in doing so there is a loss of degrees of freedom and alternate models

involving V_{cmax} or leaf N_{area} yielded poor fits to the model, invalidating the overall model (Lamb *et al.*, 2011). Based on the core model in Fig. S2.6, I evaluated the hypothesis that temperature rather than precipitation would drive both available soil water as well as photosynthetic enhancement by eCO_2. In this case, all other relationships were identical to the core model. I also substituted the S_{lim} by C_i/C_a ratio in the theoretical model in Fig. S2.6 (Fig. S2.7). Other aspects of the model are same as given in Fig. S2.6. The arrow width is proportional to the size of the standardized coefficients. The overall Chi-square of 6.45 was not significant (P = 0.26), indicating an adequate fit to the data. Based on results in Fig. 2.7, S_{lim} provided stronger descriptors for the A_{net} enhancement than Ci/Ca ratio or the absolute increase in C_i.

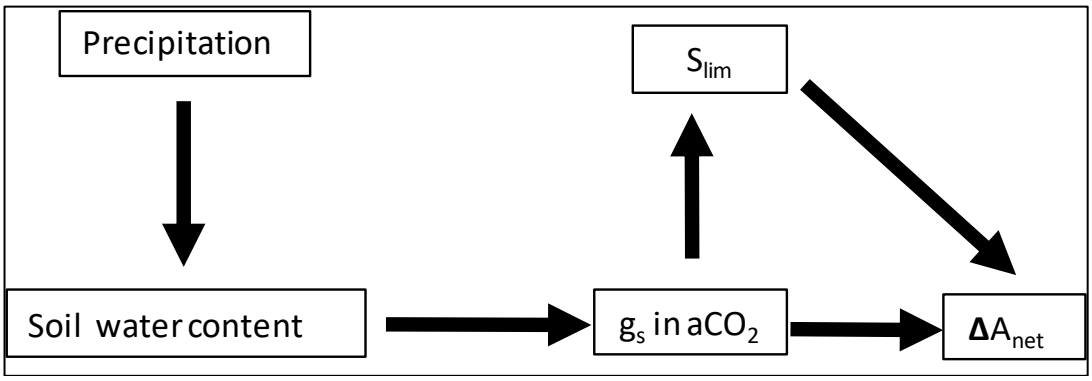

Fig. S 2.6 The basic structure of the core SEM model used to examine the multivariate regulation of photosynthetic enhancement by eCO_2 for herbaceous species at the EucFACE site.

The arrows denote a causal relationship where a change in the variable at the tail is a direct cause of changes in the variable at the head. The object ΔA_{net} denotes the absolute enhancement of A_{net} by eCO_2. Model results were very similar when A_{net} enhancement ratio for eCO_2 was used instead.

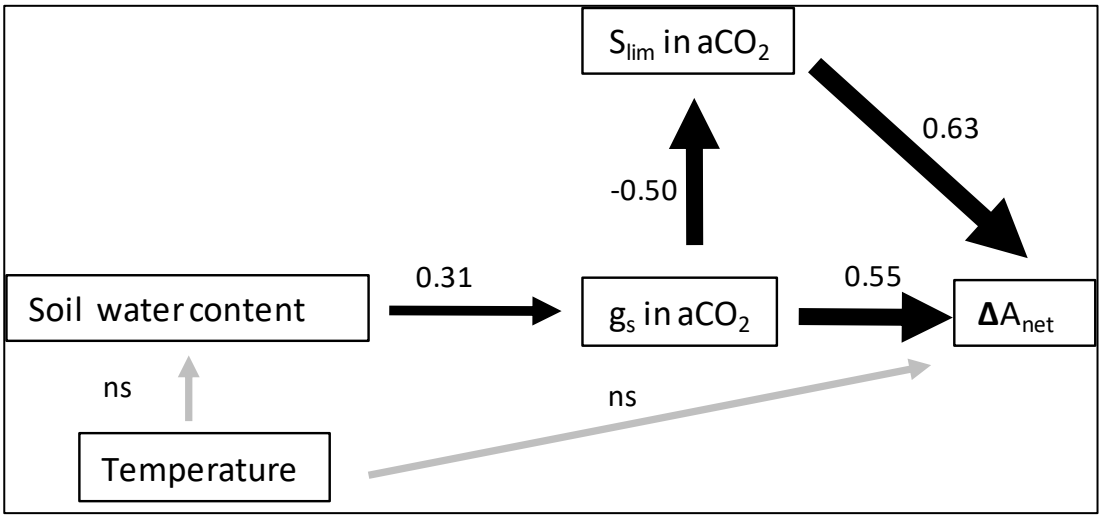

Fig. S 2.7 An alternative fitted SEM model based on the original theoretical one in Fig. S 2.6, but including the measurement temperature instead of precipitation.

Other aspects of the model are same as given in Fig. S2.6. Temperature was not significant in this model. The overall Chi-square of 6.2 was not significant ($P = 0.18$), indicating an adequate fit to the data.

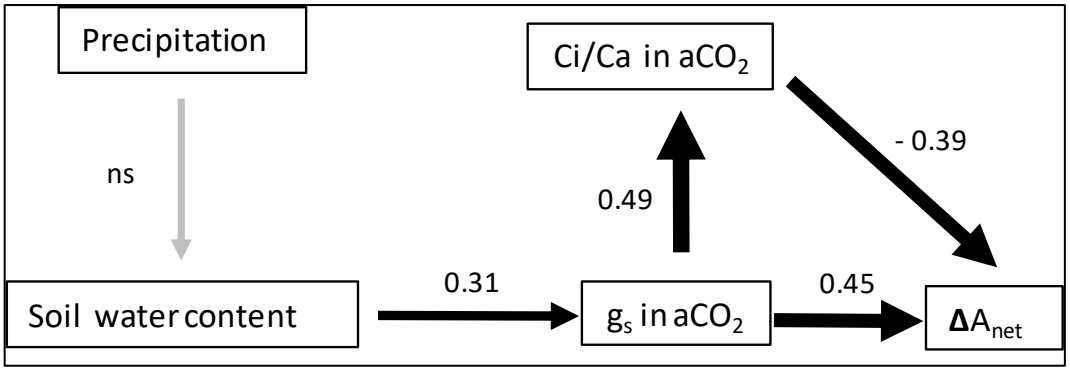

Fig. S 2.8 Another alternative SEM model similar to the theoretical model in Fig. S2.6, but replacing S_{lim} with C_i/C_a ratio.

The results are substantially similar to those in Fig. 2.7, except that there is a negative rather than positive interaction between C_i/C_a ratio and ΔA_{net}.

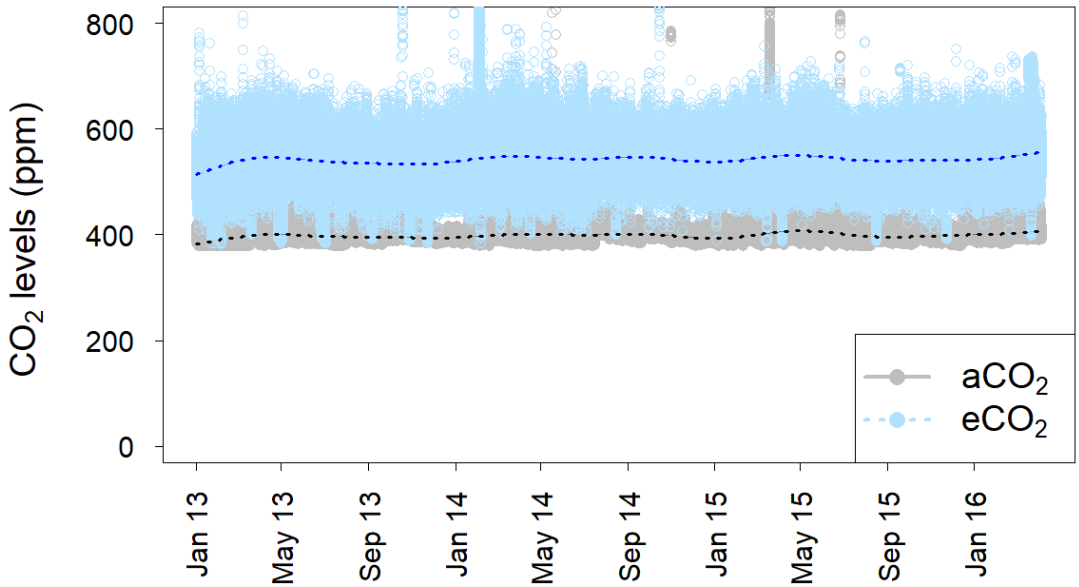

Fig. S 2.9 Atmospheric [CO_2] measured at EucFACE at 21 m above-ground for aCO_2 (gray symbols) and eCO_2 (blue symbols) plots during the first three years of CO_2 fertilisation. Data are 1-min means for [CO_2]. Smoothed regressions with 95% confidence intervals (gray areas) are shown for aCO_2 (black dashed line) and eCO_2 (blue dashed line).

2.6.4 Supplementary methods

Temperature response measurements and model fitting

We carried out temperature response measurements (data not shown) on the dominant *M. stipoides* following a procedure modified from Crous *et al.* (2013). *M. stipoides* seeds, collected from the Cumberland plain woodland, were germinated and raised in the glasshouse in 50L pots under controlled conditions (25^{o}C and 60% relative humidity). Two month old *M. stipoides* plants were then transferred to growth cabinet for temperature response measurements. A_{net}-C_i curves were measured at five specified leaf temperatures (in the sequence 18, 22, 28, 32 and 35^{o}C) starting with the lowest temperature (18 ^{o}C) and then repeated four times on the same set of leaves each time increasing the temperature. During temperature response measurements, the entire *M. stipoides* plants were exposed to similar air temperatures in the growth cabinets. At least four replicate measurements of the entire A_{net}-C_i curve per temperature were made. The temperature response of maximum carboxylation rates, (V_{cmax}; µmol m^{-2} s^{-1}), derived by using the biochemical model of Farquhar *et al*. (1980), was fit using the modified form of an Arrhenius function (peaked function; see Harley *et al.,* 1992 and Medlyn *et al.,* 2002). The resulting kinetic constants were further used to obtain temperature-normalised V_{cmax} ($V_{cmax\text{-}25}$).

N content

Leaf %N content, shown in Fig. S2.4, was then determined by using the CHNS elemental analyser (Elementar Vario Micro CHNS analyser, Hanau, Germany). Leaf N content was expressed on area (N_{area}, g m^{-2}) and mass basis (N_{mass}, mg g^{-1}).

Image 2.1 Photosynthetic gas exchange measurements, using a LiCOR-6400 at the EucFACE.

Measurements were carried out seasonally on the understory herbaceous species by the author using a set of Li-6400 photosynthesis systems. Photo courtesy: Dr Balasaheb V. Sonawane.

Chapter 3 : Photosynthetic acclimation and productivity responses of understory herbaceous species from a resource-limited *Eucalyptus* woodland

3.1 Abstract

Despite their importance for forest biodiversity and functioning, only few studies have addressed the elevated CO_2 (eCO_2) effects on photosynthesis and biomass growth of the understory species. Here, I investigated the photosynthetic and biomass responses to eCO_2 for the herbaceous understory species growing naturally in a warm, water-limited *Eucalyptus* woodland during the second and third year of CO_2 fertilisation at the *Eucalyptus* Free-Air CO_2 Enrichment Experiment (EucFACE). Photosynthetic responses to eCO_2 were measured during the spring, summer and autumn seasons each year in a dominant C_3 forb and a dominant C_3 grass. Above-ground biomass in ambient and eCO_2 was measured during the summer season of each year for all understory forbs and grasses.

Across the species and seasons, there was a significant enhancement in photosynthetic rates (A_{net}) under eCO_2 (≈ 23%). There was also evidence of down-regulation of photosynthetic capacity under eCO_2. This may have resulted in the lack of A_{net} stimulation and also lack of proportional biomass stimulation under eCO_2 that was observed during the peak seasons. In the C_3 forb, eCO_2 led to a decrease in leaf carboxylation (V_{cmax}; -30%) and electron transport capacities (J_{max}; -20%) during the spring seasons. In the C_3 grass, eCO_2 led to a decrease in leaf carboxylation (V_{cmax}; -35%) and electron transport capacities (J_{max}; -24%), but only during one spring season out of two. For the remaining measurement seasons, photosynthetic capacity and A_{net} stimulation were maintained under eCO_2 in both the species. A decrease in photosynthetic capacity under eCO_2 could be related to a protein specific down-regulation of the Rubisco enzyme. Elevated CO_2 did not significantly ($P > 0.10$) affect live above-ground forb and grass biomass, nor did it affect the total above-ground biomass (live plus senescent) measured during the summer seasons. Photosynthetic capacity down-regulation during the spring, and lack of biomass stimulation in the understory species during the following summer under eCO_2 together indicate a limited capacity of these woodland species to respond to eCO_2.

3.2 Introduction

For over two decades, long-term free-air CO_2 enrichment experiments have been conducted in different terrestrial ecosystem types (Leakey *et al.*, 2012) with a focus on the overstory tree components from forest ecosystems (Körner *et al.*, 2005, McCarthy *et al.*, 2010, Norby & Zak, 2011) and the herbaceous species from grasslands (Crous *et al*., 2010, Hovendan *et al*., 2014, Polley *et al*., 2012). Very few studies on vegetation responses to elevated CO_2 (eCO_2) focus on the understory plants, and those that have been done involve tree seedlings and shrubs (Awmack *et al.*, 2007, Kim *et al.*, 2015, Naumburg & Ellsworth, 2000, Sefcik *et al.*, 2007), resulting in less knowledge about the understory herbaceous species responses to eCO_2. Understory herbaceous vegetation represents an important component of the overall diversity and functioning and their ability to respond to eCO_2 can affect ecosystem processes such as tree seedling growth and regeneration, nutrient cycling and fire regimes (Bond & Midgley, 2012, Nilsson & Wardle, 2005, Valladares *et al.*, 2016). The understory herbaceous species may be more responsive to eCO_2 in terms of increase in photosynthesis and productivity, than those growing in open areas like grasslands, because plants growing in shaded environments may be more C-limited than those growing in open habitats (Hättenschwiler & Körner, 2000, Würth *et al.*, 1998). However, overstory dynamics (Kim *et al.*, 2015) and water and nutrient availability (Belote *et al.*, 2004, Sefcik *et al.*, 2007) may alter this expectation.

A well-documented short-term response to increase in atmospheric CO_2 is the stimulation of photosynthetic rates in the C_3 species (Ainsworth & Rogers, 2007). However, with long-term exposure to eCO_2, there may be departures from these short-term responses (Drake *et al.*, 1997), attributable to down-regulation of the biochemistry of photosynthesis. Downregulation is frequently manifested as either declines in the Rubisco carboxylation (V_{cmax}) or maximal electron transport rate (J_{max}; Ainsworth & Rogers, 2007, Ellsworth *et al.*, 2004, Stitt & Krapp, 1999), or both. Photosynthetic capacity down-regulation has been reported in field experiments on the herbaceous C_3 species (Crous *et al.*, 2010, Ellsworth *et al.*, 2004, Inauen *et al.*, 2012). These reductions have been associated to insufficient sink capacity under nutrient limitations (Stitt & Krapp, 1999), selective down-regulation of the Rubisco enzyme (Ainsworth & Rogers, 2007, Aranjuelo

et al., 2011, Long *et al.*, 2004), dilution of leaf N by carbohydrates (Deng *et al.*, 2015, Ellsworth *et al.*, 2004), differences in plant nutrient foraging capacities (Crous *et al.*, 2010) and decreases in plant N acquisition (Feng *et al.*, 2015). The ability of plants to maintain photosynthetic and biomass enhancement under eCO_2 largely depends on their ability to maintain photosynthetic capacities (Long *et al.*, 2004). If the photosynthetic capacity of plants is down-regulated under eCO_2, the ecosystem may become less responsive to eCO_2 and consequently sequester less C than it would without down-regulation (Luo *et al.*, 2003). Hence, understanding the mechanisms and extent of photosynthetic capacity down-regulation under eCO_2 in plants as well the factors affecting this response, is essential for predicting their capacity to sequester extra C in future (Bagley *et al.*, 2015, Piao *et al.*, 2008), no less important for the understory plants than the trees and the grassland species.

Seasonal variation in biotic and environmental factors like source-sink balance, species phenology, temperature, light, water and nutrient availability have all been found to affect the magnitude of photosynthetic acclimation under eCO_2 (Fatichi *et al.*, 2014, Onoda *et al.*, 2005, Sefcik *et al.*, 2006). Among these, altered source-sink balance due to seasonal variation in above and below ground growth of the evergreen species has been suggested as an important reason for seasonal photosynthetic acclimation under eCO_2 (Burnett *et al.*, 2016, Leakey *et al.*, 2009, Lewis *et al.*, 1996). A source refers to the strong net C uptake from the external environment for carbohydrate production, whereas a sink is defined as the capacity to utilize or deplete carbohydrates for growth or storage (White *et al.*, 2015). Insufficient sink capacity for additional carbohydrates produced in leaves under eCO_2 may cause carbohydrate accumulation and hence photosynthetic acclimation especially in the seasons with low temperature and low growth activity, like autumn (Fatichi *et al.*, 2014, Lewis *et al.*, 1996). Whereas, increased temperature and growth activity during the peak seasons like spring and summer, increasing possible 'sinks' for carbohydrates, may increase the response of net photosynthesis to eCO_2 (Ainsworth *et al.*, 2003b, Lewis *et al.*, 1996). Phenological shifts under eCO_2 such as early spring growth, delay or acceleration of leaf senescence (Cleland *et al.*, 2006, Onoda *et al.*, 2005, Taylor *et al.*, 2008, Zhu *et al.*, 2012) and changes in leaf ontogeny (Miller *et al.*, 1997) can also cause seasonal variation in photosynthetic capacity acclimation. For example, photosynthesis during the leaf ontogeny is characterised by phase of increasing rates to maximum

followed by phase of decreasing rates (Miller *et al.*, 1997). Elevated CO_2-induced increase in the photosynthetic rates may alter the timing of these photosynthetic stages of leaf ontogeny, possibly to an earlier onset, thus causing seasonal variation in photosynthetic acclimation (Miller *et al.*, 1997). Seasonal variation in light availability may also affect the photosynthetic capacity responses to eCO_2, particularly in the understory species (Naumburg & Ellsworth, 2000, Sefcik *et al.*, 2007). For instance, light availability in the understory is related to the dynamics of over story leaf area index (Katahata *et al.*, 2007), with reductions in the irradiance that can occur with seasonal leaf production of the overstory that progresses from spring to summer and autumn. The understory species may respond to this seasonal variation in light availability by morphological and biochemical adjustments (Hättenschwiler, 2001), thus affecting their responses to eCO_2 (Kim *et al.*, 2015). Knowledge about the seasonal variations in photosynthetic capacity under eCO_2 is important for accurate modelling of annual ecosystem carbon gain, particularly for the evergreen species growing in ecosystems where carbon uptake can occur throughout the year (Bagley *et al.*, 2015, Piao *et al.*, 2008).

Here, I investigated the seasonal effects of eCO_2 on the photosynthetic acclimation of two dominant native herbaceous evergreen species growing naturally in the understory of a sub-tropical *Eucalyptus* woodland at the *Eucalyptus* Free Air CO_2 Enrichment Experiment (EucFACE). These two species, *Microlaena stipoides* Labill. (a C_3 grass) and *Lobelia purpurascens* R.Br. (a C_3 forb) were the two most-dominant herbaceous species in the understory of the site. In Chapter 2 of the current study, I investigated the relationship between seasonal water-availability and eCO_2-induced photosynthetic enhancement for three C_3 herbaceous species (Pathare *et al.*, 2017). The proportional eCO_2-induced photosynthetic enhancement of these species was strongest during periods of low soil water content, particularly a result of eCO_2-induced boost in intercellular CO_2 concentration during dry-soil periods. However, a lack of photosynthetic stimulation by eCO_2 during wet periods in this earlier work (Pathare *et al.*, 2017) suggested a seasonal regulation of photosynthetic capacity in these species. Based on this, in the current study I sought to determine if there is evidence of down-regulation in different herbaceous plant groups and reveal its mechanism. I hypothesized that (H1) there would be larger down-regulation of photosynthetic capacity under eCO_2 during the autumn than spring and

summer. If the hypothesis was accepted, I sought to understand if growth sink capacity was related to this phenomenon.

In addition to the seasonal photosynthetic acclimation responses, I also assessed the above-ground biomass responses of total understory grasses and forbs to eCO_2. Though eCO_2 mostly leads to increase in photosynthetic rates, this may not always result in biomass enhancement because of limited ability of plant to utilize the extra carbohydrate due to soil nutrient limitations (Kirschbaum, 2011). Few previous studies addressing the effects of eCO_2 on the biomass of understory herbaceous and woody species report inconsistent responses ranging from increased biomass (Souza *et al.*, 2010) to a lack of eCO_2-induced biomass enhancement (Bandeff *et al.*, 2006, Kim *et al.*, 2015) and even a decrease in biomass under eCO_2 (Awmack *et al.*, 2007, Dawes *et al.*, 2015). This variation in understory biomass responses to eCO_2 has been hypothesized to be a result of variation in soil nutrient availability, water availability, responses of overstory canopies and growth conditions (Belote *et al.*, 2004, Kim *et al.*, 2015, Kubiske *et al.*, 2002). Based on kinetic characteristics of the Rubisco enzyme (Long, 1991), the C_3 species growing in the warmer ecosystems with higher mean annual temperatures may have a greater potential to respond positively to eCO_2 for photosynthesis and biomass, than plants growing in the cold temperate ecosystems (Cernusak *et al.*, 2013, Friend, 2010, Hickler *et al.*, 2008). Based on this, I hypothesized (H2) that, above-ground biomass of the understory herbaceous species will be higher under eCO_2 compared to ambient CO_2.

3.3 Material and methods

3.3.1 Experimental site description and species under study

We conducted this experiment on the dominant C_3 herbaceous understory species in the second and third year of the *Eucalyptus* Free-Air CO_2 Enrichment (EucFACE) experiment (Image 1.1). A detailed description of EucFACE site has been given in Chapter 2 (section 2.3.1). The total P concentration in the soil done by Kjeldahl digest and ICP analysis was 58.8 ± 7.9 mg kg^{-1} at 0 -15 cm soil depth (Crous *et al*., 2015), whereas, total soil N content was 677 mg kg^{-1} (Hasegawa *et al*., 2015).

The vegetation consists of a naturally growing open woodland (overstory leaf area index < 2, Duursma *et al*., 2016), with a substantial understory cover dominated by an evergreen native C_3 grass, *Microlaena stipoides* Labill. (≈ 70 % of total understory biomass) (Tozer, 2003). Herbaceous C_3 forbs form the second most abundant functional group in the understory (≈ 20 % of total understory biomass) and is dominated by an evergreen native shallow-rooted creeping C_3 forb, *Lobelia purpurascens* R.Br. In the current study, I focused on measuring the two dominant species: *M. stipoides* and *L. purpurascens*, as representative of two major functional groups-C_3 grass and C_3 forb respectively (see Image 1.2 and section 1.8 of Chapter 1 for species details). Of the total understory ground cover, these two species contributed 31% and 14% of the plant cover, respectively (Hasegawa, 2015). These two species are denoted in the figures by the genus initial and the first three letters of the species name.

3.3.2 Field measurements

Leaf level gas exchange measurements were performed on *L. purpurascens* as the dominant C_3 forb and *M. stipoides* as the dominant C_3 grass. Measurements were conducted during the second and third year of CO_2 fertilisation, with three time points per year and each time point representing a season of the year (spring, summer and autumn). Also, measurements were conducted during the latter half of each season, that is, October end (spring), first week of February (summer) and April end (autumn), to ensure measurements on leaves developed and acclimated to the respective growth conditions. A

set of portable infrared photosynthesis systems (Li-COR 6400XT; Li-COR Inc., Lincoln, NE, USA) with six cm^2 chambers were used for gas exchange measurements. In order to assess instantaneous and long-term effects of eCO_2 on the photosynthetic capacities of the two dominant species, photosynthetic CO_2 response curves (A_{net}-C_i curves) were measured as indicated in Chapter 2 (Pathare *et al*., 2017).. Net CO_2 assimilation rates (A_{net}), stomatal conductance (g_s) and intercellular [CO_2] (C_i) were measured at the mean growth CO_2 concentration for each treatment (≈ 400 µmol mol^{-1} for aCO_2 and ≈ 550 µmol mol^{-1} for eCO_2). T_{leaf} during the gas exchange measurement was controlled at the prevailing mean daily maximum air temperatures (T_{air}; ^oC) during each measurement season (22 ^oC for autumn, 27 ^oC for spring and 29 ^oC for summer seasons) (Chapter 2, Fig. 2.1a). Measurements were conducted during sunny mid-days (09:30-14:30) on young, fully expanded leaves exposed to sunlight. At least two measurements per CO_2 plot per species were undertaken at every season. During the A_{net}-C_i measurements, [CO_2] in the cuvette was controlled as reference. A_{net}-Ci curves were fit using the biochemical model of Farquhar *et al*. (1980), in order to obtain temperature normalized rates of maximum carboxylation ($V_{cmax-25}$; µmol m^{-2} s^{-1}) and electron transport (J_{max-25}; µmol m^{-2} s^{-1}) (see Chapter 2 for detailed procedure). While deriving the values for $V_{cmax-25}$ and J_{max-25}, C_i transition point was set at 300, since it reduced the standard error of fit by about four-fold. Also, the C_i transition point did not differ between ambient and elevated CO_2 treatments. From $V_{cmax-25}$ and corresponding leaf N content, I calculated the apparent fraction of N allocated to the active state Rubisco enzyme ($f_{N\text{-}Rubisco}$), assuming a composition of 16.67% N, eight active sites and a k_{cat} of 3.3 for the enzyme (Evans, 1989).

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, 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. Leaf samples were then freeze dried for two days at -50 ^oC and were weighed to obtain leaf mass per area (LMA; g m^{-2}). Further, the dried leaf samples were finely ground and then processed for C and N content analyses using CHNS elemental analyzer (Elementar Vario Micro CHNS analyser, Hanau, Germany). Leaf nitrogen content was expressed on

both area (N_{area}; g m^{-2}) and mass basis (N_{mass}; mg g^{-1}). Leaf P content of the dried leaf samples was determined using an X-ray fluorescence spectrometer which works on the principle of excitation of inner orbital electrons by an X-ray radiation source (Reidinger *et al*., 2012). Dried plant material was finely ground, pressed into pellets and analysed by exposing the pellets to X-rays for 30s. Certified reference materials from different plant species were used for calibration. Leaf P content was expressed on area basis as P_{area} (g m^{-2}) and mass basis as P_{mass} (mg g^{-1}). Leaf N to P ratio (N: P) was derived as N_{mass} divided by P_{mass}.

