How will the main and interactive effects of elevated \([\text{CO}_2]\) and elevated temperature affect tree response to drought and drought-induced tree mortality?

By

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as 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.

[Signature]

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List of Abbreviations

$A_{\text{sat}}$ ($\mu$mol m\(^{-2}\) s\(^{-1}\)) Leaf net photosynthesis at saturating light

$[\text{CO}_2]$ ($\mu$l l\(^{-1}\)) Atmospheric CO\(_2\) concentration

$C_a$ ($\mu$l l\(^{-1}\)) Ambient [CO\(_2\)]

$C_e$ ($\mu$l l\(^{-1}\)) Elevated [CO\(_2\)]

$D$ (cm) Stem basal diameter

$D_p$ ($\mu$m) The maximum pore diameter in the pit membrane

$E$ (mmol m\(^{-2}\) s\(^{-1}\)) Transpiration rate

$E_{\text{crit}}$ (mmol m\(^{-2}\) s\(^{-1}\)) Threshold for transpiration rate

$g$ (m s\(^{-2}\)) Gravitation constant

$g_s$ (mol m\(^{-2}\) s\(^{-1}\)) Stomatal conductance

$h$ (hour) Hour

$h$ (m) The height of the water column in the xylem

$H$ (cm) Tree height

Instantaneous WUE ($\mu$mol CO\(_2\) mol\(^{-1}\) H\(_2\)O) Instantaneous water-use-efficiency
\( K_{\text{initial}} \) (mmol s\(^{-1}\) MPa\(^{-1}\) m\(^{-1}\)) Initial/pre-flush stem hydraulic conductivity

\( K_i \) (mmol s\(^{-1}\) MPa\(^{-1}\) m\(^{-2}\)) Leaf-specific hydraulic conductance of the soil-plant-atmosphere continuum

\( K_{\text{max}} \) (mmol s\(^{-1}\) MPa\(^{-1}\) m\(^{-1}\)) Maximum stem hydraulic conductivity

\( P_{50} \) (MPa) The xylem pressure at which 50 \% loss of conductivity occurs

\( P_{88} \) (MPa) The xylem pressure at which 88 \% loss of conductivity occurs

\( P_a \) (MPa) Pressure difference between pit membranes

PLC (\%) Percentage loss of hydraulic conductivity

\( \text{PPFD} \) (\( \mu \text{mol m}^{-2} \text{s}^{-1} \)) Photosynthetic photon flux density

\( \rho_w \) (kg m\(^{-3}\)) Water density

\( Q_{10} \) Rate of change in respiration due to 10 °C increase in temperature

\( R_n \) (\( \mu \text{mol m}^{-2} \text{s}^{-1} \)) Leaf night respiration rate

\( \text{VWC} \) (m\(^3\) m\(^{-3}\)) Volumetric water content

\( T_a \) (°C) Ambient temperature

TDR Time domain reflectometer

\( T_e \) (°C) Elevated temperature

TNC Total non-structural carbohydrate

Total plant WUE (g mass kg\(^{-1}\) H\(_2\)O) Total plant water-use-efficiency

VPD (kPa) Vapour pressure deficit
\( W (g) \)  Dry mass

WTCs Whole-tree chambers

\( \theta (^\circ) \)  The contact angle between the pit membrane-air-water interface