3.3.3 Above-ground biomass measurements

To determine the peak standing above-ground biomass of the understory herbaceous species present at the EucFACE facility (Image 3.1), harvesting was carried out for two consecutive years (2015 and 2016) during the month of February which indicates the end of peak growing season (including spring and summer growth). These two years represent the second and third year of CO_2 enrichment at EucFACE. I used a clip-strip method of biomass harvest as has been applied previously at the BioCON experiment (Reich *et al.*, 2001). In particular, four narrow strips, each having a size of 1m x 10 cm, were placed in each of the CO_2 plots at least 2 m from the vertical pipes for FACE, while avoiding the understory shrubs (Image 3.1). The understory herbaceous species were clipped approximately one cm above soil level and sorted into total live grass biomass (comprising of C_3 and C_4 grasses), total live forb biomass and senesced biomass. The senesced biomass was the dead biomass still attached to the plants and did not consist of decomposing material and twigs and dead leaves of overstory trees. Biomass samples were oven dried for two days at 60 ^oC, weighed to determine the dry biomass and was expressed on g m^{-2} basis. The sampling method was robust as four clip-strips were harvested per CO_2 plot. However, the EucFACE is located in a natural, undisturbed ecosystem. As a result, the clip-strips may differ from each other in terms of number and type of species present. Thus, the biomass was separated into total grasses and total forbs.

3.3.4 Statistical analysis

Statistical analyses were performed using the R software (v3.2.2, R Foundation for Statistical Computing, Vienna, Austria). The EucFACE facility consists of three ambient and three elevated CO_2 rings or plots and hence the true number of replicates was three for each of the two levels of CO_2 treatment. For both the C_3 species, *L. purpurascens* and *M. stipoides*, gas exchange measurements were carried out in at least two locations in each of the six plots across six measurement seasons (two springs, two summer and two autumn time points) during the second and third year of CO_2 fertilisation at the EucFACE facility. Further, live above-ground biomass, for total grasses and total forbs, was measured in four locations in each of the six plots for two peak seasons, that is, summer 2015 and summer 2016. Hence, three mixed level split-plot ANOVA were performed with CO_2 treatment as a whole-plot factor and season or year as a split-plot factor. The first ANOVA compared the seasonal variation in physiological and biochemical parameters (A_{net}, C_i/C_a, N content, V_{cmax}, J_{max}, $f_{N\text{-}Rubisco}$ and LMA) for the two C_3 species across three measurement seasons and between CO_2 treatments. The second ANOVA was analysis of CO_2 treatment, year and functional type (grasses versus forbs) effects on the live above-ground biomass. The third ANOVA was analysis of CO_2 treatment effects on the total above-ground biomass (total live plus senescent), across two measurement years. 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). Student's *t*-test was used for the effect of CO_2 treatment within seasons wherein $P < 0.05$ was considered as statistically significant. For all the physiological and biochemical parameters measured, I used the Benjamini-Hochberg procedure, for the number of ANOVA tests performed, to control the false discovery rate (Benjamini & Hochberg, 1995). Based on this procedure, values of $P < 0.03$ were identified as critical. However, to avoid false negatives and also due to the low number of true replicates (n = 3) for CO_2 treatment, values of $P < 0.1$ were considered as marginally significant. We expressed the relative effect of eCO_2 on the measured variables as a percent change calculated as: Effect size = [(mean at eCO_2-mean at aCO_2)/ (mean at aCO_2)] x 100.

Table 3.1 Results of mixed level split-plot ANOVA for light saturated net photosynthetic rates (A_{net}), light saturated photosynthetic rates measured at common [CO_2] (A_{net-Ca}), temperature normalized maximum carboxylation ($V_{cmax-25}$) and electron transport rates (J_{max-25}), N content on area (N_{area}) and mass basis (N_{mass}), apparent fraction of N allocated to Rubisco ($f_{N-Rubisco}$) P content on area (P_{area}) and mass basis (P_{mass}), N to P ratio (N: P ratio) and leaf mass per area (LMA).

Numerator and denominator degrees of freedom are indicated in parentheses. CO_2 refers to ambient and elevated CO_2 treatment, season refers to spring, summer and autumn and species refers to C_3 grass (*M. stipoides*) and C_3 forb (*L. purpurascens*). Based on the Benjamini-Hochberg procedure, critical *P*-values were identified to be 0.03. However, due to the low number of true replicates for CO_2 treatment (n =3), *P*-values < 0.1 are considered as marginally significant.

	Source of variation													
	CO_2 (1, 4)		Season (2, 8)		Species (1, 12)		CO_2 x Season (2, 8)		CO_2 x Species (1, 12)		Season x Species (2, 12)		CO_2 x Season x Species (2, 12)	
Variables	*F* -value	*P* -value	*F* -value	*P* -value	*F* -value	*P* -value	*F* -value	*P* -value	*F* -value	*P* -value	*F* -value	*P* -value	*F* -value	*P* -value
A_{net}	11.330	**0.028**	31.440	**<0.001**	21.700	**0.001**	0.580	0.578	0.083	0.777	2.520	0.122	0.890	0.433
A_{net-Ca}	1.730	0.258	3.770	**<0.001**	9.530	**0.009**	2.207	0.172	0.140	0.714	1.161	0.346	0.979	0.403
$V_{cmax-25}$	1.569	0.278	12.050	**0.004**	36.726	**<0.001**	6.148	**0.024**	0.809	0.386	0.751	0.492	2.591	0.116
J_{max-25}	0.574	0.491	13.820	**0.003**	33.570	**<0.001**	4.875	**0.041**	0.000	0.996	0.326	0.728	0.837	0.456
N_{area}	1.460	0.290	8.650	**0.010**	9.670	**0.009**	0.207	0.817	1.020	0.333	1.160	0.346	1.470	0.268
N_{mass}	1.060	0.361	4.160	**0.057**	27.350	**<0.001**	2.430	0.149	0.064	0.804	1.758	0.214	1.153	0.348
$f_{N-Rubisco}$	0.037	0.857	2.770	0.121	24.930	**<0.001**	8.220	**0.011**	0.000	0.986	1.836	0.201	1.210	0.332
P_{area}	0.804	0.420	4.210	**0.056**	50.050	**<0.001**	0.130	0.878	0.298	0.594	2.174	0.156	0.140	0.870
P_{mass}	0.245	0.646	2.470	0.145	50.230	**<0.001**	0.164	0.851	0.030	0.865	0.360	0.705	0.334	0.722
N: P ratio	0.127	0.740	1.530	0.272	48.890	**<0.001**	0.008	0.991	1.150	0.304	3.790	**0.053**	0.053	0.948
LMA	2.116	0.224	25.420	**<0.001**	2.659	0.129	1.257	0.335	0.434	0.522	0.760	0.491	3.480	**0.064**

Table 3.2 Results of mixed level split-plot ANOVA for live above-ground biomass of understory species with CO_2 treatment, year and functional type (grasses versus forbs) as main effects.

df column shows numerator and denominator degrees of freedom. CO_2 refers to ambient and elevated CO_2 treatment and year refers to the second and third year of CO_2 fertilization at the EucFACE. Statistically significant *P*- values are highlighted in bold.

Source of variation	df	*F-value*	*P-value*
CO_2	1, 4	0.033	0.86
Year	1, 4	0.133	0.73
Functional type	1, 8	106	**<0.001**
CO_2 x Year	1, 4	0.07	0.805
CO_2 x Functional type	1, 8	1.62	0.24
Year x Functional type	1, 8	14.13	**0.0056**
CO_2 x Year x Functional type	1, 8	0.087	0.776

3.4 Results

3.4.1 Effects of CO_2 treatment and season on photosynthetic rates

With a +150 µmol mol^{-1} enrichment in the CO_2 concentration, there was a significant increase in light saturated net photosynthetic rates across the species and six seasons during the second and third year of CO_2 enrichment at EucFACE ($P = 0.028$, Table 1). On average, A_{net} increased by ≈ 22% and ≈ 24% under eCO_2 in *L. purpurascens* and *M. stipoides* respectively (Fig. 3.1a). The split-plot ANOVA did not detect a significant CO_2 x season interaction effect on A_{net} across the species ($P > 0.1$, Table 3.1). However, the magnitude of eCO_2-induced A_{net} enhancement varied among the six measurement seasons for the two species (Fig. 3.1a). In particular, there was no significant increase in A_{net} under eCO_2 during the two spring time points (spring 2014 and spring 2015) in *L. purpurascens* and one spring (spring 2014) and one summer (summer 2015) time point in *M. stipoides* (*t-test*, $P > 0.1$, Fig. 3.1a). For the remaining seasons, A_{net} increased significantly under eCO_2 in both the species (*t-test*, $P < 0.05$, Fig. 3.1a).

Measurement season had a highly significant effect on the net photosynthetic rates across the species ($P < 0.01$, Table 3.1). Average A_{net} across the CO_2 treatments and species was higher in the spring and summer (13.34 ± 0.48 µmol m^{-2} s^{-1}) and lower in the autumn seasons (9.45 ± 0.57 µmol m^{-2} s^{-1}). This reduction in A_{net} during the autumn probably reflects the effects of low temperature on photosynthesis. Species also differed significantly in terms of A_{net} values across the CO_2 treatments and seasons ($P = 0.001$, Table 3.1), with *L. purpurascens* having significantly higher photosynthetic rates (12.6 ± 0.71 µmol m^{-2} s^{-1}) compared to *M. stipoides* (10.5 ± 0.6 µmol m^{-2} s^{-1}). There were no significant CO_2 x species, season x species and CO_2 x season x species interaction effects on A_{net} ($P > 0.1$, Table 3.1).

Overall, the lack of statistically significant increase in A_{net} under eCO_2 during the two peak growing seasons of spring for *L. purpurascens* and one spring and one summer season for *M. stipoides*, was an indicative of downward adjustment in photosynthetic characteristics under eCO_2. This lack of photosynthetic enhancement during the peak seasons could be attributed to treatment differences associated with eCO_2-induced

stomatal closure. However, results from Chapter 2 (Pathare *et al.*, 2017), indicate no significant CO_2 treatment effect on stomatal conductance and ratio of intercellular CO_2 concentration to CO_2 outside the leaf for both *L. purpurascens* and *M. stipoides*. Thus, a lack of eCO_2-induced photosynthetic enhancement must instead be related to the biochemistry of photosynthesis. In the following sections, I examined the parameters associated with leaf photosynthetic capacity for evidence of down-regulation.

3.4.2 Effects of CO_2 treatment and season on biochemical capacity

Light saturated photosynthetic rates measured at a common CO_2 concentration of 400 µmol mol^{-1} ($A_{net\text{-}Ca}$) can be compared between CO_2 treatments to test for changes in photosynthetic capacity in response to growth in different CO_2 treatments (Fig. 3.1b), assuming the stomatal conductance is not responsive to C_a level (see Pathare *et al*., 2017 and Chapter 2). The ANOVA indicated no significant CO_2 or CO_2 x season effect on $A_{net\text{-}Ca}$ across the species ($P > 0.1$, Table 3.1). However, the percent change in $A_{net\text{-}Ca}$ due to long-term CO_2 treatment varied among the seasons for the two species (Fig. 3.1b). In particular, $A_{net\text{-}Ca}$ decreased significantly during spring 2014 in *L. purpurascens* (*t-test*, $P < 0.05$) and *M. stipoides* (*t-test*, $P < 0.05$). Furthermore, there was a marginally significant trend towards lower $A_{net\text{-}Ca}$ under eCO_2 during spring 2015 for *L. purpurascens* (*t-test*, $P < 0.1$). Seasons were different for $A_{net\text{-}Ca}$ across the CO_2 treatments and species ($P < 0.01$, Table 3.1), with mean values being significantly higher in spring and summer (12.54 ± 0.42 µmol m^{-2} s^{-1}) compared to autumn seasons (9.01 ± 0.23 µmol m^{-2} s^{-1}). Species also differed significantly in terms of $A_{net\text{-}Ca}$ values across the CO_2 treatments and seasons ($P = 0.009$, Table 3.1). There were no significant CO_2 x species, season x species and CO_2 x season x species interaction effects on $A_{net\text{-}Ca}$ ($P > 0.1$, Table 3.1).

In addition to $A_{net\text{-}Ca}$, parameters like $V_{cmax\text{-}25}$ and $J_{max\text{-}25}$ are important indicators of changes in photosynthetic capacity of plants in response to growth at eCO_2. There was no overall CO_2 treatment effect on $V_{cmax\text{-}25}$ and $J_{max\text{-}25}$ across the seasons and species ($P > 0.1$, Table 3.1). However, there was a significant CO_2 x season effect on $V_{cmax\text{-}25}$ ($P = 0.024$, Table 3.1) and a significant effect on $J_{max\text{-}25}$ across the species ($P = 0.041$, Table 3.1). In particular, values for $V_{cmax\text{-}25}$ were significantly higher under aCO_2 compared to

eCO_2 during spring 2014 in both *L. purpurascens* (*t-test*, $P < 0.05$, Fig.3.2a) and *M. stipoides* (t-test, $P < 0.05$, Fig.3.2b) and during spring 2015 in *L. purpurascens* (*t-test*, $P < 0.05$, Fig.2.2a). Similar to $V_{cmax-25}$, J_{max-25} values were lower under eCO_2 during spring 2014 in both *L. purpurascens* (*t-test*, $P > 0.1$, Fig.3.2c) and *M. stipoides* (t-test, $P < 0.05$, Fig.3.2d) and during spring 2015 in *L. purpurascens* (*t-test*, $P < 0.05$, Fig.3.2c).

Measurement season had a highly significant effect on $V_{cmax-25}$ and J_{max-25} across the CO_2 treatments and species ($P < 0.01$, Table 3.1), with mean values for $V_{cmax-25}$ and J_{max-25} being higher in spring (89 ± 9 and 115 ± 7 µmol m^{-2} s^{-1} respectively) followed by summer (73 ± 4 and 99 ± 6 µmol m^{-2} s^{-1} respectively) and autumn seasons (68 ± 4 and 92 ± 5 µmol m^{-2} s^{-1} respectively). Species also differed significantly in terms of $V_{cmax-25}$ and J_{max-25} values across the CO_2 treatments and seasons ($P = 0.009$, Table 3.1), with *L. purpurascens* having statistically higher mean $V_{cmax-25}$ and J_{max-25} (87 ± 5 and 112 ± 4.8 µmol m^{-2} s^{-1} respectively) compared to *M. stipoides* (66 ± 3.7 and 91 ± 4.7 µmol m^{-2} s^{-1} respectively). There were no significant CO_2 x species, season x species and CO_2 x season x species interaction effects on $V_{cmax-25}$ and J_{max-25} ($P > 0.1$, Table 3.1). Taken together, above results indicate a significant decrease in parameters associated with photosynthetic capacity for the dominant C_3 species particularly during the peak growing seasons of spring and coincide with the lack of eCO_2-induced A_{net} enhancement during these seasons. To determine the cause of this down-regulation in photosynthetic capacity, I further investigated the changes in leaf N content on area basis (N_{area}) and allocation of N to Rubisco under eCO_2.

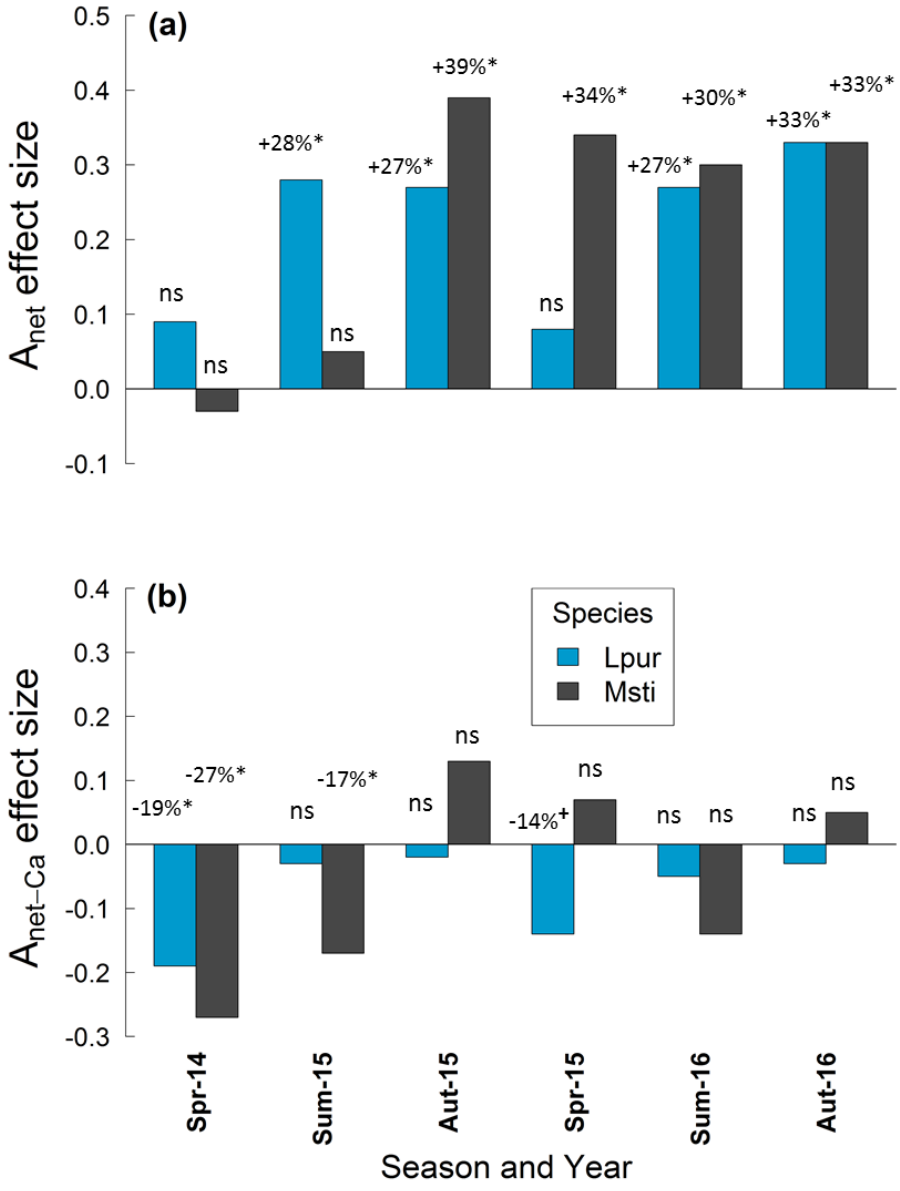

Fig. 3.1 Effects of eCO_2 on light saturated net photosynthetic rates (A_{net}) and light saturated photosynthetic rates measured at common [CO_2] (A_{net-Ca}).

Effects of eCO_2 on (a) A_{net} and (b) A_{net-Ca} are shown for *L. purpurascens* (Lpur) and *M. stipoides* (Msti) measured for six seasons. Significant differences between CO_2 treatments within season at $P \leq 0.05$ are denoted by '*', at $P \leq 0.1$ are denoted by '+' and at $P > 0.1$ are denoted by 'ns'. Bars indicate the effect sizes. Percent change is shown whenever significant. X-axis labels showing measurement seasons and years are spring 2014 (Spr-14), summer 2015 (Sum-15), autumn 2015 (Aut-15), spring 2015 (Spr-14), summer 2016 (Sum-16) and autumn 2016 (Aut-16).

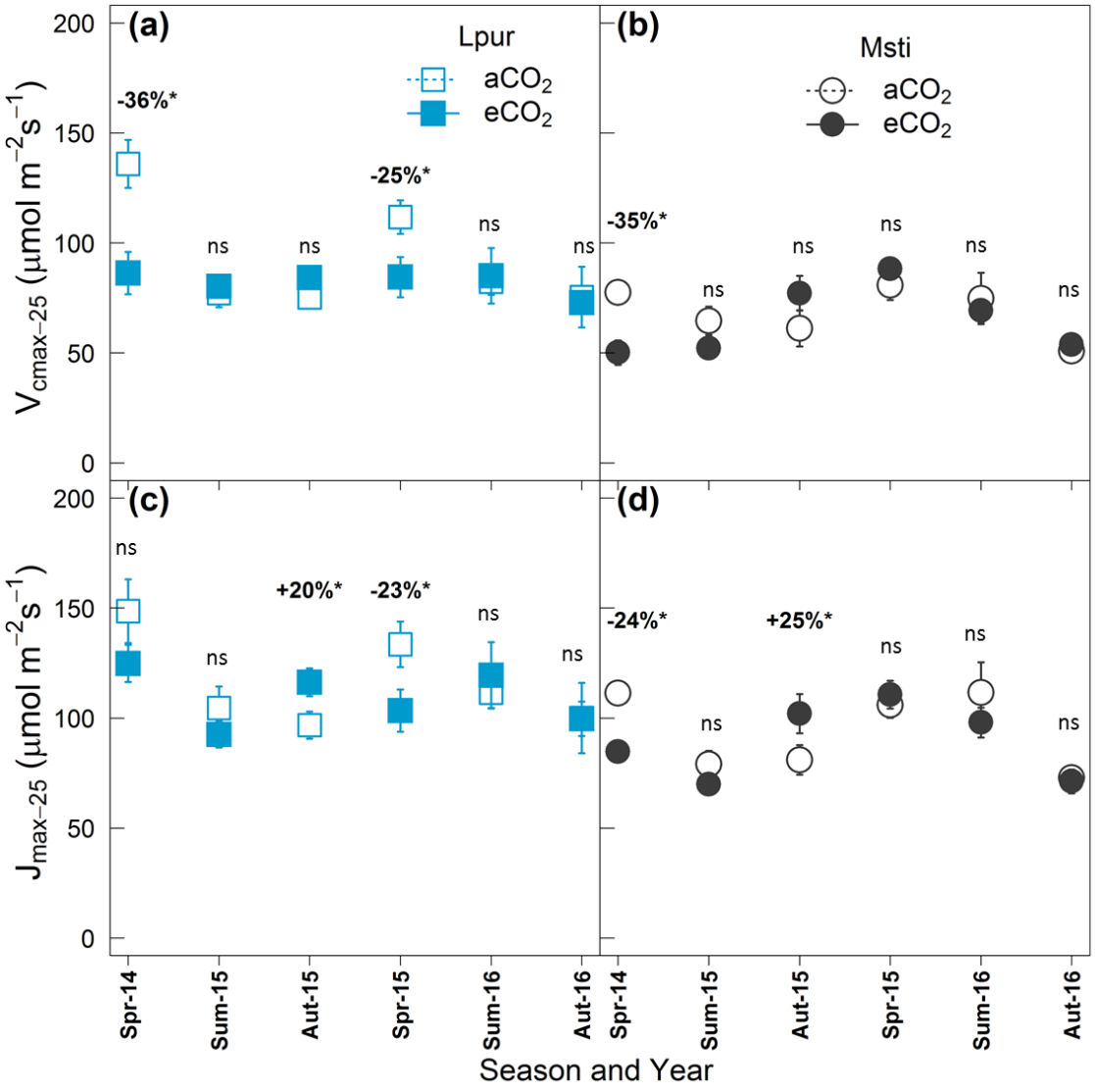

Fig. 3.2 Time course through the six measurement seasons for temperature normalised maximum carboxylation ($V_{cmax-25}$) and electron transport (J_{max-25}) as a function of CO_2 treatment.

Effects of eCO_2 on (a) $V_{cmax-25}$ and (b) J_{max-25} are shown for *L. purpurascens* (Lpur) and *M. stipoides* (Msti) measured for six seasons. Significant differences between CO_2 treatments within season at $P \leq 0.05$ are denoted by '*', at $P \leq 0.1$ are denoted by '+' and at $P > 0.1$ are denoted by 'ns'. Percent change is shown whenever significant. aCO_2 and eCO_2 in the legends denote ambient and elevated CO_2 treatments respectively.

3.4.3 Effect of CO_2 treatment and season on N content $f_{N\text{-}Rubisco}$ and P content

There was no significant overall CO_2 treatment effect on leaf N content (N_{area} and N_{mass}) across the seasons and species ($P > 0.1$, Table 3.1). Also, the split-plot ANOVA did not indicate a statistically significant CO_2 x season interaction effect on N content across the species ($P > 0.1$, Table 3.1). However, percent change in N content due to eCO_2 varied among the six measurement seasons for the two species (Fig. 3.3). In particular, N_{area} decreased significantly under eCO_2 during spring 2014 in both *L. purpurascens* (*t-test*, $P < 0.05$, Fig.3.3a) and *M. stipoides* (*t-test*, $P < 0.05$, Fig.3.3b). N_{mass} decreased significantly under eCO_2 in *L. purpurascens* (Fig. 3.3c) during spring 2014 (*t-test*, $P < 0.05$). In short, leaf N content, particularly, N_{area} showed a significant decrease under eCO_2 in both the species, but only during spring 2014 (Fig. 3.3).

There was a significant season effect on N_{area} across the CO_2 treatments and species ($P = 0.01$, Table 3.1). On average, the values for N_{area} were higher during the spring and summer (1.04 ± 0.027 g m^{-2}) compared to the autumn seasons (0.90 ± 0.03 g m^{-2}). However, for N_{mass}, there was only a marginally significant season effect ($P = 0.057$, Table 3.1). Species also differed significantly in terms of N content values across the CO_2 treatments and seasons ($P = 0.009$, Table 3.1), with *L. purpurascens* having statistically higher N_{area} and N_{mass} (1.01 ± 0.02 g m^{-2} and 32.8 ± 0.68 mg g^{-1} respectively) compared to *M. stipoides* (0.93 ± 0.018 g m^{-2} and 28.6 ± 0.48 mg g^{-1} respectively). The ANOVA did not indicate a significant CO_2 x species, season x species and CO_2 x season x species interaction effects for both N_{area} and N_{mass} ($P > 0.1$, Table 3.1). However, there was a significant decrease in N_{area} (-16%) and N_{mass} (-14%) as indicated by *t*-test ($P < 0.05$, Fig. 3.3) in *L. purpurascens* during spring 2014. Also, there was a decrease in N_{area} in *M. stipoides* during spring 2014 (-18%) and summer 2016 (-20%). These decreases in leaf N content correlate with decrease in photosynthetic capacity parameters in *L. purpurascens* and *M. stipoides* during spring 2014.

There was no overall CO_2 treatment effect on $f_{N\text{-}Rubisco}$ across the seasons and species ($P > 0.1$, Table 3.1). However, there was a significant CO x season effect on $f_{N\text{-}Rubisco}$ across the species ($P = 0.01$, Table 3.1). In particular, $f_{N\text{-}Rubisco}$ decreased under eCO_2 during spring 2014 in both *L. purpurascens* (*t-test*, $P < 0.05$, Fig.3.4a) and *M. stipoides* (*t-test*, *P*

< 0.05, Fig.3.4b) and during spring 2015 in *L. purpurascens* (*t-test*, $P < 0.05$, Fig.3.4a). This decrease in $f_{N\text{-}Rubisco}$ under eCO_2 during the peak seasons correlates with the photosynthetic capacity down-regulation observed during these seasons (Fig. 3.2). Species also differed significantly in $f_{N\text{-}Rubisco}$ across the CO_2 treatments and seasons ($P <$ 0.01, Table 3.1) as *L. purpurascens* showed higher $f_{N\text{-}Rubisco}$ (30 ± 0.01 %) compared to *M. stipoides* (25 ± 0.006%). Overall, there was no significant season, CO_2 x species, season x species and CO_2 x season x species interaction effects on $f_{N\text{-}Rubisco}$ ($P > 0.1$, Table 3.1).

Similar to leaf N content, there was no overall CO_2 treatment effect on leaf P content (P_{area} and P_{mass}) across the seasons and species ($P > 0.1$, Table 3.1). Furthermore, the split-plot ANOVA did not indicate a statistically significant CO_2 x season interaction effect on N content across the species ($P > 0.1$, Table 3.1). There was a marginally significant season effect on P_{area} across the species and CO_2 treatments ($P = 0.056$, Table 3.1), as average values for P_{area} tended to be slightly higher in spring and summer compared to autumn seasons (Fig. 3.5). Species differed significantly in terms of P_{area} and P_{mass} as there was a highly significant species effect on leaf P content across the CO_2 treatments and seasons ($P < 0.001$, Table 3.1). In particular, P_{area} and P_{mass} were higher in *L. purpurascens* (0.044 ± 0.009 g m^{-2} and 1.41 ± 0.26 mg g^{-1} respectively) compared to *M. stipoides* (0.03 ± 0.006 g m^{-2} and 1 ± 0.21 mg g^{-1} respectively). Overall, there were no statistically significant CO_2 x species, season x species and CO_2 x season x species interaction effects on P_{area} and P_{mass} ($P > 0.1$, Table 3.1). Furthermore, there was no statistically significant of CO_2, season and two-way and three-way interaction effect on N: P ratio. The average N: P ratio across the CO_2 treatment, season and species was 30 ± 4. Species differed significantly in terms of N: P ratio ($P < 0.001$, Table 3.1), as Lpur showed lower average N: P ratio (26 ± 3) compared to Msti (32 ± 3).

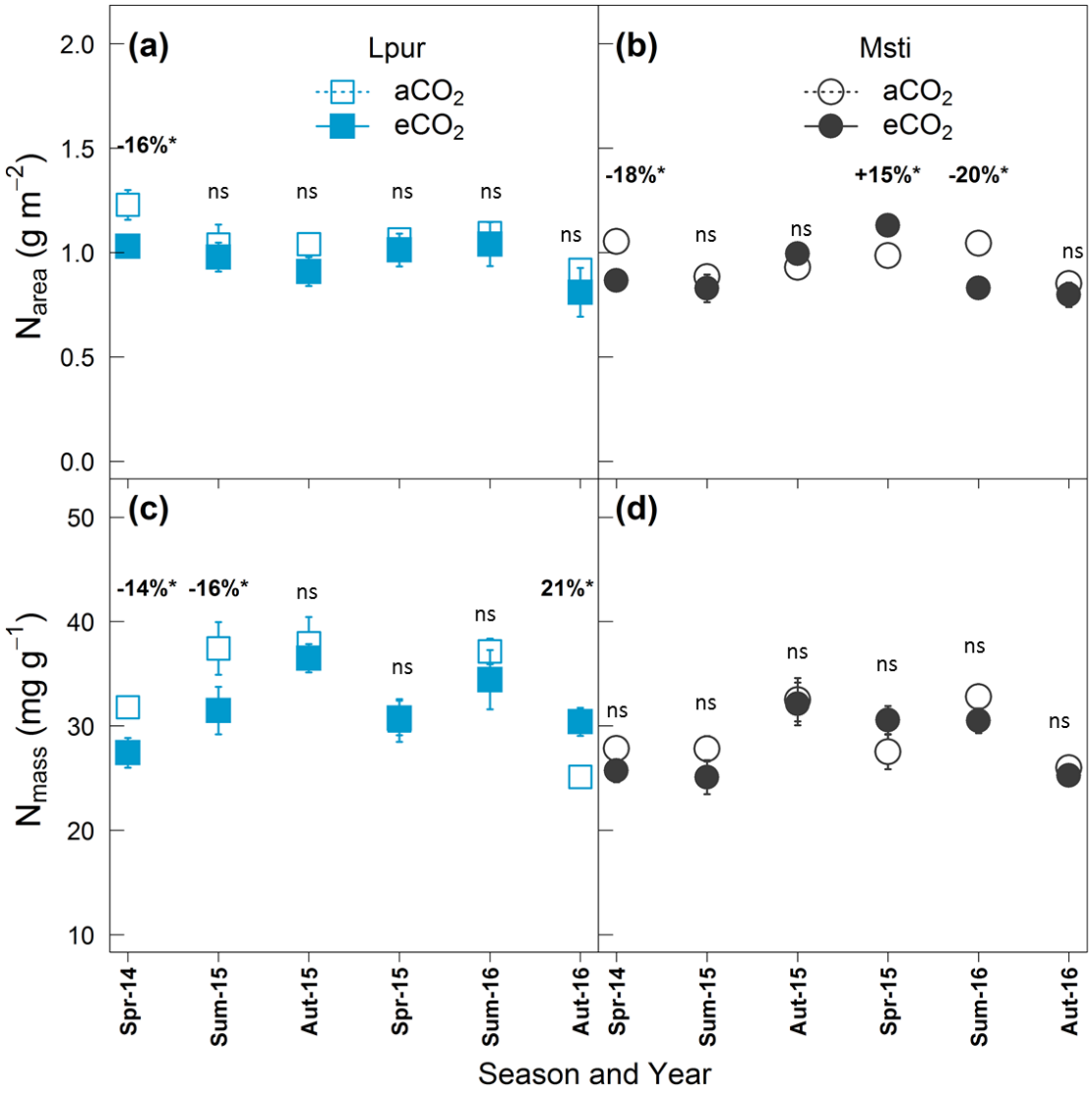

Fig. 3.3 Time course through the six measurement seasons for N content as a function of CO_2 treatment.

Effects of eCO_2 on leaf N content (a) on area basis (N_{area}) and (b) mass basis (N_{mass}) are shown for *L. purpurascens* (Lpur) and *M. stipoides* (Msti) measured for six seasons. Significant differences between CO_2 treatments within season at $P \leq 0.05$ are denoted by '*', at $P \leq 0.1$ are denoted by '+' and at $P > 0.1$ are denoted by 'ns'. Percent change is shown whenever significant. aCO_2 and eCO_2 in the legends denote ambient and elevated CO_2 treatments respectively.

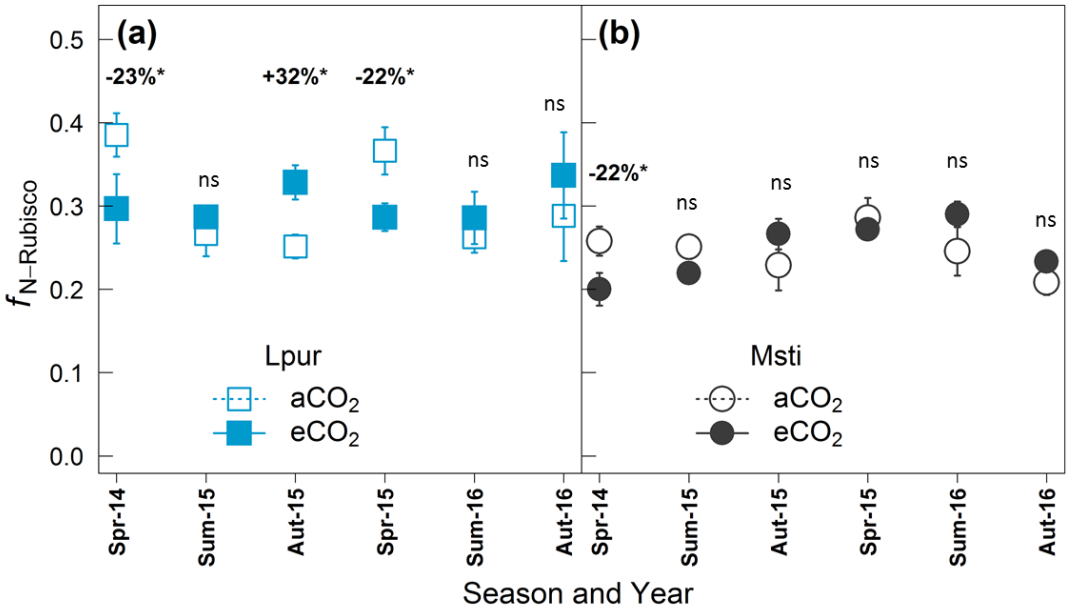

Fig. 3.4 Time course through the six measurement seasons for N allocated to Rubisco enzyme ($f_{N\text{-}Rubisco}$) function of CO_2 treatment.

Effects of eCO_2 on $f_{N\text{-}Rubisco}$ are shown for (a) *L. purpurascens* (Lpur) and (b) *M. stipoides* (Msti) measured for six seasons. Significant differences between CO_2 treatments within season at $P \leq 0.05$ are denoted by '*', at $P \leq 0.1$ are denoted by '+' and at $P > 0.1$ are denoted by 'ns'. Percent change is shown whenever significant. aCO_2 and eCO_2 in the legends denote ambient and elevated CO_2 treatments respectively.

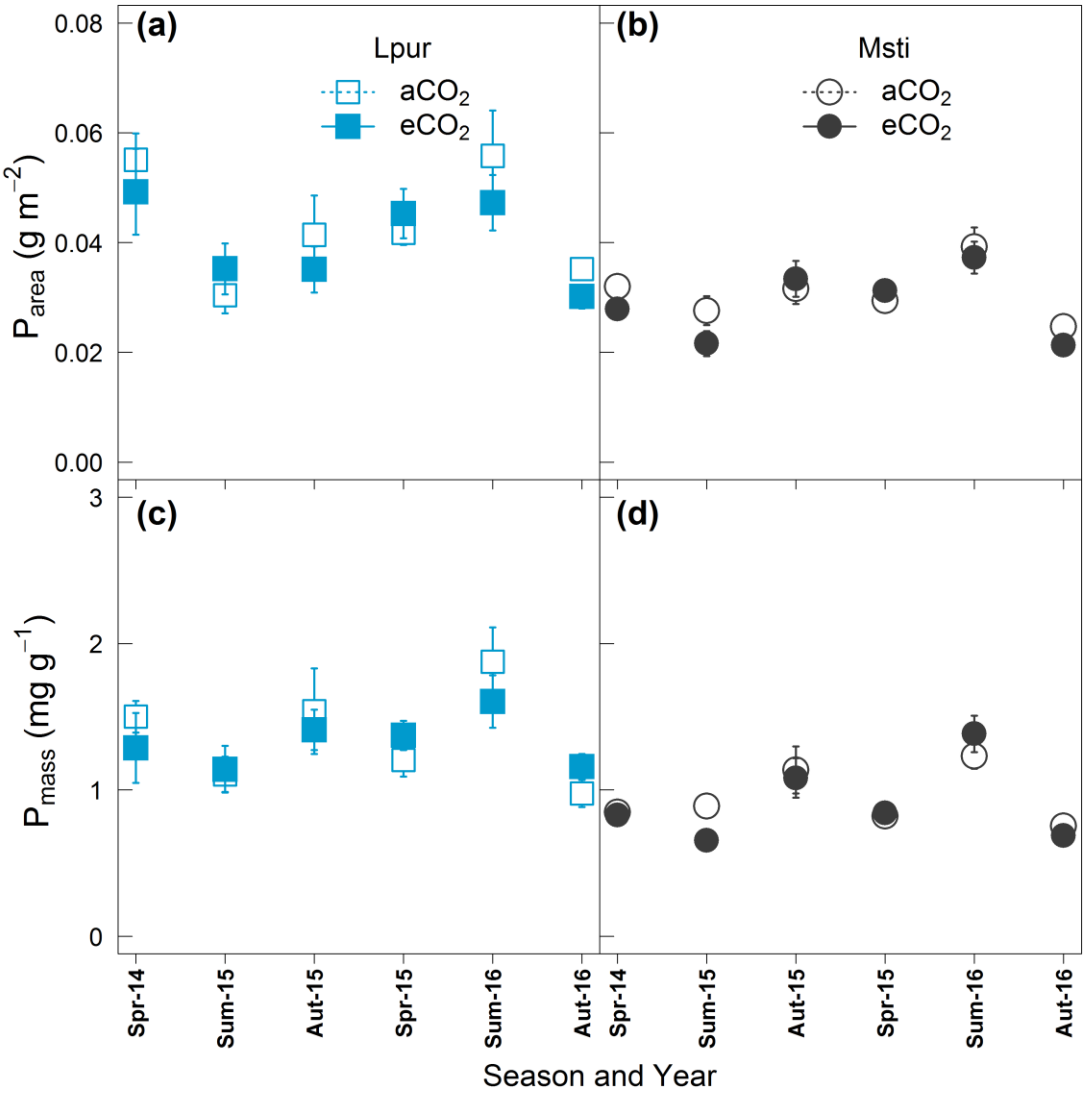

Fig. 3.5 Time course through the six measurement seasons for leaf P content as a function of CO_2 treatment.

Effects of eCO_2 on leaf P content (a) on area basis (P_{area}) and (b) mass basis (P_{mass}) are shown for *L. purpurascens* (Lpur) and *M. stipoides* (Msti) measured for six seasons. aCO_2 and eCO_2 in the legends denote ambient and elevated CO_2 treatments respectively.

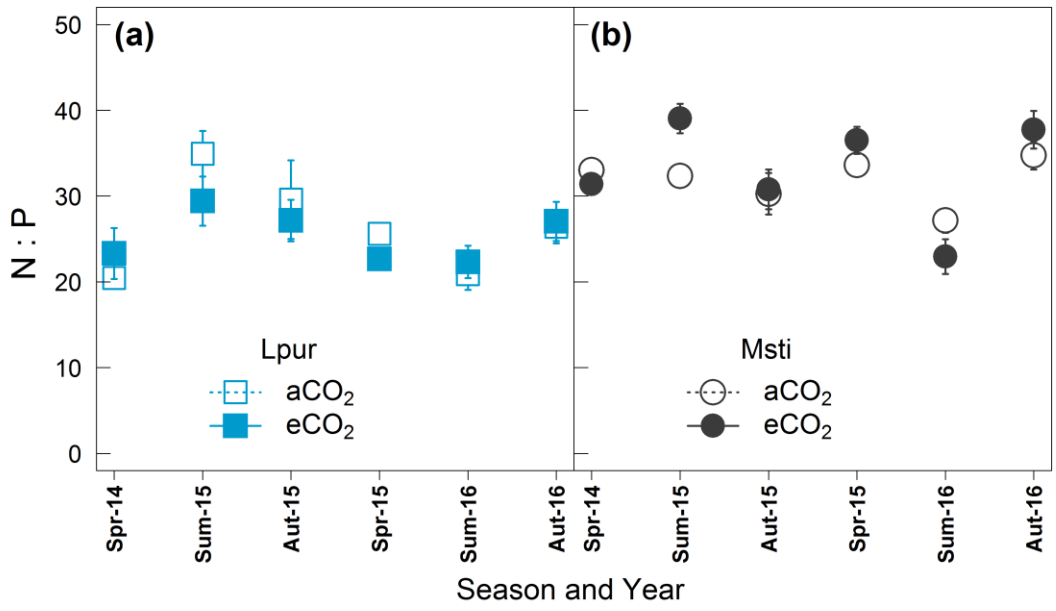

Fig. 3.6 Time course through the six measurement seasons for leaf nitrogen to phosphorus ratio (N : P) as a function of CO_2 treatment.

Effects of eCO_2 on N: P are shown for (a) *L. purpurascens* (Lpur) and (b) *M. stipoides* (Msti) measured for six seasons. aCO_2 and eCO_2 in the legends denote ambient and elevated CO_2 treatments respectively.

3.4.4 Effects on CO_2 treatment and year on above-ground biomass

For above-ground biomass measurements, species were grouped into total forbs and total grasses assuming that any eCO_2-induced changes in the understory biomass of forbs and grasses may be largely driven by responses of *L. purpurascens* and *M. stipoides*. There was no statistically significant CO_2, year and CO_2 x year interaction effect on the standing live above-ground biomass for total forbs and grasses ($P > 0.1$, Table 3.2). There was a highly significant functional type effect on live above-ground biomass ($P < 0.001$, Table 3.2), as forbs had lower above-ground biomass compared to grasses across the two years (Fig. 3.7a, b). Furthermore, there was a statistically significant year x functional group interaction effect ($P = 0.005$, Table 3.2). In particular, forbs showed lower average above-ground biomass in summer 2015 (10.7 ± 2.3 g m^{-2}) compared to summer 2016 (26.5 ± 4 g m^{-2}), whereas, grasses showed higher average above-ground biomass in summer 2015 (110 ± 12.2 gm^{-2}) compared to summer 2016 (70 ± 10 gm^{-2}). Greater live above-ground biomass during summer 2015 compared to summer 2016, particularly for the grasses, could be attributed to increase in understory light availability during this period (Duursma *et al*., 2016) as a result of major psyllid overstory defoliation at the EucFACE site (Gherlenda *et al*., 2016). There was no significant CO_2 x functional group and CO_2 x year x functional group interaction effect on live above-ground biomass ($P > 0.1$, Table 3.2). I further analysed the effects of CO_2 treatment and measurement year on the total above-ground biomass (total live plus dead biomass). Results for a mixed level split-plot ANOVA are shown in Fig. 3.7 c. There were no statistically significant CO_2, year and CO_2 x year interaction effects on total above-ground biomass ($P > 0.1$, Fig. 3.7). In summary, no significant increase in live grass and forb above-ground biomass and total above-ground biomass was observed under eCO_2 during the two measurement years.

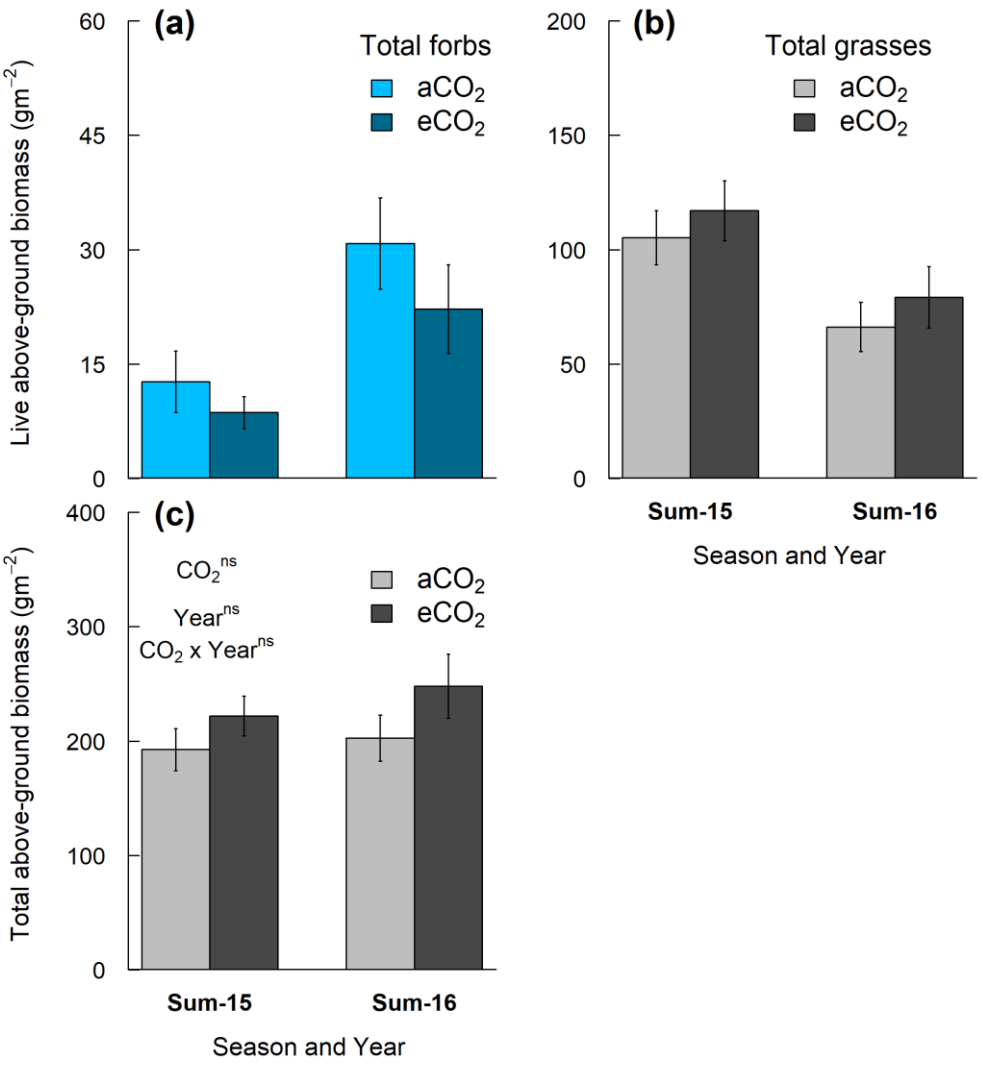

Fig. 3.7 Effects of CO_2 treatment on above-ground biomass of understory species at EucFACE.

Effects of eCO_2 are shown for above-ground biomass of (a) total forbs, (b) total grasses and (c) total above-ground biomass measured during summer 2015 (Sum-15) and summer 2016 (Sum-16). Total above-ground biomass includes total forbs, total grasses and senescent biomass. Results of spilt-plot ANOVA for total above-ground biomass are shown in panel c. Significant differences at $P < 0.05$ are denoted by * and non-significant difference are denoted by 'ns'. aCO_2 and eCO_2 in the legends denote ambient and elevated CO_2 treatments respectively.

3.5 Discussion

3.5.1 Seasonal photosynthetic down-regulation under eCO_2

In this study, I examined the seasonal photosynthetic acclimation and above-ground biomass responses to eCO_2 in the understory herbaceous species growing naturally in a warm-climate *Eucalyptus* woodland. My first hypothesis, that there would be larger down-regulation of photosynthetic capacity under eCO_2 during the autumn season than spring and summer due to the lower growth sink capacity in that season, was not supported. While the average eCO_2-induced photosynthetic enhancement across the seasons in the current study was moderate (≈ 23%), there was a considerable variation in the relative photosynthetic enhancement among the seasons as well as the species (Fig. 3.1a). During the two spring seasons, lack of photosynthetic enhancement in the C_3 forb under eCO_2 was accompanied by reductions in photosynthetic capacity (Fig. 3.1b and 3.2). Furthermore, lack of photosynthetic enhancement in the C_3 grass under eCO_2 during spring 2014 was accompanied by reductions in photosynthetic capacity (Fig. 3.1a and Fig.3.2). For the remaining measurement seasons, including autumn, there was no evidence of photosynthetic capacity down-regulation under eCO_2 for either of the species in this study. Taken together, these results provide some evidence for photosynthetic capacity down-regulation under eCO_2, during the peak growing season of spring, thus contrasting the first hypothesis (H1). Also, in contrast to the second hypothesis (H2), I did not observe a significant increase in above-ground biomass under eCO_2 for the understory grasses and the understory forbs measured in the subsequent summers (Fig. 3.5).

Earlier studies reporting seasonal photosynthetic acclimation under eCO_2 have often cited sink limitation hypothesis (Stitt, 1991) as a mechanism for lower photosynthetic capacity under eCO_2 during seasons with lowered sink capacities (Ainsworth *et al.*, 2003b, Lewis *et al.*, 1996). In particular, during active growth period like spring, sink capacity will be higher and hence there should be no down-regulation. Since autumn is characterised by low growth and hence sink capacity, I expected a significant photosynthetic capacity down-regulation (H1), and indeed lower V_{cmax} and J_{max} were exhibited in both species in this season than for the other seasons. However, in contrast to my expectation (H1) there was no evidence of photosynthetic capacity down-regulation under eCO_2 during the

autumn season in either of the species. I observed a down-regulation response during spring, a favourable growing period for these species, when growth and carbohydrate sinks are expected to be large, but N concentrations in leaves were lowest. Therefore, results from the current study suggest that sink limitation hypothesis may not be the possible explanation for down-regulation observed under eCO_2 during the physiologically active season.A general decrease in leaf N content under eCO_2 has been frequently observed in field experiments on the grasses and forbs (Aranjuelo *et al.*, 2011, Ellsworth *et al.*, 2004, Lee *et al.*, 2011) and is consistent with the results from the current study for some time points. For the dominant C_3 forb, down-regulation of photosynthetic capacity observed during spring 2014 was accompanied by significant decrease in N_{area} (-16%) and N_{mass} (-14%, Fig. 3.3a and c). For the C_3 grass, down-regulation of photosynthetic capacity observed during spring 2014 was accompanied by significant decrease in N_{area} (-18%, Fig. 3.3b). Thus, down-regulation of photosynthetic capacity under eCO_2 in the current study, observed during spring 2014 in both the dominant C_3 species, could be attributed to the decrease in leaf N concentrations, which may occur due to accumulation of soluble carbohydrates under eCO_2 (Inauen *et al.*, 2012, Long *et al.*, 2004), low soil N availability (Ellsworth *et al.*, 2004) or negative effects of eCO_2 on plant N acquisition (Feng *et al.*, 2015). However, down-regulation of photosynthetic capacity under eCO_2 was also observed during spring 2015 in the C_3 forb, but was not accompanied by a corresponding decrease in N content. This suggests that decrease in leaf N content may not be a sole mechanism of photosynthetic down-regulation under eCO_2 in the current study, although it may be an important contributing factor. A protein specific down-regulation of the Rubisco enzyme (Aranjuelo *et al.*, 2011, Rogers & Ellsworth, 2002) seems to be a more plausible explanation for the photosynthetic capacity downregulation under eCO_2 in the current study. There was a lower allocation of N to Rubisco under eCO_2 ($\approx$ -22%) during the two spring seasons in the C_3 forb and one spring season in the C_3 grass (Fig. 3.4) which corresponds to the down-regulation of photosynthetic capacity ($\approx$ -28%) during these time points (Fig. 3.2). Whereas, allocation of N to Rubisco under eCO_2 was maintained during the other seasons, including autumn, when there was no evidence of photosynthetic capacity down-regulation. This eCO_2-induced protein specific down-regulation provides

N that can be re-allocated towards other protein-requiring systems (Sage, 1994, Sharwood *et al.*, 2017).