\( \sigma (\text{N}^2 \text{m}^{-1}) \)  Surface tension of the liquid

\( \Psi_{\text{crit}} \) (MPa)  Threshold for leaf water potential

\( \Psi_l \) (MPa)  Leaf water potential

\( \Psi_{\text{md}} \) (MPa)  Mid-day leaf water potential

\( \Psi_s \) (MPa)  Soil water potential

\( \Psi_{\text{pd}} \) (MPa)  Pre-dawn leaf water potential

\( \Psi_{\text{xylem}} \) (MPa)  Xylem water potential
Abstract

Worldwide forest die-off events have been observed in a number of forest biomes due to severe droughts, rising global temperatures and associated increased vapour pressure deficit (VPD). If drought duration or severity increases with rising temperatures and increased VPD, all forest biomes may be increasingly vulnerable to drought-induced mortality. Despite the importance of forests in the biosphere and the significant potential consequences of forest die-offs, the mechanisms underpinning drought-induced tree mortality are poorly understood. In the context of climate change, elevated temperature has often been reported to exacerbate drought stress and accelerate the time-to-mortality in plants exposed to prolonged drought, while elevated [CO₂] has been proposed as a mitigating factor because it can reduce stomatal conductance ($g_s$) and thereby reduce water loss. Rarely have these three environmental factors (elevated [CO₂], elevated temperature, and drought) been studied in combination to generate a more complete assessment of the wide-ranging, long-term effects of climate change on trees. Therefore, my PhD thesis was designed to investigate the main and interactive effects of elevated [CO₂] and temperature on tree response to drought and subsequent mortality in four species representing different taxa and functional groups: *Eucalyptus globulus* Labill. (relatively isohydric, angiosperm), *Eucalyptus radiata* Sieber ex DC (relatively anisohydric, angiosperm), *Pinus radiata* D. Don (relatively isohydric, gymnosperm) and *Callitris rhomboidea*
R. Br (relatively anisohydric, gymnosperm). My goal was to use these tree species to generate improved understanding of tree physiological responses to drought and its interactions with elevated [CO₂] and temperature.

In the first experimental chapter, the main and interactive effects of elevated [CO₂] and elevated temperature on carbon dynamics in *Eucalyptus globulus* seedlings during progressive drought were examined. Seedlings were grown in two [CO₂] concentrations (400 µl l⁻¹ and 640 µl l⁻¹) and two temperatures (28/17 °C and 32/21°C) (day/night) in a sun-lit glasshouse, exposed to well-watered conditions or two progressive drought treatments having undergone two pre-treatment hardening watering regimes (i.e. *rewatered drought* and *sustained drought*). The progressive drought was imposed by replacing 80-90 % of daily gravimetical water loss, until water loss was minimal, and then watering was discontinued and plants were allowed to desiccate completely. Results showed that progressive drought in both drought treatments led to similar limitations in growth, photosynthesis and respiration, but reductions in total non-structural carbohydrate (TNC) concentration were not observed. Elevated [CO₂] ameliorated the impact of the drought during the moderate drought phase (i.e. Day 63 to Day 79) by increasing photosynthesis, relative to ambient [CO₂] and enhancing leaf and whole-plant TNC content. In contrast, elevated temperature exacerbated the impact of the drought during the moderate drought phase by reducing photosynthesis, increasing leaf respiration, and decreasing whole-plant TNC content. Extreme drought (i.e. Day 79 to Day 103) eliminated [CO₂] and temperature effects on plant growth, photosynthesis and respiration. The combined effects of elevated [CO₂] and elevated temperature on moderate drought stressed seedlings of *E. globulus* were reduced with progressive drought, with no sustained effects on growth despite greater whole-plant TNC content. Findings in
this chapter suggest that drought intensity may alter the impact of elevated [CO₂] and elevated temperature on carbon dynamics in *E. globulus* seedlings.

In the second experimental chapter, the effects of elevated [CO₂] and temperature on drought responses (i.e. water relations and carbon dynamics) and subsequent drought-induced mortality in *Eucalyptus radiata* seedlings were investigated. Seedlings were grown in two [CO₂] (400 µl l⁻¹ and 640 µl l⁻¹) and two temperature (26/16 °C and 30/20 °C) (day/night) treatments in a sun-lit glasshouse under well-watered conditions. Subsequently half of the seedlings were exposed to two controlled drying and rewatering cycles, and then water was withheld until plants died. In order to minimise the potential confounding effects of differing VPD and plant sizes among [CO₂] and temperature treatments on the rate of soil drying, soil volumetric water content (VWC) was maintained at similar levels in all treatments. Elevated temperature treatments triggered more rapid mortality (30 ~ 40 days faster) than ambient temperature treatments. Plants in elevated temperature treatments had higher water use and increased sensitivity of gas exchange traits to drought, and experienced substantial xylem cavitation earlier. Elevated [CO₂] had a negligible effect on tree seedling response to drought, and most importantly elevated [CO₂] did not ameliorate the negative effects of elevated temperature on drought stress.

Drought-induced mortality of *E. radiata* seedlings was generated primarily by hydraulic failure with limited indication of carbohydrate depletion. These findings suggest that elevated temperature and consequent higher VPD, but not elevated [CO₂], were the primary contributors affecting drought-induced tree seedling mortality in *E. radiata*.