3.5.2 Elevated CO_2 does not stimulate above-ground biomass in the herbaceous understory species

Modelling studies based on the Rubisco kinetics of the C_3 species have predicted a 35% increase in net primary productivity in the warmer ecosystems, compared to the 26% predicted for temperate ecosystems (Collatz *et al*., 1991, Farquhar *et al*., 1980, Hickler *et al*., 2008). To test the above prediction, I investigated the responses of standing above-ground biomass to eCO_2 during the two summer seasons for all the grasses and forbs growing in the understory of a warm-temperate *Eucalyptus* woodland (Fig. 3.7). Considering that *L. purpurascens* and *M. stipoides* are the most dominant species (Hasegawa *et al*., 2018), any changes in the total above-ground forb and grass biomass may be a result of responses in these two dominant species to eCO_2. There was a lack of statistically significant stimulation under eCO_2 in total live above-ground biomass of the forbs and grasses as well as total biomass (Fig. 3.7), in spite of a significant increase in photosynthetic rates of the two dominant species. Thus, results for the understory species from the current study do not support the general expectation of higher relative increase in biomass under eCO_2 for warm ecosystem species compared to cold temperate ecosystems (Cernusak *et al*., 2013). The possible factors responsible for this lack of biomass stimulation under eCO_2 for species from a warm-temperate ecosystem are discussed in the following sections.

Lack of an eCO_2-induced biomass stimulation in the understory species has often been associated with decrease in light availability because of increase in overstory biomass and leaf area (Bandeff *et al.*, 2006, Kim *et al.*, 2015, Sefcik *et al.*, 2007). Like the previous studies (Bandeff *et al.*, 2006, Kim *et al.*, 2015), I observed a lack of stimulation in above-ground biomass of the understory species in the current study. However, it is unlikely that responses of overstory trees to eCO_2 may have contributed to lack of biomass enhancement in the understory species. This is because, there was no significant decrease in light levels under eCO_2 in the current study (Fig. S. 3.1). Also, recent studies conducted at the EucFACE site suggest that eCO_2 does not cause increase in leaf area index or other

components of above-ground biomass in the overstory trees because of which understory light levels in eCO_2 plots did not decrease (Duursma *et al.*, 2016, Ellsworth *et al.*, 2017). Thus, in contrast to previous studies (Bandeff *et al.*, 2006, Kim *et al.*, 2015, Sefcik *et al.*, 2007) decreased understory light availability due to increase in overstory biomass under eCO_2 is not a possible explanation for lack of biomass enhancement in understory species in the current study.

The absence of an eCO_2 effect on biomass enhancement could instead be related to soil moisture availability (see Pathare *et al.*, 2017 and Chapter 2), if dry periods are of relatively moderate duration and intensity. Previous studies conducted on the herbaceous species particularly in the water-limited ecosystems report that, eCO_2-induced soil water savings can eliminate plant water limitation and also enhance soil nutrient availability thus supporting relatively greater biomass compared to aCO_2 (Grünzweig & Körner, 2003, Morgan *et al.*, 2011, Morgan *et al.*, 2004, Polley *et al.*, 2012a). The *Eucalyptus* woodland in the current study is seasonally water-limited, suggesting that interaction between CO_2 and water availability will have a significant effect on understory biomass response to eCO_2. In Chapter 2, I observed a larger relative enhancement of photosynthetic rates under eCO_2 during dry periods, which leads to the expectation that relative enhancement of understory biomass could also be reported during the dry periods. However, there was no significant stimulation of above-ground biomass under eCO_2 in the understory species (Fig. 3.7). One possible explanation for this finding is that the biomass harvest was conducted only during summer 2015 and summer 2016, which were relatively wet periods for this ecosystems since a cumulative precipitation of > 200 mm was received in the two summer months prior to the biomass harvest (Fig. 2.1). Thus, lack of relative increase in biomass under eCO_2 during the wet summer seasons could be attributed to greater precipitation. This leads to the question regarding the effects of eCO_2 on the understory biomass during the relatively dry periods at EucFACE site. In a recent study at EucFACE, Collins *et al*., (2018) used a repeat near-surface digital photography to quantify the effects of water availability and eCO_2 on understory live foliage biomass and biomass cover over three growing seasons. Their findings suggest that eCO_2 did not increase herbaceous cover and biomass over the duration of their three-year experiment, not even during the periods of low water availability. Taken together, findings from the current study along with those

of Collins *et al*., (2018) suggest that, though future increases in eCO_2 may cause a relative enhancement of photosynthetic rates during dry periods, this may not lead to increase in understory productivity in warm-temperate grassy woodlands of Australia.

Besides lack of soil water savings, low soil nutrient availability may also constrain the biomass enhancement under eCO_2. Since, a large proportion of leaf N is invested in the photosynthetic proteins (Evans, 1989), soil N availability has been suggested as an important determinant of photosynthesis and biomass responses to eCO_2 (Ainsworth & Rogers, 2007, Reich *et al.*, 2006a). In accordance with this expectation, earlier studies conducted in the cold temperate grasslands and forest ecosystems report a lower stimulation of biomass under eCO_2 due to insufficient N availability (Oren *et al.*, 2001, Reich & Hobbie, 2013). In the heavily weathered soils common in warmer ecosystems, P tends to be more limiting than N (Cernusak *et al.*, 2013). However, experiments addressing effects of eCO_2 on plants growing in P-limited soils are rare. Some limited number of studies conducted suggest a limited biomass stimulation under eCO_2 due to low soil P availability (Edwards *et al.*, 2005, Grünzweig & Körner, 2003, Lewis *et al.*, 2010, Shaw *et al.*, 2002). The *Eucalyptus* woodland in the current study was shown to be P-limited, with the leaf N: P ratio of ≈ 23 for the *Eucalyptus* trees (Crous *et al.*, 2015). Also, a recent study from the site (Ellsworth *et al.*, 2017), on growth responses of mature *Eucalyptus* trees to eCO_2, observed a lack of stimulation in any of the above-ground biomass components which they suggest was a result of low soil P availability. At vegetation level, N: P ratios < 10 have often been considered to indicate N-limited biomass production, whereas, ratios > 20 indicate P-limited biomass production (Güsewell, 2004). In the current study, I observed an average leaf N: P ratio of ≈ 26 and ≈ 32 for two dominant understory species, *L. purpurascens* and *M. stipoides* respectively, across the six measurement seasons and CO_2 treatments (Fig. 3.6). This N: P ratio > 20 indicates that the understory species in current study were limited by soil P availability. Thus, in addition to lack of eCO_2-induced soil water savings, greater P-limitation, could be another possible element contributing to the lack of relative increase in above-ground biomass of the understory herbaceous species in the current study. Current understanding about how low P availability constrains plant biomass responses to eCO_2 is still limited (Deng *et al.*, 2015). One of the possible explanations for this include limited rate of RuBP regeneration,

probably through inhibition of the Calvin cycle (Campbell & Sage, 2006) or reduced photorespiration under eCO_2 leading to reduced availability of photorespiration-recycled P (Ellsworth *et al.*, 2015, Harley & Sharkey, 1991).

A lack of stimulation of plant growth and biomass, despite increases in photosynthetic rates under eCO_2, has been observed previously for trees as well as herbaceous species (Ellsworth *et al.*, 2017, Norby *et al.*, 2010, Reich & Hobbie, 2013, Sigurdsson *et al.*, 2013). Similarly, lack of biomass stimulation despite increases in photosynthetic rates under eCO_2 was reported for the herbaceous understory species in the current study. This discrepancy between photosynthesis and biomass could be attributed to increase in carbohydrate availability exceeding the plants' capability to process it due to nutrient and inherent growth limitations (Kirschbaum, 2011). This disconnect between photosynthesis and biomass responses to eCO_2 leads to the question about the fate of the C assimilated under eCO_2. Some of the possible pathways for extra C assimilated under eCO_2 include: increased plant and soil respiration (Adair *et al.*, 2011, Drake *et al.*, 2016), increased root exudates and export to mycorrhiza and other microbes (Cheng *et al.*, 2012, Phillips *et al.*, 2011, Phillips *et al.*, 2012) and increased root growth under nutrient limited conditions (Inauen *et al*., 2012, Nie *et al*., 2013). Since, the soil of grassy woodland in current study is P-limited (Crous *et al.*, 2015), it is possible that the extra C assimilated is being invested in below-ground growth, although detailed discussion is beyond the scope of current study due to lack of root biomass data. Alternatively, a recent study from EucFACE (Drake *et al*., 2016) provides an evidence of initial stimulation of root and/or rhizosphere respiration thus returning the assimilated C back to the atmosphere. However, there are still open questions regarding the nature of this P limitation and how it constrains the eCO_2 response.

3.5.3 Conclusions

In summary, I investigated the effects of eCO_2 on the seasonal photosynthetic responses of two dominant understory herbaceous C_3 species and the above-ground biomass responses of total understory herbaceous species during the second and third year of CO_2 fertilisation at EucFACE. Elevated CO_2 stimulated photosynthetic rates in the dominant C_3 species by an average of 23% across the seasons. However, there was also a limited

evidence of seasonal acclimation as photosynthetic capacities decreased significantly under eCO_2 during the peak season of spring leading to lack of A_{net} enhancement. Furthermore, eCO_2 did not stimulate live above-ground forb and grass biomass as well as total above-ground biomass (live plus senescent) measured during the subsequent summer seasons. I conclude that, lack of eCO_2-induced 'water-savings effect' and higher P-limitations could be the possible elements contributing to lack of relative increases in above-ground biomass under eCO_2 in these understory species. The warm water-limited ecosystems have been predicted to be major C sinks due to greater photosynthesis and productivity responses to eCO_2 (Ahlström *et al.*, 2015). However, the lack of biomass stimulation in the understory species during the summer season, reported in the current study, indicate a limited capacity of these herbaceous species to sequester extra C in future.

3.6 Supplementary information

3.6.1 Supplementary figures

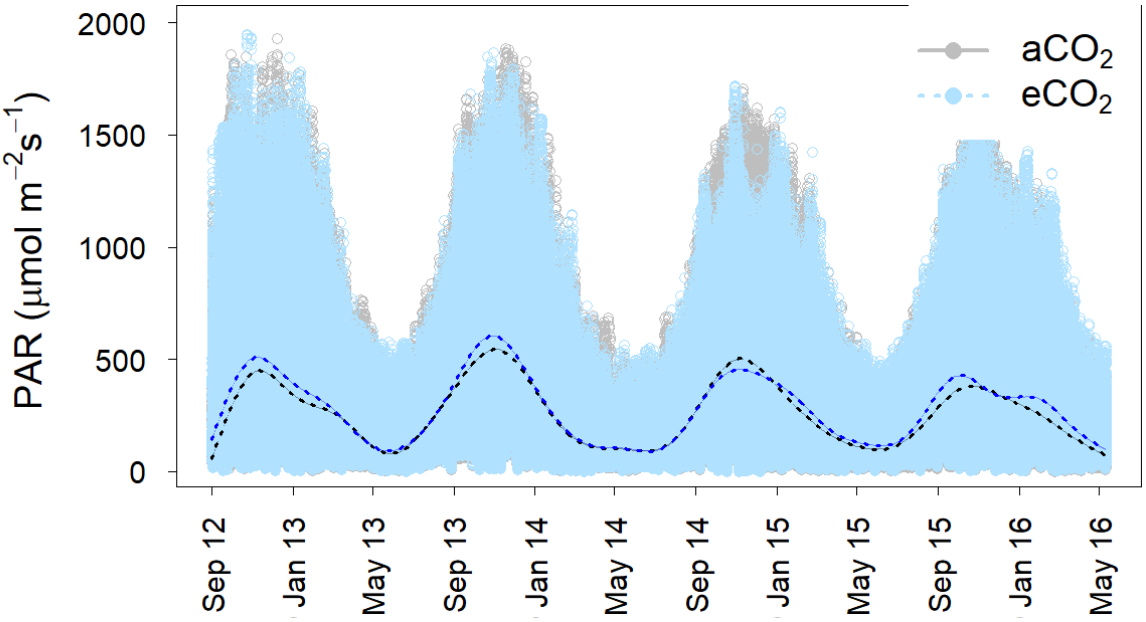

Fig. S 3.1 Understory light levels at the EucFACE site measured in aCO_2 (gray points) and eCO_2 plots (blue points). Smoothed regressions with 95% confidence intervals are shown for aCO_2 (black line) and eCO_2 (blue line).

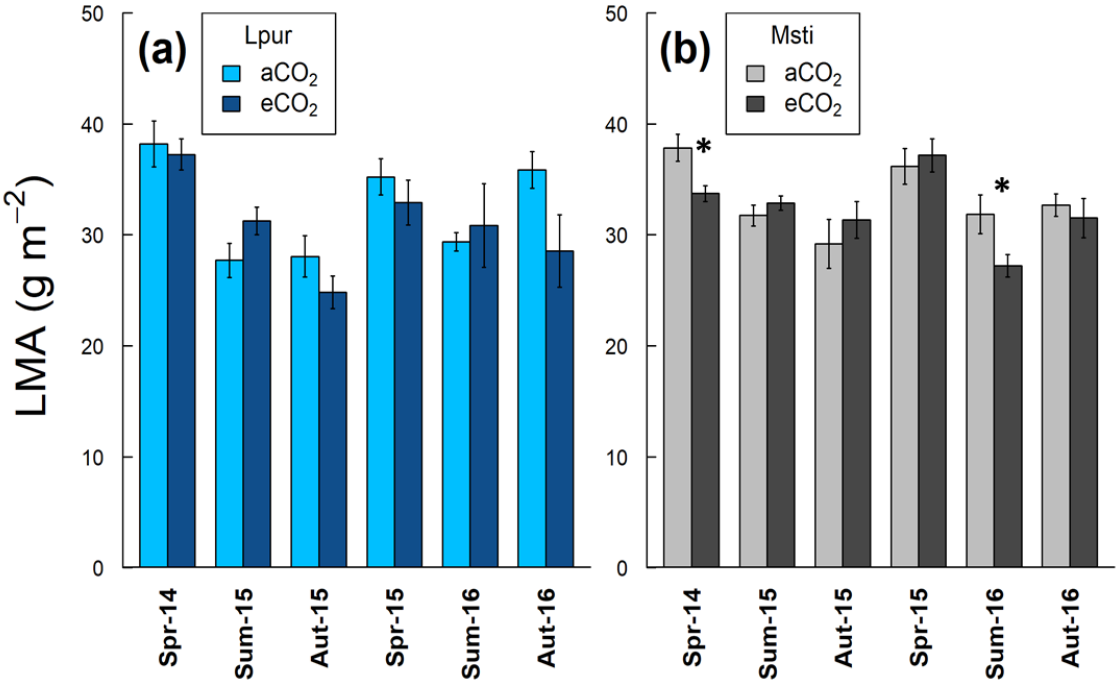

Fig. S 3.2 Time course through the six measurement seasons for leaf mass per area (LMA; g m^{-2}) as a function of CO_2 treatment.

Effects of eCO_2 on LMA are shown for (a) *L. purpurascens* (Lpur) and (b) *M. stipoides* (Msti) measured for six seasons. aCO_2 and eCO_2 in the legends denote ambient and elevated CO_2 treatments respectively. Significant differences at $P < 0.05$ are denoted by *.

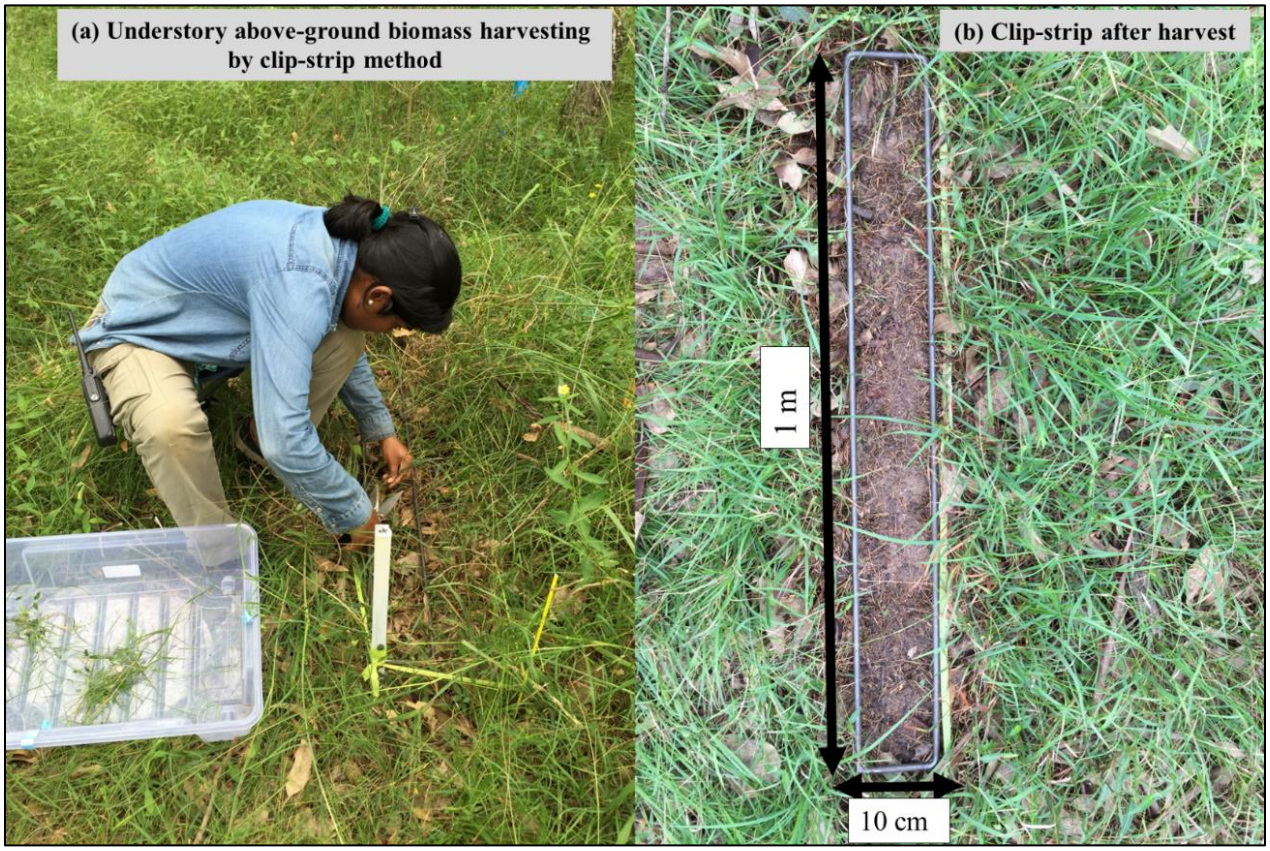

Image 3.1 Above-ground biomass harvest of understory species at EucFACE.

(a) The author carrying out above-ground biomass harvest of the understory species growing naturally at the EucFACE facility using the clip-strip method. (b) View of one clip-strip after the harvest is completed. Biomass harvest was carried out at four different locations in each CO_2 plot using a steel frame of 1m x 10 cm dimensions in order to maintain the accurate size of each strip. Photo courtesy: Mr Sachin Chavan.

Chapter 4 : Differential photosynthetic and biomass responses of C_3 grasses and C_3 forbs to elevated CO_2

4.1 Abstract

Understanding how a set of coexisting species, provided with similar resources for growth, may respond differently to eCO_2 is critical for predicting future ecosystem composition. I designed a glasshouse experiment to test whether the species or functional groups differed in their photosynthetic or biomass allocation and growth responses to eCO_2 for two C_3 forbs and two C_3 grasses. The four species were grown as monocultures under limited nutrient availability and unlimited water supply. C_3 forbs exhibited a strong down-regulation in photosynthetic capacity under eCO_2, for parameters like maximum carboxylation (V_{cmax}, -48%) and electron transport capacity (J_{max}, -41%), whereas grasses did not. Consequently, photosynthetic rates were markedly enhanced for C_3 grasses (+68%) in 570 ppm eCO_2, but not significantly enhanced for C_3 forbs. Photosynthetic down-regulation under eCO_2 in the C_3 forbs could not be attributed to decrease in N content (N_{area}), since N_{area} was maintained under eCO_2 in the C_3 forbs but decreased in C_3 grasses. Average N_{area} was lower in the C_3 forbs (0.70 g m^{-2}) compared to the C_3 grasses (1.05 g m^{-2}). Also, apparent fraction of N allocated to Rubisco enzyme ($f_{N\text{-}Rubisco}$) under eCO_2 decreased in the C_3 forbs (-50%) but was maintained in the C_3 grasses. Above differences in average leaf N content and allocation of N to portions of the photosynthetic apparatus might be responsible for differences in CO_2 responsiveness in the C_3 forbs and C_3 grasses. C_3 forbs also differed from the C_3 grasses in terms of above-ground biomass allocation responses to eCO_2, as leaf area ratio decreased significantly under eCO_2 in the C_3 forbs (-48%), but increased in the C_3 grasses (+80%). Total biomass remained unchanged under eCO_2 in the C_3 grasses, but decreased significantly in the C_3 forbs (-37%). Differences in photosynthesis and biomass allocation responses to eCO_2 between the C_3 forbs and C_3 grasses suggest that the grasses might obtain greater dominance in C_3-dominated herbaceous ecosystems under eCO_2.

4.2 Introduction

A well-documented effect of ongoing rise in atmospheric CO_2 levels is an increase in leaf net photosynthetic rates (Ainsworth & Rogers, 2007, Leakey *et al.*, 2009, Long *et al.*, 2004). Since the C_3 photosynthetic pathway is CO_2-limited at current atmospheric [CO_2], C_3 plants have been expected to photosynthesize at higher rates under elevated CO_2 (eCO_2) because of increase in the carboxylation of Rubisco enzyme and decrease in the photorespiratory carbon loss (Drake *et al.*, 1997, Long *et al.*, 2004). However, the magnitude of photosynthetic stimulation observed under eCO_2 in the C_3 species does not always match theoretical expectations (Ainsworth & Rogers, 2007, Lee *et al.*, 2011, Nowak *et al.*, 2004). Whilst all C_3 plants contain essentially the same Rubisco enzyme which is highly conserved (Andersson & Backlund, 2008), species still vary significantly in their responses to eCO_2 (Ainsworth & Rogers, 2007, Lee *et al.*, 2001, Reich *et al.*, 2004). Considering the wide variety of plant species, some kind of grouping based on plant traits has been viewed essential for generalizing about their responses to eCO_2 (Poorter & Navas, 2003, Reich *et al.*, 2001). In accordance with this, different types of plant species have been grouped into broad functional groups depending on the key structural and functional traits like photosynthetic rates, specific leaf area, root foraging capacities, plant nutrient content and ability to fix atmospheric N (Lavorel *et al.*, 1997, Reich *et al.*, 2001). These traits are also central to how different species respond to rising atmospheric CO_2 (Poorter & Navas, 2003, Woodward & Cramer, 1996), because they influence the carbon acquisition and storage capacities, water uptake and use, nutrient acquisition and allocation patterns and plant relative growth rates (Adler *et al.*, 2014, Ali *et al.*, 2013). Consequently, functional groups have been used for capturing the aggregated responses of different types of species to eCO_2 by both empirical and modelling studies (Crous *et al.*, 2010, Poorter & Navas, 2003, Wullschleger *et al.*, 2014, but see Hovenden & Williams, 2010, Lee *et al.*, 2011).

Theory and empirical evidence suggest that enhancement of plant photosynthesis and growth under eCO_2 will be sustained only under sufficient nutrient availability (Rastetter & Shaver, 1992, Reich *et al.*, 2006a). For example, in contrast to high nutrient availability, low nutrient availability may result in down-regulation of photosynthetic capacity, lower

stimulation of photosynthetic rates (Ainsworth & Rogers, 2007, Crous *et al.*, 2010, Ellsworth *et al.*, 2004), lack of an increase in biomass and altered above and below ground biomass allocation in C_3 plants growing under eCO_2 (Inauen *et al.*, 2012, Reich *et al.*, 2006a). However, these responses do not typify all plant functional groups, because even plants growing under similar soil nutrient availability have been reported to differ in their responses to eCO_2, possibly due to differences in nutrient acquisition and allocation capacities (Ainsworth *et al.*, 2003b, Crous *et al.*, 2010, Ellsworth *et al.*, 2004). In a study conducted on trees growing in N-limited conditions, Ellsworth *et al*., 2012 reported photosynthetic down-regulation under eCO_2 in *Pinus taeda*, but not in the co-occurring deciduous species which they attributed to different strategies for acquiring and allocating N to photosynthesis. Furthermore, some studies on herbaceous species suggest that the photosynthesis and biomass responses of C_3 forbs may be more sensitive to eCO_2 compared to grasses (Polley *et al.*, 2012b, Polley *et al.*, 2003, Teyssonneyre *et al.*, 2002). For instance, growth at eCO_2 increased total biomass by 31% in C_3 forbs, but only by 9% in C_3 grasses and the response was independent of soil N supply (Reich *et al.*, 2001). Furthermore, eCO_2 coupled with infrequent cutting significantly increased the proportion of forbs and reduced that of grasses (Teyssonneyre *et al.*, 2002). In contrast to the positive CO_2 effects on C_3 forbs in some studies (Reich *et al.*, 2001, Teyssonneyre *et al.*, 2002), Crous *et al*., 2010 observed a strong down-regulation of photosynthetic capacity accompanied by lack of photosynthetic enhancement in response to eCO_2 in the C_3 forbs, but not in the C_3 grasses. How or why might C_3 forbs respond differently to eCO_2 than other functional groups like grasses? Examination of key structural and functional traits, associated with biomass allocation patterns and nutrient content and use capacities, shared by the species in functional groups may help us better understand the causes for these differential responses (Crous *et al.*, 2010, Poorter & Bongers, 2006).

The goal of my experiment was to examine how co-existing C_3 grasses and C_3 forbs, provided with similar water inputs and nutrient supply from the soil for growth, may respond differently to eCO_2. To address this goal, I examined the photosynthesis and biomass growth responses of two C_3 grasses and two C_3 forbs growing in a low-nutrient soil in an environmentally controlled glasshouse experiment. The C_3 species used in this study are ecologically important and common to the understory of a nutrient-limited

Eucalyptus woodland ecosystem (Tozer, 2003). The soil of this *Eucalyptus* woodland is P-limited (Crous *et al.*, 2015). But, since photosynthetic acclimation under eCO_2 in the C_3 species is often associated with changes in leaf N content and allocation patterns (Ellsworth *et al.*, 2004, Lee *et al.*, 2011, Long *et al.*, 2004), I hypothesized that (H1), photosynthetic capacity down-regulation under eCO_2 will be manifested as decrease in leaf N content and/or protein specific down-regulation of Rubisco enzyme. I further hypothesized that (H2), down-regulation of photosynthetic capacity under eCO_2 would result in little or no enhancement of photosynthetic rates and biomass in a nutrient poor soil. The species were grouped into C_3 grasses and C_3 forbs in order to assess whether these groupings represent species responses within their respective functional group. An examination of the species and functional group responses to eCO_2 under common resource availability and climate may improve our capacity to generalize herbaceous plant community responses to climate change (Poorter & Navas, 2003, Wullschleger *et al.*, 2014).

4.3 Material and Methods

4.3.1 Species under study and growth conditions

The glasshouse experiment was designed to simulate the growth conditions experienced by the herbaceous species growing in local Cumberland plain woodland as a major ecosystem type in the region. I selected four evergreen herbaceous species, two C_3 grasses- *M. stipoides*, *L. purpurascens* and two C_3 forbs- *S. madagascariensis* and *N. neesiana*, for this study (see Image 1.2 and section 1.8 of Chapter 1 for details). These four species occur in the understory of nearby CPW on a nutrient poor soil that is P-limited (Crous *et al.*, 2015). In the figures, the four species are denoted by the genus initial and the first three letters of the species name.

For the glasshouse experiment, seeds of three species were collected from a local patch of CPW, whereas *L. purpurascens* was propagated through cuttings collected from CPW. Due to the longer time required for establishment, cuttings of *L. purpurascens* were planted 20 days before seeds of other species were sown for germination. This facilitated simultaneous transplanting of 40 days old *L. purpurascens* cuttings and 20 days old seedlings of other species germinated from seeds. Seeds were sown in seedling trays containing seed germination mixture.. During the duration of experiment, all the seedlings were germinated and maintained in naturally lit glasshouse chambers using temperatures of 26 ^{o}C during the day (10 am to 4 pm) and 16 ^{o}C at night (8 pm to 6 am) with a step transition of 20 ^{o}C in between (see Fig. S4.2), similar to spring-time temperatures in the locality. After establishment, the seedlings were transplanted in 10 L cylindrical polyvinyl chloride pots in four glasshouse chambers located at the Western Sydney University, Richmond, NSW, Australia.

Out of the four chambers, two were set at ambient CO_2 (aCO_2) concentrations (400 µmol mol^{-1}) and other two at elevated CO_2 (eCO_2) concentrations (550 µmol mol^{-1}) during the duration of experiment (see Fig. S4.1a). The eCO_2 treatment represents the predicted atmospheric CO_2 concentrations by 2050 (IPCC, 2013) as well as the target eCO_2 concentrations at the EucFACE experiment (see Gimeno *et al*., 2016). The CO_2 concentration was continuously monitored and controlled (Argus Control Systems Ltd,

White Rock, BC1). Humidity levels in the chambers were maintained at 60 % during the day (8 am to 4 pm, see Fig. S4.1b) using a centrifugal humidifier (HumiDisk 65, Carel industries, Padova – Italy) . Average daily light levels in the glasshouse during the period from 10 am to 2 pm were ≈ 500 µmol quanta m^{-2} s^{-1} (see Fig. S4.1c) with highest intensity light levels (>1000 µmol quanta m^{-2} s^{-1}) lasting at least 15 min per day. As the experiment was conducted in autumn through winter months, additional light of about 200 µmol quanta m^{-2} s^{-1} was supplemented late during the day (from 3 pm to 5 pm) in order to extend day length.

The aim of current experiment was to study the photosynthetic and biomass responses to eCO_2 for some key C_3 grasses and C_3 forbs, on a low-nutrient soil. Given that the CPW tract near Richmond, NSW Australia (33° 37' S, 150° 44.3' E) was found to be P-limited (Crous *et al.*, 2015), I used the surface soil excavated from the top 30 cm layer in a remnant patch of CPW. I did not fertilize the soil during the whole duration of our experiment in order to maintain the nutrient-limited conditions. The soil was sieved to remove plant parts, homogenized and then filled into pots with a diameter of 15 cm and height of 40 cm. The pot size was assumed to be sufficient enough for the species in this study, as the fully-grown individuals of these species have relatively small dry mass (less than 4 g per individual). The weight of each pot was recorded and then 11 kg of air-dry soil was filled. Field capacity was identified to be 16.5% gravimetric soil moisture (G_{SWC}) which corresponded to 23.5 % volumetric soil water content (V_{SWC}) for this bulk density of soil. The field capacity of EucFACE soil (18 % V_{SWC}) was different from that of the soil used in glasshouse study (23.5 % V_{SWC}). This is because soil used in the glasshouse experiment was not excavated from EucFACE, but from an open area adjacent to EucFACE. Also, in the process of mixing and sieving soil for potting, its aggregation structure is destroyed, dead plant material is removed and soil in compacted into pots. These factors can lead to differences in field capacity between the EucFACE soil and the soil used in glasshouse. All the pots were watered to 92% field capacity (15.4% G_{SWC}) and then 20 day old seedlings of the three species and the cuttings of *L. purpurascens* were transplanted into the pots as monocultures of four individuals of same species in each pot. Measurements were conducted on the species individual growing in the center of the pot.

I also included a water treatment in the overall experiment though I selected the well-watered conditions here as there was no significant effect of water treatment in the experiment. During the first three weeks of the experiment, the pots were maintained at 15.4% G_{SWC} to ensure seedling establishment. Drought treatment was initiated at the end of third week following the procedure used in Djikstra *et al*., 2010 with some modifications. The drought treatment, not reported further in this manuscript, was initiated by discontinuing the watering until half of the pots in each chamber dried down to 9.12 % of G_{SWC} (≈13% of V_{SWC}), whereas the other half were maintained at 15.4% G_{SWC} (≈ 22 % of V_{SWC}). 15.4 % of G_{SWC} (Water sufficient/ WS) and 9.12 % of G_{SWC} (Water limited/ WL) corresponded to ≈ 92% and ≈ 55% of field capacity respectively. During the 10 week duration of experiment, the plants were subjected to water-limited conditions for seven weeks in total. A soil moisture probe was used to determine the V_{SWC} at biweekly intervals up to a depth of 20 cm (HydroSense II, Campbell Scientific). Across all the four glasshouse chambers, I had ten pots of each treatment combination (ten pots x four species x two CO_2 treatments x two water treatments = 160 pots). Pots were arranged in blocks with ten blocks per chamber (five WS plus five WL blocks) and each block consisting one replicate pot per species. To reduce chamber effects, I swapped the pots (while maintain the block arrangement) between the chambers with respective CO_2 treatments once in every 15 days during the whole duration of this experiment.

4.3.2 Gas exchange measurements

To assess the effects of eCO_2 on the photosynthetic parameters of the C_3 species growing in nutrient-limited soil, gas exchange measurements were conducted using the Li-6400 portable photosynthesis system (LI-6400XT, Li-Cor, Lincoln, USA) at the mean growth CO_2 concentration for each treatment (≈ 410 µmol mol^{-1} for aCO_2 and ≈ 560 µmol mol^{-1} for eCO_2). To determine the effects of eCO_2 on photosynthetic capacities of the species, photosynthetic CO_2 response curves (A_{net}-Ci curves) were measured. Gas-exchange measurements were carried out five days before the final harvest. Measurements were taken around mid-day (10:00-14:00) on top-most fully-expanded leaves. Multiple non-overlapping leaves were placed across the Li-COR chamber and a minimum time of 15-min was allowed for stabilisation of gas exchange before commencing measurements.

After stabilisation, an initial measurement of net CO_2 assimilation rate (A_{net}; µmol m^{-2} s^{-1}) was conducted at growth CO_2 concentration (≈ 400 µmol mol^{-1} for aCO_2 and ≈ 550 µmol mol^{-1} for eCO_2), followed by the A_{net}-C_i response curves. A_{net}-C_i response curves for the four species were done with ten different steps of CO_2 concentrations (40, 150, 210, 300, 420, 590, 1000, 1200, 1500 and 1800 µmol mol^{-1}) while maintaining saturating light conditions (photon flux density of 1500 µmol m^{-2} s^{-1}), 55 – 65 % relative humidity and leaf temperatures of 26 ^oC and allowing a stabilization time of two-three minutes after each step change in [CO_2]. During the A_{net}-C_i measurements, [CO_2] in the cuvette was controlled as reference. A minimum 3-5 replicate plants per treatment condition were measured. In total 36 A_{net}-C_i response curves were measured in three days using three Li-6400s at the rate of four A_{net}-C_i response curves per Li-6400 per day (see Chapter 2 for calibration details). 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, 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.

The A_{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_{cmax}; µmol m^{-2} s^{-1}) and electron transport (J_{max}; µmol m^{-2} s^{-1}) as outlined in Chapter 2 (Pathare *et al*., 2017). While deriving the rates of V_{cmax} and J_{max}, I used a fixed mesophyll conductance value of 0.2 mol m^{-2} s^{-1} bar $^{-1}$for the evergreen herbaceous species (Flexas *et al.*, 2008) to reflect the finite characteristics of this trait. The temperature responses of V_{cmax} and J_{max} were carried out previously in Chapter 2 (see section 2.6.4). The resulting kinetic constants derived by fitting the modified Arrhenius function for V_{cmax} were used in the fitacis function in plant ecophys package (Duursma, 2015). From V_{cmax} and corresponding leaf N content, I calculated the apparent fraction of N allocated to the active state Rubisco enzyme ($f_{N\text{-}Rubisco}$), assuming a composition of 16.67% N, eight active sites and a k_{cat} of 3.3 for the enzyme (Evans, 1989). $f_{N\text{-}Rubisco}$ indicates the carboxylation per unit leaf N and the possibility of protein specific down-regulation (Rogers & Ellsworth, 2002).4.3.3 Chemical and morphological traits

I harvested all the pots 70 days after transplanting. During the harvesting, each plant species was separated into leaves (green and senescent), stems and roots. Root system was carefully washed of soil particles. While doing so, roots were placed in a sieved tray (4 mm) to avoid loss of fine roots. Root system was over dried for two days at 60 ^{o}C. Green leaves were scanned using leaf area meter LI-3100 (Li-Cor, Lincoln, NE, USA) to determine the leaf area, oven dried for two days at 60 ^{o}C and weighed to obtain leaf area ratio (LAR). LAR ($cm^2\ g^{-1}$) was expressed as total leaf area divided by total plant biomass (Funk, 2008). Above-ground and below-ground samples, oven dried at 60 ^{o}C for 48 hours, were weighed to obtain root and shoot biomass which were expressed on g pot $^{-1}$ basis. Leaves used for gas exchange measurements were further processed for C, N and P content analyses. In particular, leaves were oven dried, finely ground and then processed for C and N content analyses using (Elementar Vario Micro CHNS analyser, Hanau, Germany). Green leaf N content was expressed on area basis as N_{area} ($g\ m^{-2}$). N_{area} was calculated as N_{mass} ($g\ g^{-1}$) x Leaf mass per area ($g\ m^{-2}$). Leaf P content was determined using an X-ray fluorescence spectrometer which works on the principle of excitation of inner orbital electrons by an X-ray radiation source (Reidinger *et al.*, 2012). Dried plant material was finely ground, pressed into pellets and analysed by exposing the pellets to X-rays for 30s. Certified reference materials from different plant species were used for calibration. Leaf P content was expressed on area basis as P_{area} ($g\ m^{-2}$).

4.3.4 Statistical analysis

Statistical analyses were performed using the R software (v3.2.2, R Foundation for Statistical Computing, Vienna, Austria). The experiment consisted of two aCO_2 chambers and two eCO_2 chambers and hence the true number of replicates was two for each of the two levels of CO_2 treatments. A split-plot ANOVA was performed with CO_2 treatment (aCO_2 vs. eCO_2) as whole-plot factor and plant species as a split-plot factor. CO_2 chamber number and block number were included in the random effects part of the model. 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. Linear mixed-effects models were used to test for the main effects of CO_2 and plant functional type (package nlme in R, Pinheiro *et al.*, 2016). For

all the parameters measured in this experiment, I used the Benjamini-Hochberg procedure for the number of ANOVA tests I did to control the false discovery rate (Benjamini & Hochberg, 1995). Based on this procedure, values of $P \leq 0.041$ were identified as critical. However, values of $P \leq 0.1$ were considered as marginally significant to avoid false negatives. Student's *t*-test was used for testing the effect of CO_2 treatment on measurement variables of individual species wherein $P < 0.05$ was considered as statistically significant. I expressed the effect of eCO_2 on photosynthetic variables of each species as percent change, calculated as CO_2 effect = [(mean at eCO_2-mean at aCO_2)/ (mean at aCO_2)] x 100.

Table 4.1 Results of mixed level split-plot ANOVA with CO_2 and plant species as main effects for net photosynthesis in respective growth CO_2 levels (A_{net}), net photosynthesis at a common CO_2 level ($A_{net\text{-}Ca}$), maximum carboxylation (V_{cmax}) and electron transport rates (J_{max}), leaf nutrient content on area basis (N_{area} and P_{area}), fraction of N allocated to Rubisco ($f_{N\text{-}Rubisco}$) morphological traits (total leaf dry weight, leaf area ratio and biomass).

Critical *P*-value identified through the Benjamini-Hochberg procedure is 0.04. Numerator and denominator degrees of freedom (df) are indicated in parenthesis next to the main and interaction effects.

Source of variation									
	CO_2			Species			CO_2 x Species		
Variable	df	*F*-value	*P*-value	df	*F*-value	*P*-value	df	*F*-value	*P*-value
A_{net}	1,2	1.33	0.368	3,11	11.13	**0.021**	3,11	25.6	**0.005**
g_s	1,2	5.49	0.144	3,11	36.92	**0.007**	3,11	14.1	**0.028**
C_i	1,2	55.02	**0.018**	3,11	20.04	**0.017**	3,11	5.5	0.097
$A_{net\text{-}Ca}$	1,2	4.20	0.177	3,11	8.83	**0.031**	3,11	22.1	**0.006**
$V_{cmax\text{-}25}$	1,2	4.27	0.175	3,11	6.79	0.048	3,11	19.8	**0.007**
$J_{max\text{-}25}$	1,2	0.65	0.504	3,11	6.88	0.047	3,11	20.9	**0.007**
N_{area}	1,2	0.4	0.583	3,11	15.4	**0.021**	3,11	11.8	**0.030**
$f_{N\text{-}Rubisco}$	1, 2	9.8	0.110	3,11	5.6	0.065	3,11	55.8	**0.001**
P_{area}	1,2	34.8	**0.028**	3,11	24.9	**0.013**	3,11	15.7	**0.024**
Total leaf biomass	1,2	2.14	0.281	3,11	30.84	**<0.001**	3,11	6.2	**0.009**
LAR	1,2	0.03	0.876	3,11	19.31	**<0.001**	3,11	13.9	**<0.001**
LMA	1,2	17.27	0.053	3,11	84.48	**<0.001**	3,11	3.6	**0.024**
Shoot biomass	1,2	3.31	0.210	3,11	136.32	**<0.001**	3,11	3.5	0.050
Root biomass	1,2	9.31	0.120	3,11	28.13	**<0.001**	3,11	11.2	**0.001**
Total biomass	1,2	12.17	0.073	3,11	114.70	**<0.001**	3,11	8.2	**0.003**

4.4 Results

4.4.1 Effects of CO_2 treatment on photosynthetic rates and stomatal conductance

The CO_2 enrichment of ambient + 150 µmol mol^{-1} did not have a significant overall effect on net photosynthetic rates across the functional groups ($P > 0.1$, Table 4.1). However, the species differed in their responses to eCO_2 for most of the variables associated with photosynthesis. The ANOVA indicated a significant CO_2 x species interaction effect on A_{net} ($P = 0.005$, Table 4.1). Photosynthetic rates increased significantly by 100% and 43% in *M. stipoides* and *N. neesiana* respectively (*t*-test, $P < 0.01$, Fig.4.1a), whereas decreased significantly by 56% (*t*-test, $P = 0.02$, Fig.4.1a) or remained unchanged for *L. purpurascens* and *S. madagascariensis*, respectively. There was a significant species effect ($P = 0.021$, Table 4.1) as greater average A_{net} values were reported for *N. neesiana* followed by *L. purpurascens*, *S. madagascariensis* and *M. stipoides*. The average values of A_{net} reported in the current study for *M. stipoides* and *L. purpurascens* (8.5 µmol m^{-2} s^{-1} and 12 µmol m^{-2} s^{-1} respectively) were comparable to the average values reported previously in the field study at the EucFACE (9.4 µmol m^{-2} s^{-1} and 12.5 µmol m^{-2} s^{-1} respectively; see Pathare *et al*. 2017 and Chapter 2). However, *S. madagascariensis*, showed lower average values of A_{net} under eCO_2 in the current study (10 µmol m^{-2} s^{-1}) compared to the field conditions (18.5 µmol m^{-2} s^{-1}; see Pathare *et al*. 2017 and Chapter 2). In summary, lack of eCO_2-induced photosynthetic enhancement in *L. purpurascens* and *S. madagascariensis* observed in this study was an indicative of the downward adjustment in photosynthetic capacities. Hence, I further analyzed the parameters associated with photosynthetic capacity for evidence of down-regulation under eCO_2.

Changes in stomatal conductance (g_s) in response to eCO_2 is an important factor affecting photosynthetic responses. Hence, I further analysed the effects of CO_2 treatment on g_s in the C_3 grasses and C_3 forbs (Fig. 4.1b). There was no overall CO_2 treatment effect on gs across the four species ($P = 0.144$, Table 4.1). However, there was a statistically significant CO_2 x species interaction effect on g_s ($P = 0.028$, Table 4.1). In particular, g_s decreased significantly under eCO_2, but only in *L. purpurascens* (-80%, t-test, $P < 0.001$, Fig. 4.1b), whereas, g_s increased significantly in M. stipoides under eCO_2 (+46%, t-test, $P < 0.001$, Fig. 4.1b) and remained unchanged in *N. neesiana* and *S. madagascariensis*

(Fig. 4.1b). Species also differed significantly in terms of g_s across the CO_2 treatments (*P* = 0.007, Table 4.1). Highest average g_s was reported for *N. neesiana*, followed by *S. madagascariensis*, *L. purpurascens* and *M. stipoides*. The volumetric soil water content did not respond to eCO_2 treatment (Fig. S4.1), thus suggesting a lack of soil water savings.

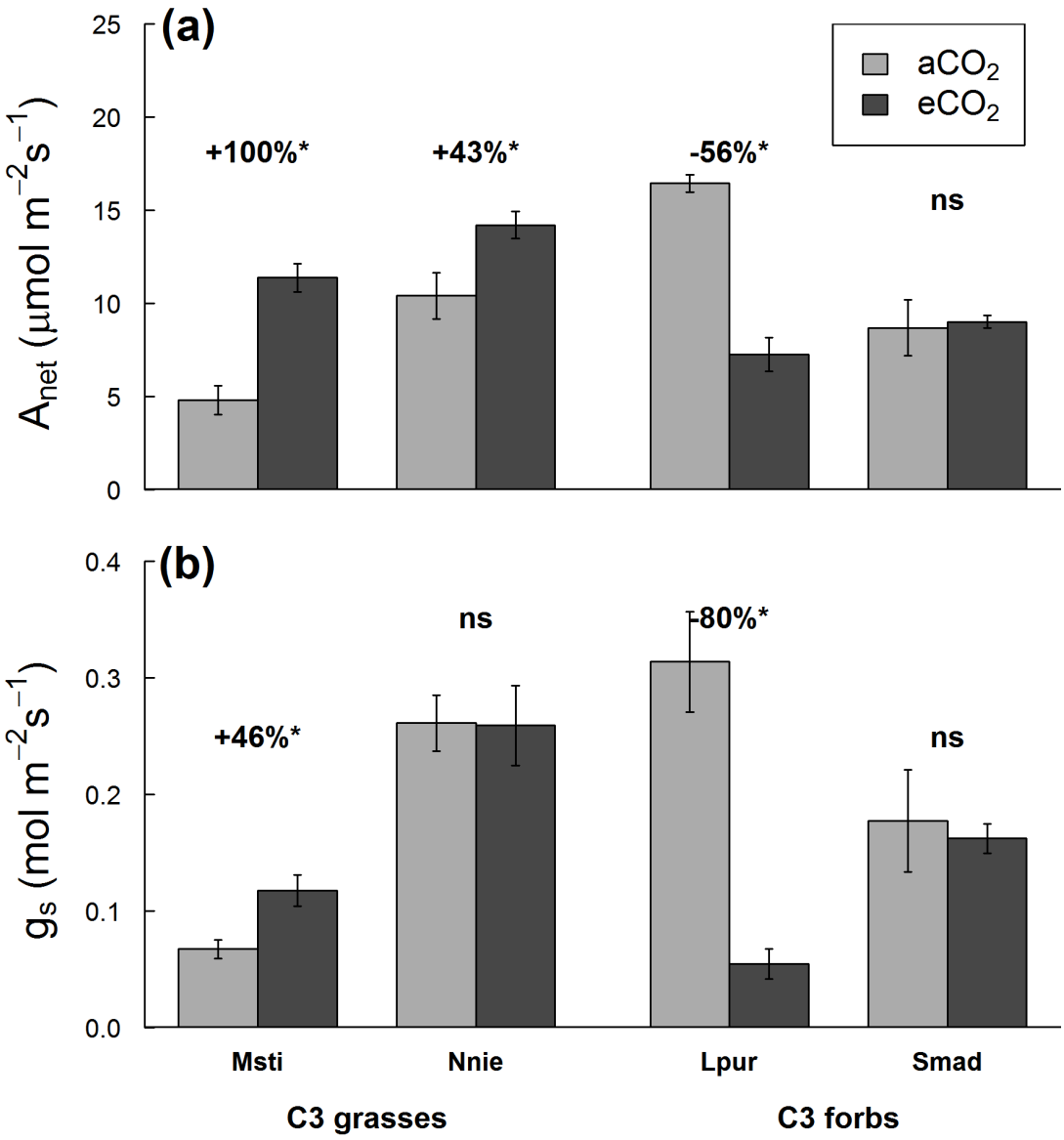

Fig. 4.1 Effects of CO_2 treatment on (a) net photosynthetic rates on area basis (A_{anet}) and (b) stomatal conductance (g_s) in two C_3 grasses (Msti and Nnie) and two C_3 forbs (Lpur and Smad).

Grey bars indicate ambient CO_2 and black bars indicate elevated CO_2. The percentages above a pair of columns denote changes with eCO_2. Within a species, differences in parameter between CO_2 treatments (paired *t*-test) are denoted by '*' when $P \leq 0.05$ and 'ns' when $P > 0.1$.

4.4.2 Effects of CO_2 treatment on photosynthetic capacity

Photosynthesis measured at common CO_2 levels ($A_{net\text{-}Ca}$) can be compared to test for changes in photosynthetic capacity in response to growth at eCO_2. The CO_2 treatment had no significant effect on $A_{net\text{-}Ca}$ (P = 0.17, Table 4.1), but showed a significant CO_2 x species interaction effect (P = 0.006, Table 4.1). $A_{net\text{-}Ca}$ increased significantly by 62% (*t*-test, P = 0.01, Fig.4.2a) and non-significantly by 18% (*t*-test, P > 0.1, Fig.4.2a) in *M. stipoides* and *N. neesiana* respectively. In case of the C_3 forbs, $A_{net\text{-}Ca}$ decreased by 69% in response to eCO_2 in *L. purpurascens* (*t*-test, P < 0.024, Fig.4.2a) and by 28% in *S. madagascariensis* (*t*-test, P < 0.03, Fig.4.2a).

The biochemical parameter, V_{cmax}, did not show a significant CO_2 treatment effect (P = 0.17, Table 4.1). However, there was a highly significant CO_2 x species interaction effect on V_{cmax} (P = 0.007, Table 4.1). V_{cmax} increased significantly by 37% under eCO_2 (*t*-test, P < 0.01, Fig.4.2b) in *M. stipoides* and remained unchanged in *N. neesiana*. In case of the two C_3 forbs, V_{cmax} decreased significantly by 57% and 33% under eCO_2 (*t*-test, P < 0.01, Fig. 4.2b) in *L. purpurascens* and *S. madagascariensis* respectively. Average values of V_{cmax} reported in the current study for *M. stipoides* and *L. purpurascens* (50 µmol m^{-2} s^{-1} and 60 µmol m^{-2} s^{-1} respectively) were comparable to the average values reported previously in the field study at the EucFACE (50.5 µmol m^{-2} s^{-1} and 67 µmol m^{-2} s^{-1} respectively; see Pathare *et al*. 2017 and Chapter 2). However, *S. madagascariensis*, showed lower average values of V_{cmax} under eCO_2 in the current study (35 µmol m^{-2} s^{-1}) compared to field conditions (100 µmol m^{-2} s^{-1}).

Similar responses to eCO_2 were observed for J_{max}. There was no overall CO_2 treatment effect on J_{max} across the functional groups (P =0.50, Table 4.1). However, there was a significant CO_2 x species interaction effect on J_{max} (P = 0.007, Table 4.1). J_{max} increased significantly by 58% under eCO_2 (*t*-test, P < 0.01, Fig. 4.2c) in *M. stipoides* and remained unchanged in *N. neesiana*. In case of the two C_3 forbs, V_{cmax} decreased significantly by 48% and 29% under eCO_2 (*t*-test, P < 0.02, Fig. 4.2c) in *L. purpurascens* and *S. madagascariensis* respectively. Average values of J_{max} reported in the current study for *M. stipoides* and *L. purpurascens* (80 µmol m^{-2} s^{-1} and 110 µmol m^{-2} s^{-1} respectively) were comparable to the average values reported in the field study at the EucFACE (85 µmol m^{-}

^{2} s^{-1} and 100 µmol m^{-2} s^{-1} respectively; see Pathare *et al*. 2017 and Chapter 2). However, *S. madagascariensis*, showed lower average values of J_{max} under eCO_2 in the current study (75 µmol m^{-2} s^{-1}) compared to field conditions (110 µmol m^{-2} s^{-1}). In summary, in the current study measures of photosynthetic capacity, that is, $A_{net\text{-}Ca}$, V_{cmax} and J_{max}, were reduced significantly under eCO_2 but only in *L. purpurascens* and *S. madagascariensis*. There was a marginally significant species ($P < 0.1$, Table 4.1) on the variables associated with photosynthetic capacities as highest values for $A_{net\text{-}Ca}$, V_{cmax} and J_{max} were observed in *N. neesiana* followed by *L. purpurascens, M. stipoides* and *S. madagascariensis*. Furthermore, compared to field conditions, parameters associated with photosynthetic capacity (V_{cmax} and J_{max}) were lower in the glasshouse study especially for *S. madagascariensis*. This lower photosynthetic capacity in *S. madagascariensis* reported in the glasshouse study could be related to greater root restrictions in pots and greater competition for resources due to four *S. madagascariensis* individuals per pot.

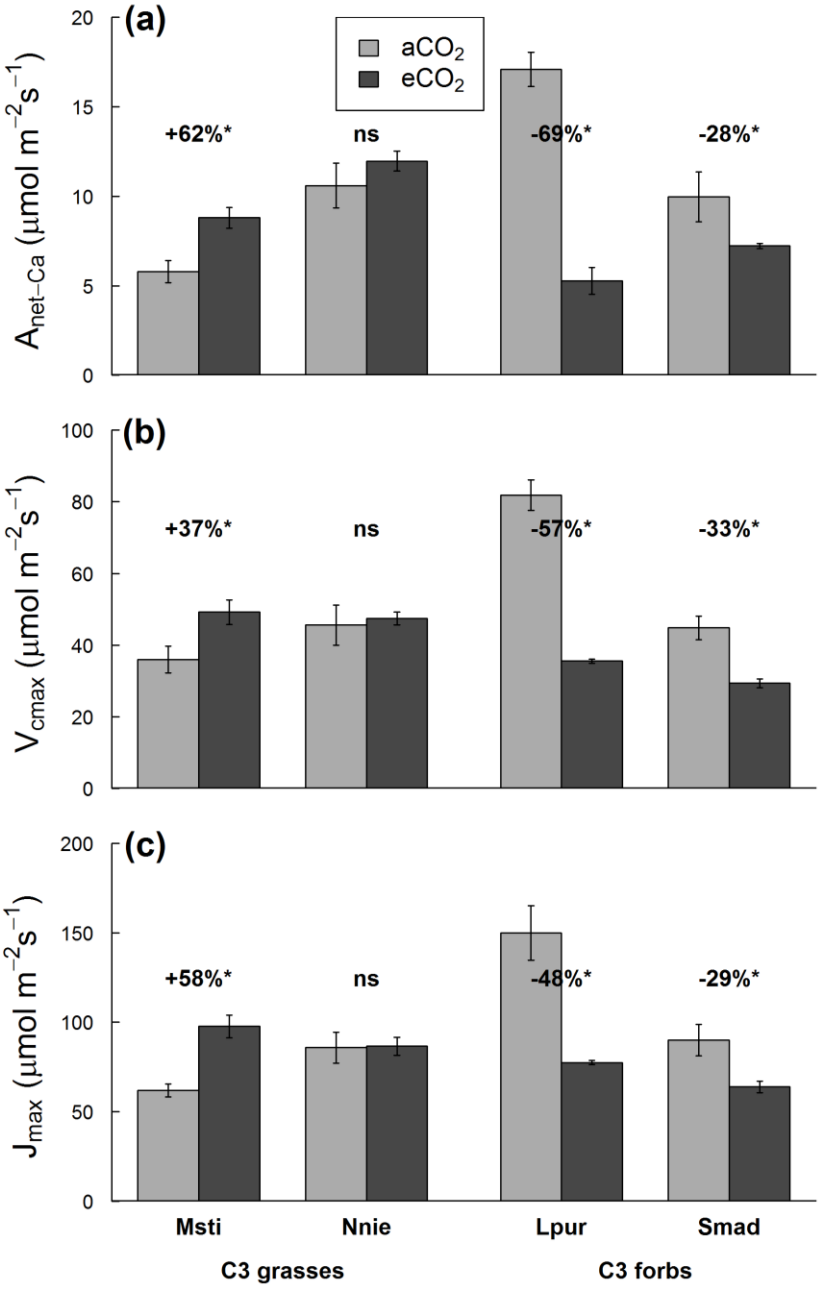

Fig. 4.2 Effects of eCO_2 on parameters associated with photosynthetic capacity.

Effects of CO_2 treatment on (a) net photosynthetic rates on area basis at common CO_2 levels ($A_{net\text{-}Ca}$), (b) maximum carboxylation rates (V_{cmax}) and (c) maximum electron transport rates (J_{max}) in C_3 grasses (Msti and Nnie) and C_3 forbs (Lpur and Smad). Grey bars indicate ambient CO_2 and black bars indicate elevated CO_2. The percentages above a pair of columns denote changes with eCO_2. Within a species, differences in parameter between CO_2 treatments (paired *t*-test) are denoted by '*' when $P \leq 0.05$ and 'ns' when $P > 0.1$.

4.4.3 Effects of CO_2 treatment on leaf N content,N allocation to Rubisco and leaf P content

To gain perspective on observed photosynthetic capacity responses to eCO_2, I examined the leaf N content and N allocation to Rubisco ($f_{N\text{-}Rubisco}$) in the four species (Fig. 4.3). Though there was no significant CO_2 treatment effect on N content (N_{area}) across the species (P = 0.58 0.1, Table 4.1), a significant CO_2 x species interaction effect was observed (P = 0.03, Table 4.1). There was 27% and 22% decrease in N_{area} under eCO_2 in *M. stipoides* and *N. neesiana* respectively (*t*-test, P < 0.02, Fig.4.3a), whereas N_{area} increased by 94 % in *L. purpurascens* (*t*-test, P < 0.01, Fig.4.3a) but remained unchanged in *S. madagascariensis*. Species also differed significantly in N content across the CO_2 treatments (P < 0.02, Table 4.1), with highest N_{area} observed in *N. neesiana* followed by *M. stipoides, L. purpurascens* and *S. madagascariensis* (Fig. 4.3a). Average values of N_{area} reported in the current study for *M. stipoides* (0.90 g m^{-2}) were comparable to the average values reported in the field study at the EucFACE (0.91 g m^{-2}; see Pathare *et al*. 2017 and Chapter 2). However, *L. purpurascens* and *S. madagascariensis*, showed lower average values of N_{area} under eCO_2 in the current study (0.75 g m^{-2} and 0.6 g m^{-2} respectively) compared to field conditions (1.0 g m^{-2} and 1.1 g m^{-2} respectively).

There was no overall CO_2 treatment effect on $f_{N\text{-}Rubisco}$ (P =0.11, Table 4.1, Fig.4.3b) across the species. However, we observed a highly significant CO_2 x species interaction effect on $f_{N\text{-}Rubisco}$ (P = 0.001, Table 4.1). In particular, $f_{N\text{-}Rubisco}$ increased by 89% in *M. stipoides* but remained unchanged in *N. neesiana*. In case of two C_3 forbs, $f_{N\text{-}Rubisco}$ decreased by 78% and 27% in *L. purpurascens* and *S. madagascariensis* respectively. Taken together, despite the significant decrease in N_{area} under eCO_2 in the two C_3 grasses, N allocation to Rubisco was maintained under eCO_2 conditions. This contrasts with the C_3 forbs, which showed decrease in allocation of N to Rubisco under eCO_2, despite the maintenance of total leaf N levels.

I further examined the effects of CO_2 treatment on leaf P content (P_{area}) for the four species. There was a statistically significant overall CO_2 treatment effect (P = 0.028, Table 4.1) and a CO_2 x species (P = 0.024, Table 4.1) effect on P_{area}. In particular, P_{area} decreased under eCO_2 by 69% and 62% in *M. stipoides* and *N. neesiana* respectively, but remained

unchanged in *L. purpurascens* and *S. madagascariensis* (Fig.4.4). Species also differed significantly in P_{area} across the CO_2 treatments. Average P_{area} was higher in the two grasses compared to the two forbs (Fig. 4.4).

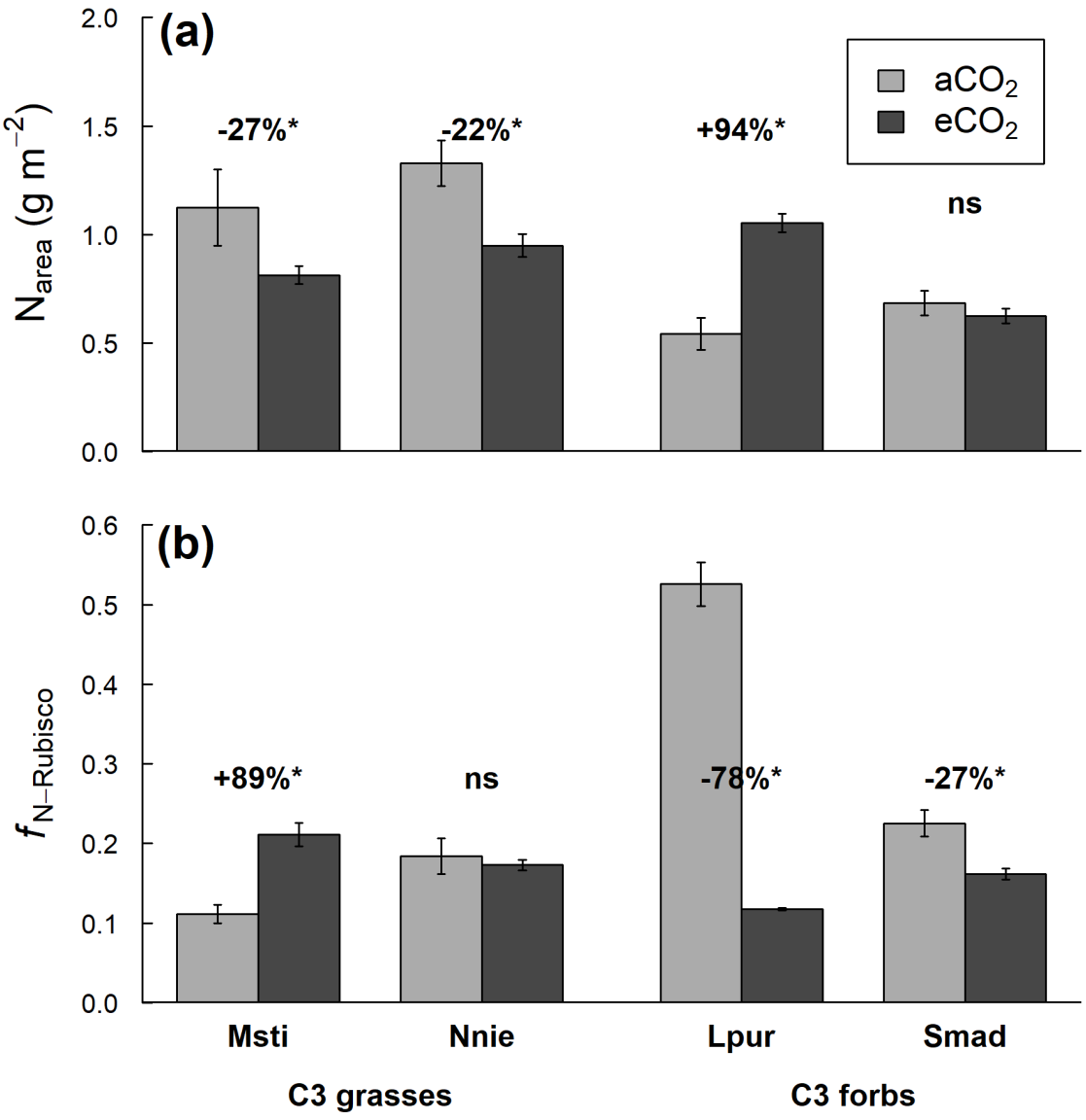

Fig. 4.3 Effects of CO_2 treatment on (a) N content on area basis (N_{area}) and (b) N allocation to Rubisco ($f_{N\text{-}Rubisco}$) in C_3 grasses (Msti and Nnie) and C_3 forbs (Lpur and Smad).

Grey bars indicate ambient CO_2 and black bars indicate elevated CO_2. The percentages above a pair of columns denote changes with eCO_2. Within a species, differences in parameter between CO_2 treatments (paired *t*-test) are denoted by '*' when $P \leq 0.05$ and 'ns' when $P > 0.1$.

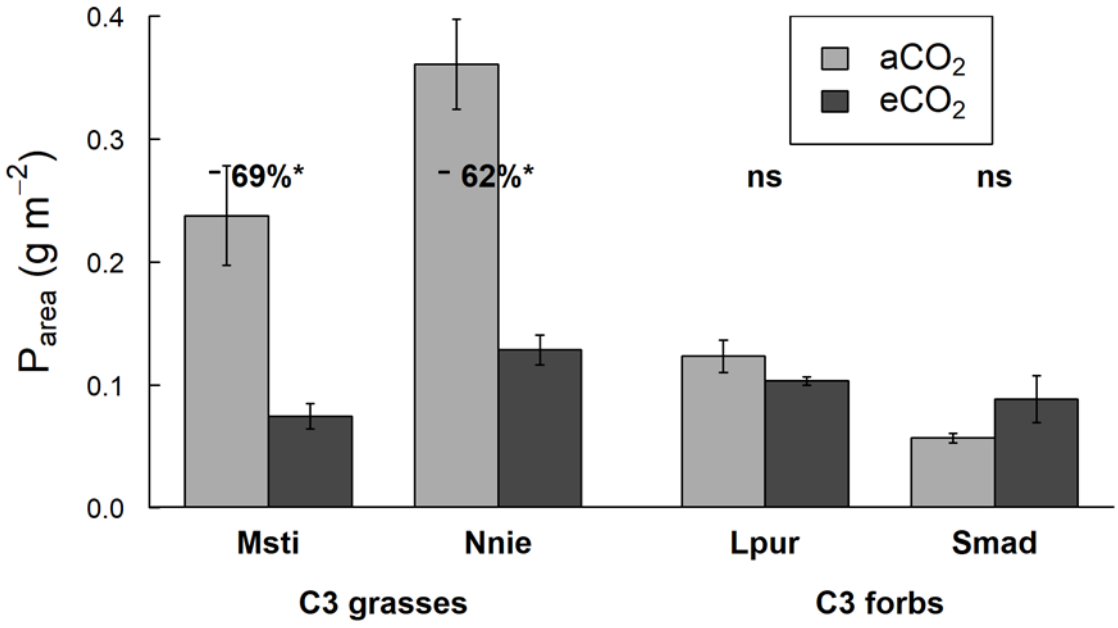

Fig. 4.4 Effects of CO_2 treatment on P content on area basis (P_{area}) in C_3 grasses (Msti and Nnie) and C_3 forbs (Lpur and Smad).

Grey bars indicate ambient CO_2 and black bars indicate elevated CO_2. The percentages above a pair of columns denote changes with eCO_2. Within a species, differences in parameter between CO_2 treatments (paired *t*-test) are denoted by '*' when $P \leq 0.05$ and 'ns' when $P > 0.1$.

4.4.4 Effects of eCO_2 on the morphological traits

We examined morphological traits associated with leaf area adjustments and biomass allocation patterns. A marginally significant CO_2 treatment effect was observed for LMA across the four species ($P = 0.053$, Table 4.1), whereas, a highly significant CO_2 x species interaction effect was observed for LMA ($P = 0.024$, Table 4.1). In particular, LMA decrease significantly under eCO_2 in *M. stipoides* (-33%), *N. neesiana* (-28%) and *L. purpurascens* (-20%), but remained unchanged in *S. madagascariensis* (Fig. 5a). Species also differed significantly in terms of LMA across the CO_2 treatments ($P < 0.001$, Table 4.1). Average LMA was highest in *N. neesiana* followed by *M. stipoides, S. madagascariensis* and *L. purpurascens*. I further analysed the responses of leaf area ratio and leaf biomass to CO_2 treatment. There was no overall CO_2 treatment effect on leaf area ratio and total leaf biomass across the species ($P > 0.1$, Table 4.1, Fig.4.5b, c). However, a significant CO_2 x species interaction effect was observed for both leaf area ratio and total leaf biomass ($P < 0.01$, Table 4.1). Leaf area ratio increased by 53% and 65% under eCO_2 in *M. stipoides* and *N. neesiana* respectively (*t*-test, $P < 0.03$, Fig. 4.5b), and decreased by 59% and 37% in *L. purpurascens* and *S. madagascariensis* respectively (*t*-test, $P < 0.02$, Fig. 4.5b). Total leaf biomass remained unchanged in response to CO_2 treatment in both the C_3 grasses (Fig. 4.5c). However, there was a significant decrease of 82% and 40% in total leaf biomass under eCO_2 in *L. purpurascens* and *S. madagascariensis* respectively (*t*-test, $P < 0.02$, Fig. 4.5c). Taken together, growth at eCO_2 resulted in significant decrease in leaf area ratio and total leaf biomass in the C_3 forbs, but not in the C_3 grasses.

The ANOVA did not indicate a significant CO_2 effect on shoot biomass and root biomass across the species ($P > 0.1$, Table 4.1). However, there was a marginally significant CO_2 x species interaction effect on shoot biomass ($P = 0.05$, Table 4.1), due to a decrease of 39% and 31% in shoot biomass under eCO_2 in *L. purpurascens* and *S. madagascariensis* respectively (Fig. 4.6a). Furthermore, there was a highly significant CO_2 x species interaction effect on root biomass ($P = 0.001$, Table 4.1), as root biomass decrease by 45% and 82% in *M. stipoides* and *L. purpurascens* respectively (Fig. 4.6b). There was a marginally significant CO_2 effect on total biomass ($P = 0.075$, Table 4.1), but a highly

significant CO_2 x species interaction effect (P = 0.003, Table 4.1). Total biomass decreased by 68% and 19% in *L. purpurascens* and *S. madagascariensis* respectively (Fig. 4.6c). Overall, growth at eCO_2 resulted in a significant decrease in total biomass in the C_3 forbs, whereas, total biomass of the two C_3 grasses remained unchanged.

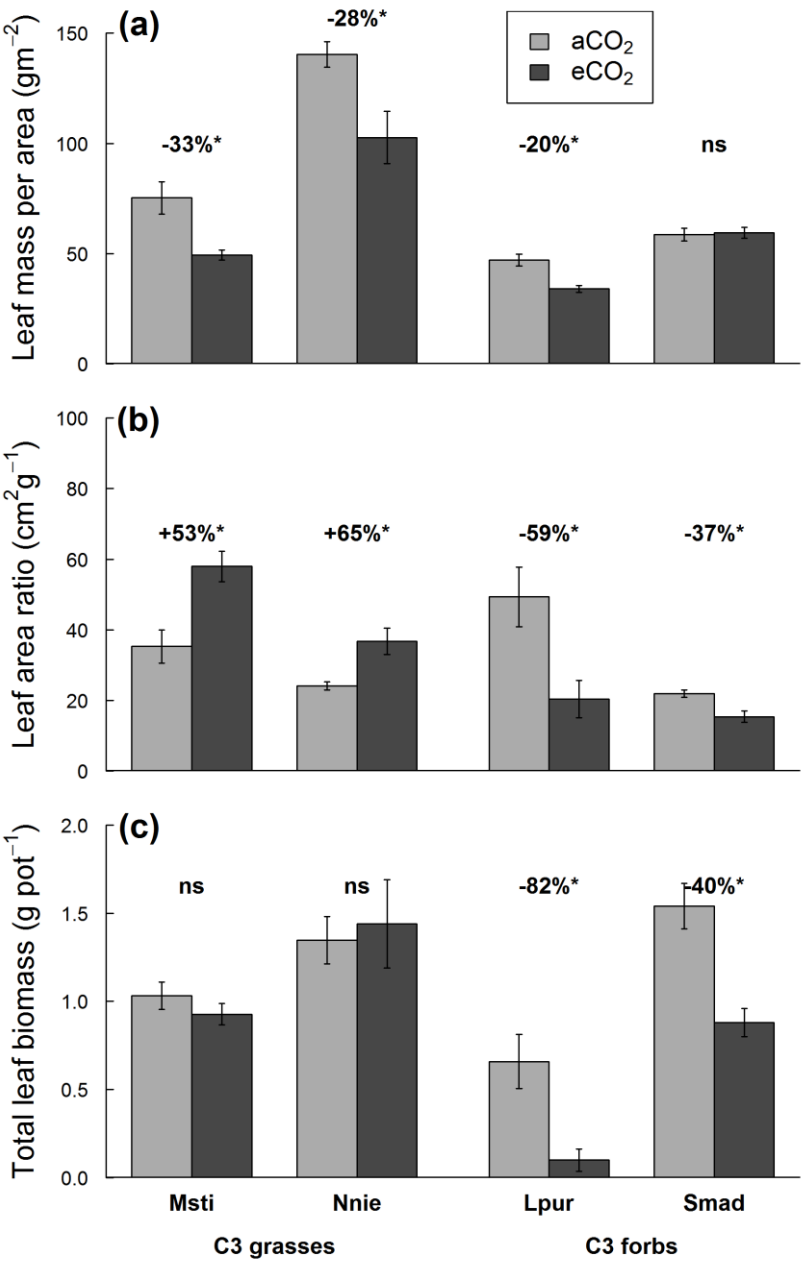

Fig. 4.5 Effects of CO_2 treatment on (a) leaf mass per area, (b) leaf area ratio and (c) total leaf dry biomass in C_3 grasses (Msti and Nnie) and C_3 forbs (Lpur and Smad).

Grey bars indicate ambient CO_2 and black bars indicate elevated CO_2. The percentages above a pair of columns denote changes with eCO_2. Within a species, differences in parameter between CO_2 treatments (paired *t*-test) are denoted by '*' when $P \leq 0.05$ and 'ns' when $P > 0.1$.

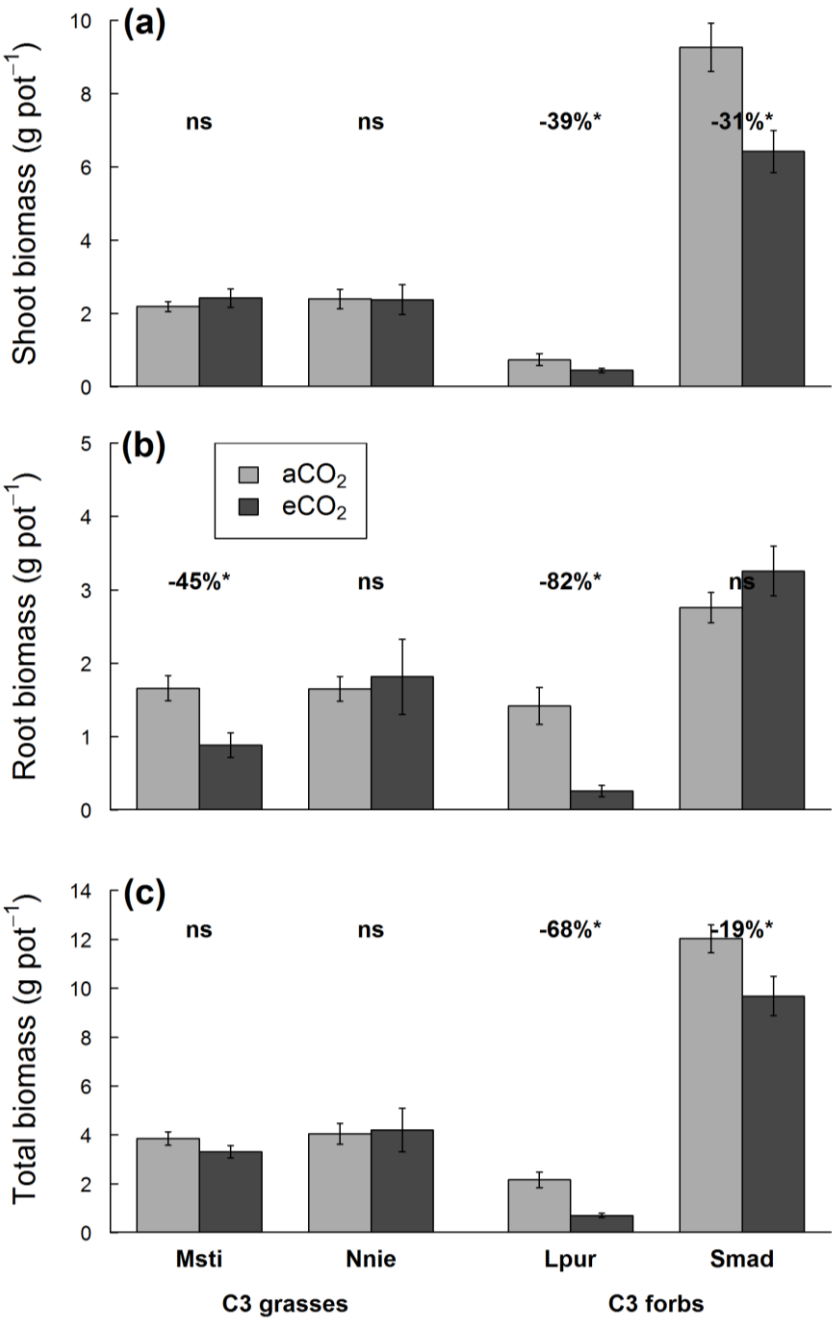

Fig. 4.6 Effects of CO_2 treatment on (a) shoot biomass, (b) root biomass and (c) total biomass in C_3 grasses (Msti and Nnie) and C_3 forbs (Lpur and Smad).

Grey bars indicate ambient CO_2 and black bars indicate elevated CO_2. The percentages above a pair of columns denote changes with eCO_2. Within a species, differences in parameter between CO_2 treatments (paired *t*-test) are denoted by '*' when $P \leq 0.05$ and 'ns' when $P > 0.1$.

4.5 Discussion

4.5.1 Photosynthetic capacity downregulation under eCO_2 is evident only in the C_3 forbs

Though eCO_2 is expected to increase photosynthetic rates in the C_3 species (Drake *et al.*, 1997, Long *et al.*, 2004), this expectation may not be always be realized even under similar resource supply due to differences in the nutrient acquisition and allocation capacities among the plant species (Ainsworth & Rogers, 2007, Ellsworth *et al.*, 2004, Lee *et al.*, 2011). In the current study, I observed strong differences in photosynthetic and biomass allocation responses to eCO_2 among four C_3 species growing under similar soil nutrients and unlimited water availability. Variables associated with photosynthetic capacity, stomatal conductance, $A_{net\text{-}Ca}$, V_{cmax} and J_{max}, showed a significant increase under eCO_2 in *M stipoides* and remained unchanged in *N. neesiana* (Fig. 4.2). This was accompanied by a significant stimulation of net photosynthetic rates under eCO_2 in the two C_3 grasses, that is, *M. stipoides* and *N. neesiana* (Fig.4.1). In contrast to the C_3 grasses, there was a significant decrease in $A_{net\text{-}Ca}$, V_{cmax} and J_{max} under eCO_2 in the two C_3 forbs, that is, *L. purpurascens* and *S. madagascariensis* (Fig. 4.2). Furthermore, there was a significant decrease in g_s under eCO_2 only in *L. purpurascens* (Fig. 4.1b). Decrease in photosynthetic capacity under eCO_2 in *S. madagascariensis* correlated with the lack of stimulation in net photosynthetic rates under eCO_2 (Fig. 4.1). Whereas, for *L. purpurascens*, the decrease in photosynthetic rates under eCO_2 correlate with the decrease in g_s. Based on these evidences I conclude that my first hypothesis- nutrient limited conditions will result in a decrease in the photosynthetic capacity in plants growing under eCO_2- was partially supported. Photosynthetic capacity down-regulation under eCO_2 was observed only for the C_3 forbs. My second hypothesis-photosynthetic down-regulation would result in little or no enhancement of photosynthetic rates and biomass-was also partially supported. There was a lack of stimulation in photosynthetic rates under eCO_2 in the C_3 forbs, but not in the C_3 grasses. Also, total biomass of the two C_3 forbs decreased significantly under eCO_2, but remained unchanged in the C_3 grasses.

Though higher stimulation of photosynthetic rates and biomass under eCO_2 in the forbs, compared to the grasses, has been reported by earlier studies (Lee *et al.*, 2011, Reich *et al.*, 2001), some studies have found a significant photosynthetic capacity down-regulation

and lack of or even negative biomass responses under eCO_2 in the forbs (Ainsworth & Long, 2005, Crous *et al.*, 2010, Huxman & Smith, 2001, Inauen *et al.*, 2012). The results of my study are consistent with photosynthetic responses under eCO_2 reported for C_3 species from a nutrient-limited prairie grassland (Crous *et al.*, 2010) and for a C_3 grass and C_3 forbs from Mojave desert (Huxman & Smith, 2001) and with the biomass responses under eCO_2 reported for C_3 forbs from glacier fore-field (Inauen *et al.*, 2012). In particular, Crous *et al.*, (2010) observed a significant photosynthetic capacity down-regulation under eCO_2 in the C_3 forbs, but not in the C_3 grasses, even under N sufficient conditions. This down-regulation response in the C_3 forbs under eCO_2 was attributed to the differences in functional traits, especially, lower root foraging capacities resulting in lower leaf N content. In the current study, I further examined whether differences in key functional traits, like overall leaf N content and changes in leaf N content and allocation under eCO_2, were responsible for photosynthetic capacity adjustments observed in the C_3 forbs.

4.5.2 Possible reasons for photosynthetic capacity down-regulation in the C_3 forbs

Photosynthetic capacity down-regulation under eCO_2 has often been related to plant N status and reduction in leaf N concentrations and N assimilation capacity under eCO_2, since N-containing amines are required for synthesizing and maintaining photosynthetic proteins (Ainsworth & Rogers, 2007, Bloom *et al.*, 2010, Ellsworth *et al.*, 2004). Many previous studies on the herbaceous species have observed dilution of leaf N concentrations under eCO_2, which has been largely attributed to lower soil N availability coupled with increased plant N demands, higher leaf carbohydrate content and decreased N uptake capacities under eCO_2 (Ainsworth & Rogers, 2007, Crous *et al.*, 2010, Ellsworth *et al.*, 2004, Feng *et al.*, 2015). I investigated whether dilution of leaf N content under eCO_2 was responsible for the photosynthetic capacity adjustments observed in the C_3 forbs. The species differed significantly in terms of leaf N responses to eCO_2. In particular, leaf N content increased significantly in *L. purpurascens* and remain unchanged in *S. madagascariensis* (Fig. 4.3a). Whereas, leaf N content decreased significantly under eCO_2 in the two C_3 grasses (Fig.4.3a), without any evidence of photosynthetic capacity down-regulation (Fig. 4.2). Similar to leaf N content, leaf P content decreased

significantly under eCO_2 in the C_3 grasses, but remained unchanged in the two C_3 forbs (Fig. 4.4). Taken together, these results suggest that eCO_2-induced decrease in leaf N and P content was not the possible reason for photosynthetic capacity down-regulation under eCO_2 observed in the C_3 forbs. Also, despite the increase N_{area} under eCO_2 in *L. purpurascens* (+94%, Fig. 4.3), photosynthetic rates decreased significantly (-56%, Fig. 4.1a). This decrease in photosynthetic rates under eCO_2 in L. purourascens could be attributed to eCO_2-induced decrease in g_s (Fig. 4.1b). In terms of whether leaf nutrient status under eCO_2 was responsible for the photosynthetic capacity adjustments observed under eCO_2 in the C_3 forbs, but not grasses, a key finding is that leaf N per unit area was lower in forbs compared to grasses even though they were grown in the same planting medium (Fig. 4.3a). Furthermore, average leaf P content per unit area was also lower in both the C_3 forbs compared to the C_3 grasses (Fig.4.4). Lower overall leaf N and P content compared to the C_3 grasses, could be responsible for photosynthetic capacity adjustments observed under eCO_2 in the C_3 forbs.

Causes for differential photosynthetic responses to eCO_2 in the C_3 forbs and C_3 grasses may also include differences in N allocation patterns. In particular, protein specific down-regulation of the Rubisco enzyme under eCO_2 can result in significant adjustments in photosynthetic capacity (Rogers & Ellsworth, 2002). To test the possibility of a protein specific down-regulation, I examined the apparent fraction of N allocated to Rubisco ($f_{N\text{-}Rubisco}$). Despite increase in leaf N levels in *L. purpurascens* and maintenance of leaf N in *S. madagascariensis* under eCO_2, there was a significant decrease in the amount of N allocated to Rubisco under eCO_2 in both the C_3 forbs (Fig.4.3). In contrast to the C_3 forbs, leaf N content decreased under eCO_2 in the C_3 grasses (Fig.4.3). However, $f_{N\text{-}Rubisco}$ increased under eCO_2 in the C_3 grasses thus indicating that C_3 grasses were able to increase their allocation of N to photosynthetic capacity. Taken together, the evidence suggests that protein specific down-regulation of the Rubisco enzyme under eCO_2 was the possible reason for photosynthetic down-regulation observed in the two C_3 forbs. This decrease in $f_{N\text{-}Rubisco}$ may provide N that can be re-allocated towards other protein-requiring systems (Drake *et al.*, 1997, Sage, 1994).

4.5.3 Biomass and allocation responses to eCO_2

Previous studies have reported increased C_3 forb biomass in response to eCO_2 (Polley *et al.*, 2003, Reich *et al.*, 2001, Teyssonneyre *et al.*, 2002). However, lack of response (Dijkstra *et al.*, 2010, Polley *et al.*, 2012a) or even reduced biomass and relative abundance under eCO_2 in the forbs has also been reported (Niklaus & Körner, 2004, Zavaleta *et al.*, 2003). In the current study, the C_3 grasses and C_3 forbs varied significantly in overall biomass as well as biomass allocation responses to eCO_2. Specifically, leaf area ratio and leaf biomass decreased significantly under eCO_2 in the two C_3 forbs (Fig.4.5). In contrast, there was a significant decrease in leaf mass per area and increase in leaf area ratio under eCO_2 in the two C_3 grasses, which was accompanied by no change in the leaf biomass as well as total biomass (Fig.4.5 and Fig. 4.6). These results suggest that under eCO_2, adjustments in leaf area occur in the C_3 grasses which may help the plants in optimizing resource capture and use in response to changes in resource availability (Poorter *et al.*, 2012, Tilman & Wedin, 1991). In particular, decrease in LMA and increase in leaf area ratio without a corresponding increase in total leaf biomass indicates a decrease in leaf density or the production of thin leaves in the C_3 grasses under eCO_2. This decrease in LMA could also be responsible for decrease in leaf N as well as P content under eCO_2 especially in the two C_3 grasses (Fig. 4.3 and Fig. 4.4). Since, the Rubisco enzyme represents a larger fraction of leaf N in thin leaves (Hassiotou *et al.*, 2010, Poorter & Evans, 1998), the decrease in LMA under eCO_2 in the current study suggests a strategy of the C_3 grasses to allocate leaf N efficiently to photosynthesis under eCO_2. In accordance with this I observed higher $f_{N\text{-}Rubisco}$ under eCO_2 in the grasses, despite decreases in N_{area} (Fig.4.3). Taken together, results from the current study suggest that differences in $f_{N\text{-}Rubisco}$ responses to eCO_2 coupled with changes in above-ground biomass allocation patterns via leaf area adjustments likely affected the CO_2 responsiveness in these species, in particular ability to maintain $f_{N\text{-}Rubisco}$ and avoid down-regulation by the grasses but not by the forbs. These differences in N allocation patterns and leaf area adjustments among different species have important implications for nitrogen-use efficiency and species responses to eCO_2 in nutrient-limited sites (Ellsworth *et al.*, 2004).

Despite stimulation of photosynthetic rates and maintenance of photosynthetic capacity, there was no significant increase in total biomass in the C_3 grasses under eCO_2 (Fig. 4.6c). Such discrepancy between photosynthesis and biomass responses to eCO_2 has been observed previously for trees as well as herbaceous species (Ellsworth *et al.*, 2017, Norby *et al.*, 2010, Reich & Hobbie, 2013, Sigurdsson *et al.*, 2013) and could be attributed to increase in carbohydrate availability exceeding the plants' capability to utilise it due to nutrient and inherent growth limitations (Kirschbaum, 2011). In contrast to the C_3 grasses, total biomass decreased significantly under eCO_2 in the C_3 forbs (Fig. 4.6c) and was correlated with the lack of stimulation in photosynthetic rates (Fig. 4.1) and significant down-regulation of photosynthetic capacity (Fig. 4.2). Overall, results from the current study suggest that eCO_2 had a negative effect on biomass of the two C_3 forbs, but not grasses. A negative eCO_2 effect on biomass has rarely been reported, and if so, has been observed under low nutrient availability (Inauen *et al.*, 2012, Zavaleta *et al.*, 2003). Under low soil nutrient availability, plants exposed to eCO_2 may allocate more biomass to roots in order to increase the root foraging capacity (Sigurdsson *et al.*, 2001, Suter *et al.*, 2002). For instance, Inauen *et al*., 2012 observed a significant decrease in above-ground biomass under eCO_2 in glacier fore-field forb plants, which they indicated was a consequence of higher biomass partitioning to roots. In the current study, there was a significant decrease in shoot biomass under eCO_2 in both the C_3 forbs. However, I did not observe a concurrent increase in the root biomass under eCO_2 (Fig. 4.6b; Piñeiro *et al*., unpublished data). Thus, there was no evidence of biomass partitioning in favour of root growth under eCO_2 in the C_3 forbs in current study.

4.5.4 Conclusions

In summary, the main goal of my experiment was to examine the differential photosynthesis and biomass responses to eCO_2 in the dominant C_3 grasses and C_3 forbs growing under similar nutrient availability and unlimited water inputs. The results suggest that magnitude of eCO_2 effect on photosynthesis and hence biomass accumulation varied among the species. Photosynthetic capacity and total biomass decreased in the C_3 forbs under eCO_2, but were maintained in the C_3 grasses. Lower leaf N content and inability to maintain allocation of N to Rubisco may be responsible for decrease in photosynthetic

capacity and biomass in the two C_3 forbs under eCO_2, as was found by Crous *et al*. (2010) previously. Such differences in photosynthesis and biomass responses to eCO_2, between the C_3 grasses and C_3 forbs, may lead to less diverse herbaceous communities, possibly dominated by grasses.

4.6 Supplementary information

4.6.1 Supplementary figures

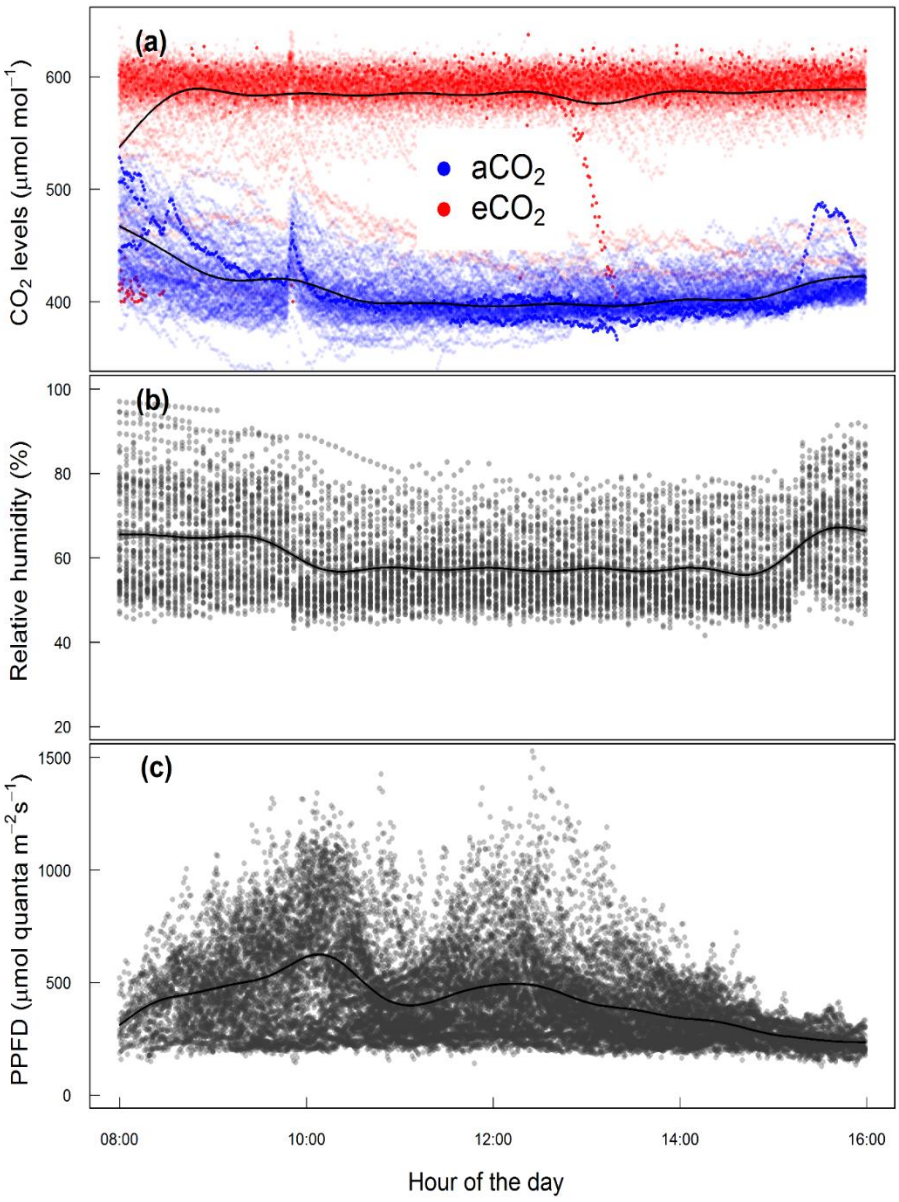

Fig. S 4.1 Glasshouse growth conditions for the daily time period from 8 am to 4 pm during the duration of experiment.

Panel (a) shows CO_2 levels under the ambient (aCO_2, blue dots) and elevated CO_2 (eCO_2, red dots) treatments; (b) shows relative humidity averaged across all the glasshouse chambers and (c) shows PPFD averaged across all the glasshouse chambers. Black solid lines indicate the gam fits with shaded confidence interval of 95%.

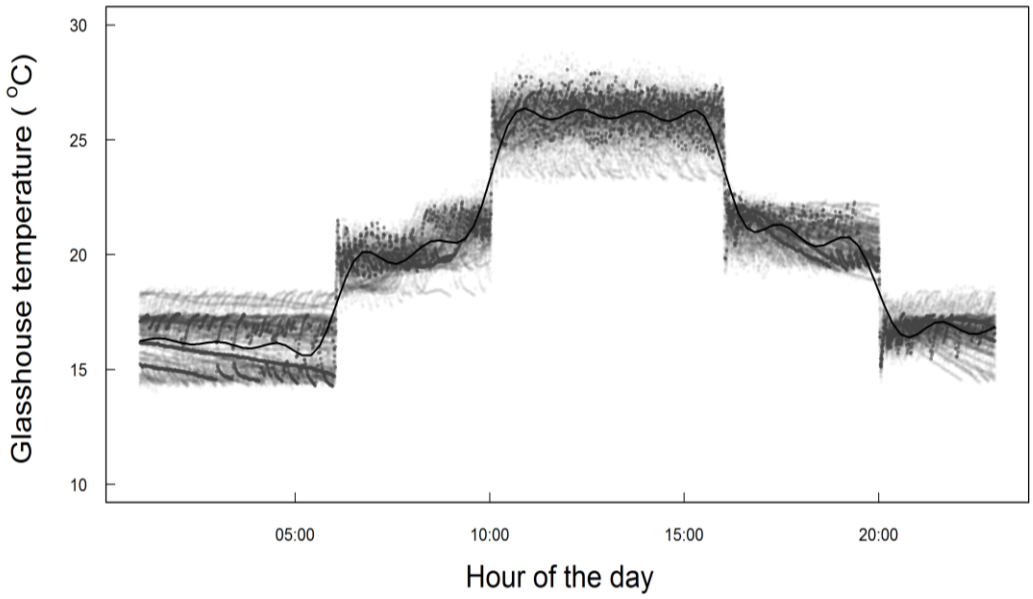

Fig. S 4.2 Daily glasshouse temperatures during the duration of experiment across all the four glasshouse chambers.

Black solid line indicates the gam fit with shaded confidence interval of 95%.

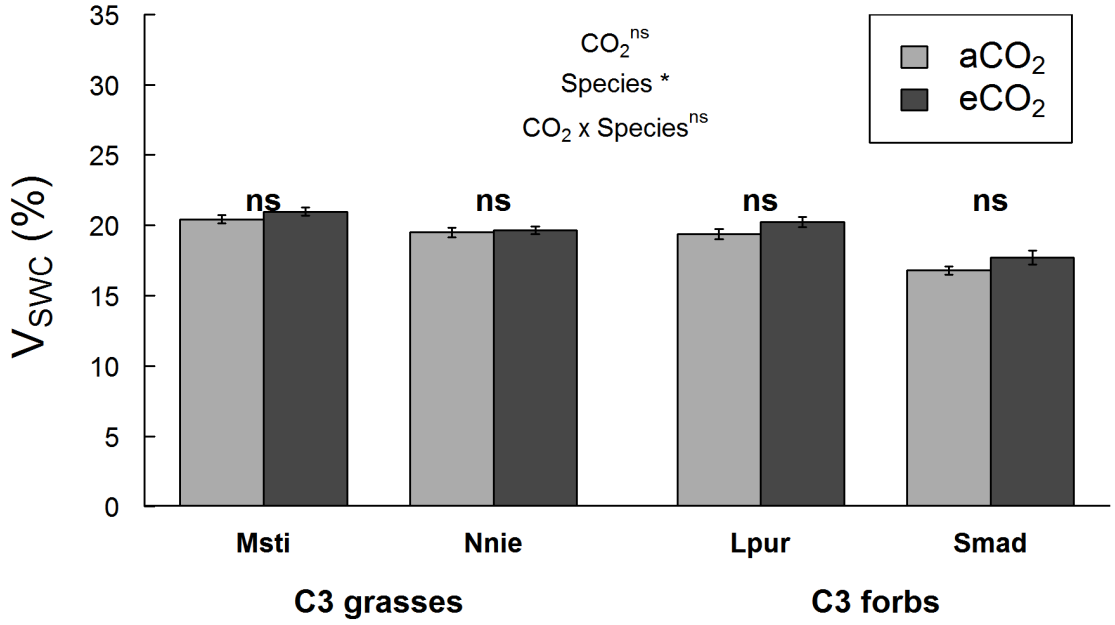

Fig. S 4.3 Effects of CO_2 treatment on volumetric soil water content (V_{SWC}) in C_3 grasses (Msti and Nnie) and C_3 forbs (Lpur and Smad).

Grey bars indicate ambient CO_2 and black bars indicate elevated CO_2. The percentages above a pair of columns denote changes with eCO_2. Results of split plot ANOVA with CO_2 and species as main effects are shown in the panel. Within a species, differences in parameter between CO_2 treatments (paired *t*-test) are denoted by '*' when $P \leq 0.05$ and 'ns' when $P > 0.1$.

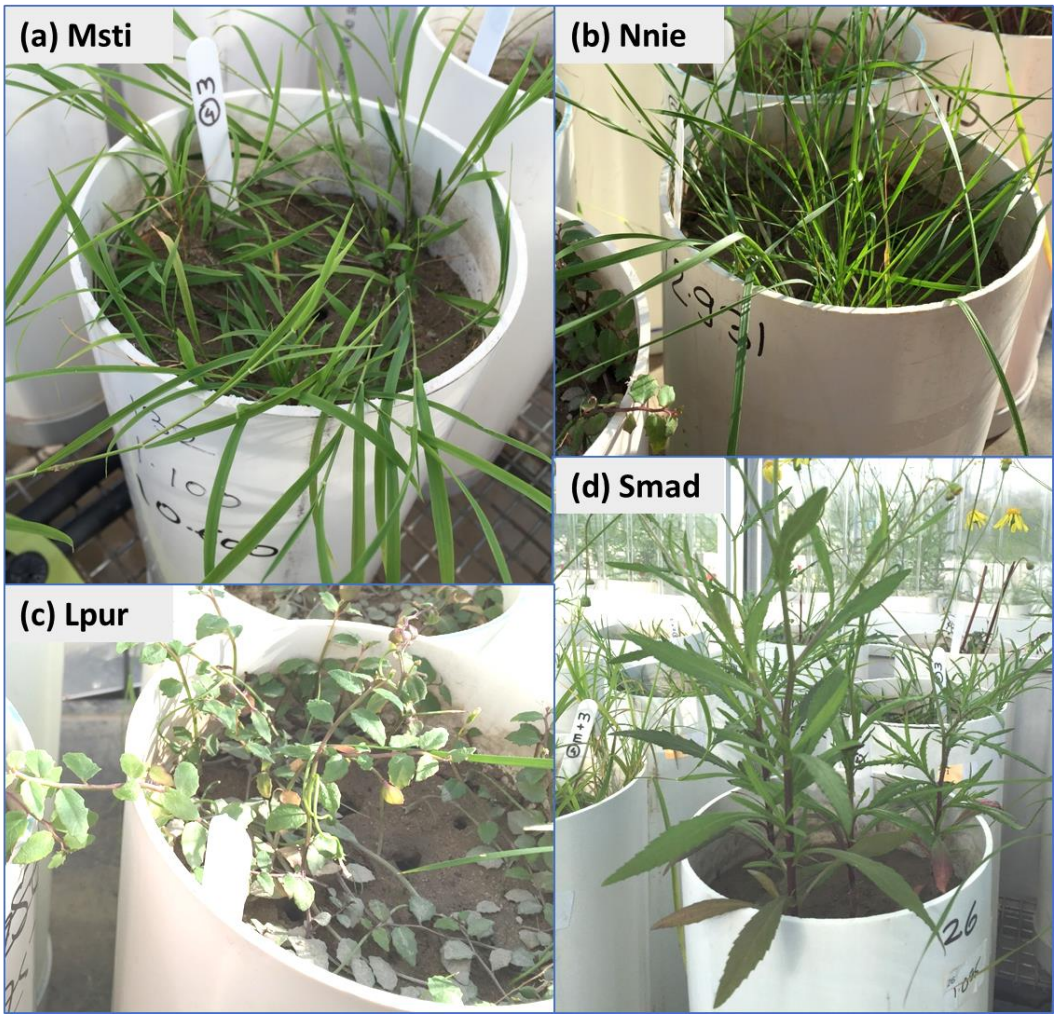

Image 4.1 Four herbaceous plant species growing in pots in the glasshouse during the current study.

(a) *Microlaena stipoides* Labill. (Msti) - a native C_3 grass (b) *Nasella neesiana* (Trin. & Rupr.) Barkworth (Nnie) - an invasive C_3 grass (c) *Lobelia purpurascens* R.Br (Lpur). - a native C_3 forb (d) *Senecio madagascariensis* Poir (Smad). - an invasive C_3 forb. Images were photographed by Ms. Varsha Pathare when the plants were 45 days old.

Chapter 5 : Synthesis

5.1 Background overview and project aims

Despite the large number of studies about photosynthetic and productivity responses to eCO_2, our ability to predict responses of terrestrial ecosystems to eCO_2 remains incomplete due to lack of studies in the warmer-climate ecosystems which includes the warm-temperate, sub-tropical and tropical ecosystems (Cernusak *et al.*, 2013, Leakey *et al.*, 2012). Consequently, expected impacts of eCO_2 on these warm ecosystems have been modelled or predicted based on findings from the well-studied cold temperate ecosystems (Hickler *et al.*, 2008, Leakey *et al.*, 2012, Norby *et al.*, 2016). The warmer ecosystems differ from the cold temperate ecosystems in important attributes beyond the obviously higher mean annual temperatures. These other differences include water availability, type of nutrient limitation and vegetation type, suggesting different responses to eCO_2 (Cernusak *et al.*, 2013, Hickler *et al.*, 2008). Furthermore, despite their importance for forest biodiversity and functioning, only few studies have addressed the eCO_2 effects on photosynthesis and biomass growth of the understory species and mostly focus on responses of the woody seedlings and/or shrubs (Dawes *et al.*, 2015, Hättenschwiler & Körner, 2000, Kim *et al.*, 2015), resulting in less knowledge about understory herbaceous species responses to eCO_2. The overall aim of this dissertation was to quantify the photosynthesis and productivity responses of an understory plant community from an ecosystem to eCO_2. In order to address this aim, I undertook a study of the evergreen C_3 herbaceous species from the understory of a *Eucalyptus* woodland. The climate of this grassy woodland ecosystem is warm-temperate to subtropical with a mean annual temperature of 17^{o}C and a mean daily maximum temperature of 30^{o}C during warmest month thus resulting in year round growth in the evergreen C_3 species. Furthermore, the woodland is characterised by seasonal water- limitation and low soil nutrient availability, particularly low P (Crous *et al.*, 2015). Consequently, this model grassy woodland ecosystem provided an opportunity to test the predictions of eCO_2 effects under water- and P-limited conditions. The three experimental chapters of this thesis (Chapter 2, 3 and 4) were designed to address the key predictions discussed in the following sections.

5.2 Key findings and general discussion

Detailed results and discussion of the findings have already been covered in each experimental chapter. In the following sections I highlight the key findings of each experimental chapter and discuss the implications of this study.

5.2.1 Elevated CO_2-induced A_{net} enhancement is a decreasing function of seasonal water availability

In Chapter 2, I investigated the effects of seasonal water availability on eCO_2-induced A_{net} enhancement in three C_3 herbaceous species growing at the EucFACE facility across the first three years of CO_2 fertilisation. Results demonstrate that eCO_2-induced A_{net} enhancement is a decreasing function of soil water availability, as highest proportional increase in A_{net} under eCO_2 was evident during the driest periods. Elevated CO_2 overcomes the higher stomatal limitations during water-limited periods by increasing C_i and thus leads to proportional increase in photosynthetic rates. Furthermore, eCO_2 did not result in decrease in g_s and increase in V_{SWC}, thus 'water-savings effect' of eCO_2 was absent in this warm water-limited ecosystem. As a result, 'water-savings effect' was not responsible for higher eCO_2-induced A_{net} enhancement observed during water-limited periods.

One of the most consistent responses of plant species to eCO_2 is a decrease in stomatal conductance (Ainsworth & Rogers, 2007, Xu *et al*., 2016). However, in contrast to this general trend, I did not observe a decrease in stomatal conductance for the herbaceous species in the current study. The mechanism underlying lack of stomatal response to eCO_2 still remains to be clarified further (Xu *et al*., 2016). Earlier studies (Jarvis & Davies 1998, Jarvis *et al*., 1999) suggest that stomatal conductance response to CO_2 is a function of the photosynthetic capacity of plants and how close the realized rates of photosynthesis are to this maximum photosynthetic capacity. That is, stomatal conductance may respond to eCO_2 when photosynthetic rates are closer to the photosynthetic capacity than when they are not. In addition to seasonal variation in water availability, the mean daily maximum temperature of the *Eucalyptus* woodland also varies (18, 22, 27 and 29 ^oC for winter, autumn, spring and summer respectively). The biochemical model of Farquhar *et al*.,

(1980), based on the kinetic properties of Rubisco, suggests that an increase in A_{net} with an increase in the [CO_2] will be greater at higher than lower temperatures. Hence, I also investigated whether seasonal variation in temperature affected the magnitude of eCO_2-induced A_{net} enhancement. Results from chapter 2 indicate that temperature was not a significant predictor of eCO_2-induced A_{net} enhancement (Chapter 2, Fig. S7).

5.2.2 Growth at eCO_2 causes down-regulation of photosynthetic capacity in the C_3 herbaceous species

Though eCO_2 results in significant stimulation of photosynthetic rates, lower or lack of stimulation of A_{net} during some time points observed in Chapter 2 (Pathare *et al.*, 2017) provides preliminary evidence for photosynthetic capacity down-regulation in these species. Also, the absence of an eCO_2 effect on g_s (Chapter 2) suggests that photosynthetic capacity down-regulation must instead be related to the biochemistry of photosynthesis. Accordingly, in Chapter 3, I investigated the seasonal effects of eCO_2 on photosynthetic capacity of two dominant C_3 species to determine if there was down-regulation and the possible mechanisms involved. The two species, including a dominant C_3 grass (*M. stipoides*) and a dominant C_3 forb (*L. purpurascens*), were measured for six seasons (two years over each spring, summer and autumn seasons) in the second and third years of CO_2 enrichment at EucFACE. Results from Chapter 3 demonstrate that eCO_2 elicits down-regulation of photosynthetic capacity in the dominant C_3 herbaceous species, especially during the peak growing season of spring. A decrease in V_{cmax} and J_{max} along with a lack of significant stimulation in A_{net} under eCO_2 was evident during one spring season in the C_3 grass and two spring seasons in the C_3 forb. For the summer and autumn periods, photosynthetic capacities of both the species were maintained under eCO_2 and there was an average 30% stimulation of A_{net} across the species.

Chapter 4 involved a glasshouse study designed to simulate the soil conditions at the EucFACE facility with well-watered conditions. In Chapter 4, I demonstrated that growth at eCO_2 significantly increases A_{net} in the C_3 grasses, but not C_3 forbs. Also, there was no 'water-savings effect' of eCO_2 in the glasshouse experiment (Fig. S4.3). The lack of significant stimulation of A_{net} under eCO_2 in the C_3 forbs coincided with biochemical indicators of photosynthetic capacity down-regulation. This down-regulation of

photosynthetic capacity under eCO_2 observed in C_3 forbs was a result of inability to maintain the fraction of N allocated to photosynthesis. In contrast to forbs, photosynthetic capacity, fraction of N allocated to Rubisco and stimulation of A_{net} under eCO_2 was maintained in the C_3 grasses. Overall, based on key findings from Chapter 3 and 4 I conclude that the down-regulation of photosynthetic capacity under eCO_2 occurs in the C_3 species from a grassy woodland, though the seasons or C_3 grasses and C_3 forbs may differ in this regard.

It has been expected that photosynthetic capacity down-regulation under eCO_2 will be greater in the low N conditions compared to the high N conditions (Long *et al.*, 2004, Moore *et al.*, 1999) and some evidences support these expectations (Ellsworth *et al.*, 2004). However, some studies conducted in the N sufficient ecosystems (Inauen *et al.*, 2012) or N fertilized conditions (Crous *et al.*, 2010, Lee *et al.*, 2011, Ruiz-Vera *et al.*, 2017) have also reported a down-regulation of photosynthetic capacity under eCO_2. The current study has been conducted in a relatively N sufficient ecosystem (Crous *et al.*, 2015) compared to the cold-temperate ecosystem (Schulze *et al.*, 1994) and there was evidence of photosynthetic acclimation. In the field experiment, photosynthetic down-regulation under eCO_2 was evident during the peak growing season of spring (Chapter 3), when photosynthetic acclimation under eCO_2 is less expected due to higher growth sink capacities of plants (Lewis *et al.*, 1996). Also, in the glasshouse study, photosynthetic down-regulation under eCO_2 was evident only in C_3 forbs but not in the C_3 grasses, in spite of being grown under similar supply of soil nutrients and water (Chapter 4). Taken together, findings from the current study support the previous reports of photosynthetic down-regulation under eCO_2 even under sufficient N supply (Inauen *et al.*, 2012, Lee *et al.*, 2011). The ability of plants to maintain biomass enhancement under eCO_2 largely depends on their ability to maintain photosynthetic capacities (Long *et al.*, 2004). If the photosynthetic capacity of plants is down-regulated under eCO_2, the ecosystem may become less responsive to eCO_2 and consequently sequester less C than it would without down-regulation (Luo *et al.*, 2003).

Although the results from current study suggest limited evidence for photosynthetic capacity adjustment with long-term eCO_2, the $[CO_2]$ of ambient +150 ppm (≈ 550 ppm)

was used to addresses the effects of eCO_2 on the understory herbaceous species. However, with the rates of CO_2 emissions steadily increasing, (Peters *et al*., 2012), values of CO_2 up to 1000 ppm have been considered as realistic experimental treatments for studying plant responses to higher [CO_2] (Franks *et al*., 2013). However, at [CO_2] greater than 550 ppm, most of the C_3 plant species will be CO_2 saturated and operating in the asymptotic part of the A_{net}-C_i response curve and hence will be largely limited by RuBP regeneration capacity. This could result in a different pattern of acclimation of photosynthetic capacity compared to that observed at ambient +150 ppm [CO_2]. Thus, any further increase in atmospheric [CO_2] (> 550 ppm) may not result in increases in photosynthetic rates. Furthermore, though rise in atmospheric [CO_2] may cause competitive inhibition of oxygenation of Rubisco, this may not always be beneficial to the plants in terms of increase in photosynthetic rates. For instance, recent studies suggest photorespiration is important for nitrate assimilation (Bloom *et al*., 2014) as well as P recycling in P-limited conditions (Ellsworth *et al*., 2015). Thus, increase in atmospheric [CO_2] (> 550 ppm) will result in decrease in photorespiration and may further exacerbate N and P-limitation of photosynthesis and growth.

5.2.3 Elevated CO_2 does not increase biomass of the understory herbaceous species component from a water -limited ecosystem

To assess the effects of eCO_2-induced A_{net} enhancement on biomass responses of the herbaceous species, I investigated the above-ground biomass of the total grasses and forbs in the EucFACE experiment (Chapter 3) and the total biomass of C_3 grasses and forbs in the glasshouse experiment (Chapter 4). Results from EucFACE facility (Chapter 3) demonstrated a lack of significant CO_2 effect on the above-ground biomass of the herbaceous species. The EucFACE provides direct field insights into the effects of eCO_2 on the above-ground biomass responses of herbaceous species growing in a warm-temperate ecosystem. However, being located in a natural and undisturbed ecosystem, it was difficult to isolate the root biomass of the dominant species. Consequently, a glasshouse study was undertaken to study the effects of eCO_2 on the root biomass response of the dominant species along with other photosynthetic and above-ground traits. Results like the EucFACE were reported for total biomass of herbaceous plants in the glasshouse

study (Chapter 4). Here total biomass of C_3 grasses remained unchanged under eCO_2, whilst there was a significant decrease in total biomass of C_3 forbs in eCO_2 relative to aCO_2 (Chapter 4). The glasshouse study provided some evidence that the below-ground biomass of the dominant herbaceous species from a warm-temperate ecosystem may not increase under eCO_2. Further work in the field is needed to corroborate this evidence.It has been expected that C_3 species growing in the warm, water-limited ecosystems will have a greater potential to respond positively to eCO_2 in terms of relative increase in biomass, compared to the species from cold temperate ecosystems (Morgan *et al.*, 2011, Hickler *et al.*, 2008, Morgan *et al.*, 2004, Long, 1991). However, results for the herbaceous species from a warm water-limited grassy woodland ecosystem in the current study contrast above expectations as there was a lack of relative biomass stimulation under eCO_2.

Lack of an eCO_2-induced biomass enhancement observed previously in the understory species has been attributed to increases in overstory biomass and leaf area leading to decreases in understory light availability (Bandeff *et al.*, 2006, Kim *et al.*, 2015). However, this could not be a possible explanation for lack of eCO_2-induced biomass enhancement in understory species in the current study, because eCO_2 did not cause increase in leaf area index or other components of above-ground biomass in the overstory trees at the site (Duursma *et al.*, 2016, Ellsworth *et al.*, 2017). Lack of a biomass response to eCO_2 in the current study could be explained by lack of eCO_2-induced 'water-savings effect' and low soil nutrient availability. Previous studies, both modelling and empirical, based on water-limited ecosystems suggest that, eCO_2-induced soil water savings can eliminate plant water limitation and enhance soil nutrient availability thus supporting relatively greater biomass under eCO_2 compared to aCO_2 (Fatichi *et al.*, 2014, Grünzweig & Körner, 2003, Morgan *et al.*, 2011). However, in the current study there was a lack of eCO_2-induced 'water-savings effect' which may have resulted in lack of relative increase in above-ground biomass (Chapter 3). Another possibility is that lack of biomass stimulation under eCO_2 is a consequence of limitation by some soil nutrient (Ellsworth *et al.*, 2017, Reich & Hobbie, 2013). Previous studies, particularly from the cold-temperate ecosystems, suggest N as an important nutrient affecting plant photosynthesis and biomass responses to eCO_2 (Ellsworth *et al.*, 2004, Reich *et al.*, 2006a). The grassy woodland

ecosystem in the current study is relatively N sufficient compared to the cold temperate ecosystems (Crous *et al.*, 2015, Schulze *et al.*, 1994). The soil of grassy woodland ecosystem in the current study is strongly P-limited (Crous *et al.*, 2015). Earlier studies indicate the potential for limited photosynthesis and productivity responses to eCO_2 under low P availability (Cernusak *et al.*, 2013, Ellsworth *et al.*, 2017). Also, the average N: P ratio of ≈ 30 reported for the dominant understory species in the current study indicates that these species are P-limited (Güsewell, 2004). Regardless of the exact cause, lack of relative increase in biomass under eCO_2 observed for the understory herbaceous species from a warm, water-limited woodland, suggests a limited capacity of these species to respond to eCO_2. As root biomass data for the herbaceous understory species at EucFACE is not available for the time points when above-ground biomass harvest was performed, I do not know if below-ground productivity of the understory species was influenced by eCO_2-induced photosynthetic enhancement. However, lack of root biomass stimulation under eCO_2 observed in the glasshouse experiment (Chapter 4) provides some evidence about the possible lack of root biomass stimulation due to eCO_2 at EucFACE as well.

Stimulation of photosynthesis under eCO_2 does not always translate into stimulation of plant growth and biomass (Ellsworth *et al.*, 2017, Norby *et al.*, 2010, Reich & Hobbie, 2013, Sigurdsson *et al.*, 2013). In my study, though the magnitude of eCO_2-induced A_{net} enhancement varied with time points (Chapter 2 and 3) and species (Chapter 3 and 4), the overall trend was towards higher photosynthetic rates under eCO_2 across the dominant C_3 herbaceous species. However, I did not observe relative stimulation of understory above-ground biomass (Chapter 3) as well as total biomass (Chapter 4) under eCO_2. These findings about lack of significant biomass stimulation under eCO_2, despite evidence of increases in photosynthetic rates, lead to the question about the fate of extra carbon assimilated under eCO_2 (Fatichi & Leuzinger, 2013, Fatichi *et al.*, 2014). Some of the possible pathways for extra C assimilated under eCO_2 include: increased plant and soil respiration (Adair *et al.*, 2011, Drake *et al.*, 2016), increased root exudates and export to mycorrhiza and other microbes (Cheng *et al.*, 2012, Phillips *et al.*, 2011, Phillips *et al.*, 2012) and increased root growth (Inauen *et al.*, 2012, Nie *et al.*, 2013).

5.2.4 Differences and similarities in field and glasshouse results

Two field experiments and one glasshouse experiment were conducted in the current study. Results from the field experiments varied from those of the glasshouse experiment for many measured parameters, particularly for the dominant species, *M. stipoides* and *L. purpurascens*. For instance, stomatal conductance for both the dominant species did not respond to eCO_2 in the field (Fig. 2.1). However, in the glasshouse experiment, stomatal conductance increased significantly under eCO_2 in *M. stipoides* but decrease in *L. purpurascens* (Fig. 4.1). Furthermore, at the field level there was an overall increase in photosynthetic rates under eCO_2 across the seasons in *L. purpurascens* (Fig. 2.1). Photosynthetic rates of *L. purpurascens* did not increase in response to eCO_2 in the glasshouse experiment (Fig. 4.6). Leaf N content showed significant seasonal variation in response to eCO_2 in the field for both the species (Fig. 3.3 and 3.5). Leaf P content did not respond to eCO_2 in both the species (Fig. 3.5). However, in the glasshouse experiment, leaf N and P content decreased under eCO_2 in *M. stipoides* (Fig. 4.3 and 4.4), whereas, for *L. purpurascens* leaf N increased and leaf P remained unchanged in response to eCO_2. Similar to leaf N, $f_{N\text{-}Rubisco}$ showed seasonal variation in responses to eCO_2 for both the species (Fig. 3.4). Whereas, in the glasshouse experiment $f_{N\text{-}Rubisco}$ increased in *M. stipoides* and decreased *in L. purpurascens* (Fig. 4.3). Furthermore, total above-ground biomass for grasses and forbs did not respond to eCO_2 in the field (Fig. 3.7). In the glasshouse, total biomass of *M. stipoides* remained unchanged under eCO_2, but decreased significantly in *L. purpurascens*. In sum, it can be difficult to replicate field conditions in the glasshouse, and hence difficult to compare results from field experiments to those obtained from glasshouse study, as in the latter case complex plant-environment interactions are eliminated. The glasshouse study was conducted only for one season with plants growing as monocultures under unlimited water supply, in contrast to the field study where water availability, light, temperature and competition for resources varied seasonally. Hence, plant-soil equilibrium over decades of growth in the field may not be well-replicated in the glasshouse and as a result the nutritional status of *M. stipoides* and its eCO_2 response was not matched in the glasshouse relative to the field site. Despite the differences between the field and glasshouse study, some common and important findings

from both the studies are- lack of 'water-savings effect' of eCO_2 and no stimulation of biomass of the herbaceous species despite significant increases in photosynthetic rates.

5.3 Implications of the current study for the Australian ecosystems

An important goal of this thesis was to contribute towards the understanding of eCO_2 effects on the understory herbaceous species of grassy woodlands, which are important ecosystems that occupy a large area in Australia (Australian Government Department of Agriculture, Fisheries and Forestry, 2012). Native Australian species have not been extensively investigated in eCO_2 (Hovenden and Williams, 2010). A characteristic feature of the grassy woodlands is the co-existence of trees and herbaceous species, with seasonal water limitations and fire as the major drivers of tree-grass dynamics (Baudena *et al.*, 2015). Grassy woodlands are expected to undergo imminent ecological changes due to eCO_2 because of increases in soil water content and plant biomass (Baudena *et al.*, 2015, Bond & Midgley, 2000, Scheiter *et al.*, 2015), that will be largely influenced by responses of the understory herbaceous species to eCO_2 (Nilsson & Wardle, 2005, Valladares *et al.*, 2016). In this study, I investigated the main pretext for this, that eCO_2 causes decrease in g_s of the herbaceous understory species and increase in soil water content (Chapter 2). In contrast to the expectations, I did not observe a significant decrease in g_s under eCO_2 in the dominant herbaceous species nor an increase in soil water content, even during the most water-limited periods (Chapter 2). These results suggest that changes in the tree/shrub-grass competition for water, via eCO_2-induced increase in soil water content, may not occur under future rise in CO_2 for ecosystems similar to the one I studied.

Fire is an important determinant of tree-grass interactions in the grassy woodlands whereas grasses constitute most of the fuel load (Baudena *et al.*, 2015). Elevated CO_2 has been thought to increase grass biomass (in both C_3 and C_4) due to a 'water-savings effect' (Bond & Midgley, 2012, Kgope *et al.*, 2010, Morgan *et al.*, 2011) and/or increase C_3 grass biomass due to direct stimulation of photosynthetic rates (Long *et al.*, 2004, Morgan *et al.*, 2004, Polley, 1997). In contrast, eCO_2 may also cause decrease in C_4 grass biomass given that C_3 species are favoured by direct stimulation effect of eCO_2 over the C_4 species (Bazzaz, 1990, Pearcy & Ehleringer, 1984). Such eCO_2-induced changes in grass fuel

along with the delayed drying due to a 'water-savings effect', have been expected to alter the fire regimes in ecosystems like savannas and grassy woodlands characterised by fire as an important driver of ecosystem processes (Baudena *et al.*, 2015, Kgope *et al.*, 2010). In the current study, there was no 'water-savings effect' of eCO_2 (Chapter 2) nor a significant stimulation of biomass under eCO_2 in the herbaceous species (Chapter 3 and 4). Results from my study suggest that eCO_2 may not alter the fire regimes in a fire prone Australian grassy woodland via increase in fuel load or increase in soil water availability, though changes in fire regimes via other mechanisms like altered litter flammability under eCO_2 may be possible (Cary *et al.*, 2012, Manea *et al.*, 2015).

5.4 Implications of the current study for the Earth system models

My research provides information that could be used to validate the previous predictions related to eCO_2 effects on photosynthesis and productivity responses of the herbaceous C_3 species from a warm grassy woodland growing in water and P-limited conditions (Medlyn *et al.*, 2016). Specifically, the negative relationship of eCO_2-induced A_{net} enhancement with seasonal water availability, absence of a 'water-savings effect' (Chapter 2) and lack of significant biomass stimulation under eCO_2 observed in the herbaceous species of a water and P-limited grassy woodland ecosystem (Chapter 3) may be of particular importance.

For instance, previous modelling studies have predicted a trend towards 'global greening' in the warm semi-arid regions (Donohue *et al.*, 2013), which has been attributed to eCO_2-induced 'water-savings effect' thought to enable plants to use less water and therefore stay greener (Lu *et al.*, 2016). Furthermore, Fatichi *et al.*, (2016) used an ecohydrological model to determine the relative importance of direct (enhanced photosynthetic rates) and indirect effects (soil water savings) of eCO_2 on NPP and evapotranspiration. Their results predicted a larger effect of eCO_2 (28% stimulation of NPP) for water-limited ecosystems mediated via increase in soil water content. Using a dynamic global vegetation model (LPJ-GUESS) and model inter-comparison, Alhström *et al.*, (2015) predicted a greater mean C sink capacity under eCO_2 for the warm semi-arid ecosystems. Experimental findings from the current study contrast the previous modelling predictions (Ahlström *et*

al., 2015, Fatichi *et al.*, 2016), as there was there was no 'water-savings effect' of eCO_2 nor biomass stimulation for the herbaceous species in a warm water-limited grassy woodland.

An elevated CO_2-induced increase in soil water content and plant biomass has also been proposed to cause significant changes in the tree-grass interactions and fire regimes in the grass-dominated and fire prone ecosystems like savannas and grassy woodlands (Baudena *et al.*, 2015, Scheiter *et al.*, 2015). This study reports a lack of 'water-savings effect' of eCO_2 (Chapter 2) and no increase in the herbaceous species biomass under eCO_2 (Chapter 3 and 4) in a seasonally water-limited and fire prone Australian grassy woodland. Data generated from this study may help validate the assumptions pertaining to eCO_2 effects on woody thickening process and fire regimes in grassy woodlands like the one in the current study, particularly in the Australian context (Baudena *et al.*, 2015, Scheiter *et al.*, 2015).

5.5 Overall conclusions

The main aspects arising from the thesis can be briefly summarised as follows:

(1) Results obtained from this study support the general view that responses to eCO_2, especially photosynthetic ones, are strongest during the drier conditions (Morgan *et al.*, 2004, Nowak *et al.*, 2004). In particular, for the C_3 species from a seasonally water-limited ecosystem, the proportional eCO_2-induced A_{net} enhancement was a decreasing function of seasonal water availability.

(2) Following this, seasonal water availability is important in determining the photosynthetic responses of herbaceous species to eCO_2, as has been recently reported for herbaceous biomass responses to eCO_2 (Hovenden *et al.*, 2014).

(3) Elevated CO_2 did not decrease g_s and increase in soil water content, even during the dry periods when the magnitude of a 'water-savings effect' was expected to be larger. Thus, eCO_2 has the potential to alter the functioning of periodically water-limited grassy woodland ecosystems, though not via a 'water-savings effect' as is usually observed in temperate grasslands (Blumenthal *et al.*, 2013, Morgan *et al.*, 2011).

(4) The proportional eCO_2-induced A_{net} enhancement observed during dry periods was a result of amelioration of drought-induced stomatal limitations via increase in C_i.

(5) There was evidence of photosynthetic capacity down-regulation in the dominant understory C_3 herbaceous species, particularly during the peak growing seasons.

(6) Plant species differed in their photosynthetic and biomass allocation responses to eCO_2, which may be a result of differences in nutrient acquisition and use strategies.

(7) There was no significant stimulation of biomass under eCO_2 in the understory herbaceous species from a warm water and nutrient-limited grassy woodland ecosystem.

5.6 Limitations of the study

In chapter 2 and 3, I report the findings from field experiments conducted at the EucFACE facility. A FACE experiment is indeed an effective platform to investigate plant eco-physiological responses to eCO_2, as it provides a direct field insight into effects of eCO_2 on the complex terrestrial ecosystem functioning without disturbing natural ecosystem level processes (Nowak *et al*., 2004). However, one of the typical issues involved in these large-scale, expensive experiments is the low number of true replicates, that is, $n = 3$ in case of EucFACE (and many of the other FACE experiments worldwide). Consequently, ability to detect CO_2 effects is often constrained by reduced statistical power, especially when there is naturally substantial between-ring variation. In chapter 3, I report the effects of eCO_2 on the above-ground biomass of the herbaceous understory species for two-time points only, with one time-point per year. The *Eucalyptus* woodland ecosystem in this study experiences considerable seasonal variation in environmental factors like temperature, precipitation and nutrient availability, which can interact with eCO_2 thus resulting in significant seasonal variation in CO_2 effects on biomass. This study could have benefitted more from multiple seasonal above-ground biomass harvests. However, being located in the native endangered ecosystem, frequent destructive harvesting at the EucFACE facility was not feasible, and it would compromise a number of other scientific studies in the plots. Another limitation of the current study was the inability to estimate

below-ground biomass of individual species, as the species at the site grow naturally in competition. One of the important limitations of the glasshouse experiment (Chapter 4) was the inability to capture interactions of eCO_2 effects with seasonal fluctuations in climate. A second key limitation of the glasshouse study was that the N and P status of plants in the field were not well-matched in the glasshouse even when the same soil was used in pots in the glasshouse.

5.7 Future work needed

My research provides important information pertaining to photosynthesis and productivity responses of herbaceous C_3 species from a warm, water and P-limited grassy woodland ecosystem. However, there are many avenues and options for further research to be undertaken. Following are some of the areas of research identified for possible further work relevant to the effects of eCO_2 on herbaceous understory species:

(1) Does eCO_2 alter biomass flammability?: Results from this thesis suggest that eCO_2 does not cause increase in soil water content (Chapter 2) as well as biomass (Chapter 3 and 4) in the herbaceous species of a grassy woodland. Consequently, eCO_2 may not alter the fire regimes via delayed drying and increased fuel load. However, earlier studies suggest that eCO_2 can change the litter flammability through effects of leaf chemical composition (Cary *et al.*, 2012, Manea *et al.*, 2015). Thus, in future studies it would be interesting to investigate if eCO_2 has altered the leaf chemical properties and hence flammability of herbaceous species biomass in this fire prone grassy woodland ecosystem.

(2) Measurement of root biomass in synchronization with shoot biomass: In this study I measured only the above-ground biomass of herbaceous species at the EucFACE facility (Chapter 3). Lack of data on root biomass and shoot biomass measured in synchronization precludes a definitive answer to the question on effect of CO_2 on total productivity of a warm ecosystem. Lack of root biomass stimulation under eCO_2 observed in the glasshouse experiment for the dominant species in this study (Chapter 4), provides preliminary evidence that eCO_2 may not stimulate herbaceous species root biomass in this P-limited ecosystem. Thus,

upcoming shoot and root biomass measurements at the EucFACE facility should be carried out in synchronization with each other.

(3) **Does eCO_2 alter the composition of herbaceous community?** In this study, I did not observe a significant CO_2 effect on above-ground biomass of grasses and forbs (Chapter 3) measured during the second and third year of CO_2 fertilisation at EucFACE. However, there was a non-significant trend towards lower above-ground biomass (-30%) under eCO_2 in the forbs measured at EucFACE, in contrast to a non-significant increase in grass biomass (+14%). This trend was also supported by the total biomass data from the glasshouse study, where the grass biomass did not respond to eCO_2 whereas a significant decrease in forb biomass was observed under eCO_2 (Chapter 4). These results suggest that grasses and forbs may respond differently to eCO_2 with implications for altered understory composition in this grassy woodland. However, responses of community composition and abundance are likely to operate on long-time frames, longer than the 3-year observational span of the current study. This is particularly true considering the large seasonal variation in environmental conditions like water and nutrient availability and temperature that can influence eCO_2 effect on biomass and species composition. Thus, long-term measurement of grass and forb biomass at the EucFACE is indeed required to assess changes in community composition. For instance, work by Morgan *et al*., 2011, in the first three years of the Prairie Heating and CO_2 Enrichment (PHACE) experiment, showed an overall increase in productivity under eCO_2 conditions. However, a study extending over the eight-year duration of PHACE experiment showed that eCO_2 reduced biomass production of dominant species, particularly in later years (Zelikova *et al*., 2014). Thus, under long-term, plant responses to eCO_2 can diminish over time. To accurately predict the change in plant community composition under eCO_2, studies addressing the responses of dominant, subdominant as well as invasive plant species and extending for at least 10 years of duration may be necessary. Furthermore, it would also be interesting to assess the photosynthetic and biomass responses to eCO_2 by grouping the understory herbaceous species of the *Eucalyptus* woodland into native and invasive. Results from the current study

indicate that invasive C_3 grasses may have advantage over the invasive forbs as well as native forbs. However, detailed study of photosynthesis and productivity of all the invasive species is essential to make definitive predictions about future invasions. This would be helpful in assessing the vulnerability of this grassy woodland ecosystem to plant invasions under climate change.

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