In the third experimental chapter, water relations and carbon dynamics during drought-induced mortality in two gymnosperms with contrasting stomatal response
strategies (i.e. relatively isohydric *Pinus radiata* and relatively anisohydric *Callitris rhomboidea*) were examined. This chapter aimed to explore how elevated [CO$_2$] and temperature would modify time to mortality in these two gymnosperms, again under contrasting [CO$_2$] and temperature treatments. Seedlings of these two gymnosperms were grown in two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperatures (26/16 °C and 30/20 °C) (day/night) in a sun-lit glasshouse under *well-watered* conditions. Half of the seedlings in each gymnosperm species were then subjected to an acute *drought* treatment (i.e. irrigation was withheld) until mortality. *Pinus radiata* and *C. rhomboidea* exhibited differing responses of water relations to soil drying. However, leaf desiccation and catastrophic xylem cavitation eventually occurred in both species prior to mortality. There were only limited changes in TNC, depletion of leaf starch were offset by increases of leaf soluble sugar in *C. rhomboidea*; thus, there was no evidence to support carbon starvation as a mechanism contributing to mortality, but it was evident that hydraulic failure played the primary role during drought-induced mortality in both species. Elevated temperature treatments led to faster mortality in *P. radiata*, associated with more rapid declines in leaf water potential, but they did not modify the time-to-mortality in *C. rhomboidea*. Elevated temperature treatments did not induce earlier onset of stomatal closure at any given soil VWC. Furthermore, elevated [CO$_2$] alone did not alter physiological responses (i.e. gas exchange and leaf water potential) to progressive drought nor did elevated [CO$_2$] modify time-to-mortality in either species. Elevated [CO$_2$] did not ameliorate the negative effects of elevated temperature on drought mortality. These findings indicate that hydraulic failure played the primary role in the mortality of *P. radiata* and *C. rhomboidea* during severe droughts, irrespective of stomatal response strategies. Elevated temperature and consequent higher VPD were the dominant
factors affecting water relations and carbon dynamics during drought-induced mortality in *P. radiata*, but had minimal effects on *C. rhomboidea*.

In conclusion, this PhD research addressed the main and interactive effects of elevated [CO2] and temperature on tree response to drought and drought-induced tree mortality, by linking water relations and carbon dynamics in four tree species representing different taxa (angiosperms and gymnosperms) and functional groups (relatively isohydric and anisohydric). The study confirmed that hydraulic failure was the dominant mechanism underpinning tree mortality during severe droughts regardless of species or stomatal response strategy. Increasing temperature (ambient + 4 °C) and consequent higher VPD exacerbated drought stress and led to more rapid mortality through hydraulic failure in most species in this study. Rising [CO2] (ambient + 240 µl l⁻¹) ameliorated moderate drought stress in *E. globulus*, but the positive effects of rising [CO2] were eliminated by increasing drought intensity. Further, elevated [CO2] did not ameliorate drought stress in *E. radiata*, *P. radiata* and *C. rhomboidea* or delay the time-to-mortality. These results suggest that elevated [CO2] may not ameliorate drought or temperature stress in these tree species, particularly when drought is prolonged and severe. Elevated [CO2] partially offset the negative effects of elevated temperature during moderate drought stress in *E. globulus*, but did not ameliorate drought response to elevated temperature in the other three species in this study. This study suggests that rising temperatures and associated higher VPD may be the predominant contributing factors to drought-induced mortality. Global forests maintain very narrow hydraulic safety margins and these findings raise concern that under future climate scenarios, characterised by rising temperatures and changing drought frequency and intensity, forests will be increasingly vulnerable to large scale mortality events with associated changes in the
cycle of mass and energy with ecosystems and the provision of vital ecosystem services.
Chapter 1

General introduction

1.1 Background

Global climate change factors such as rising atmospheric CO$_2$ concentration ([CO$_2$]), increasing air temperature and drought, are globally important drivers of change due to their potential effects on ecosystems and human society. Fossil fuel consumption and land use changes have increased [CO$_2$] from pre-industrial values of about 280 $\mu l$ l$^{-1}$ to 397 $\mu l$ l$^{-1}$ in 2013 (IPCC, 2007; Mauna Loa Observatory data source). [CO$_2$] is projected to reach between 500 and 1000 $\mu l$ l$^{-1}$ by the end of 21$^{st}$ century (Franks et al., 2013). Meanwhile, global mean temperature has risen by 0.76 °C relative to the pre-industrial value and is projected to increase by 1 °C -6.4 °C (Christensen et al., 2007; IPCC, 2007). In Australia, mean air temperature has increased by 0.9 °C in the period of 1910 to 2011 and is projected to rise by 2 °C to 5 °C by 2070 (CSIRO, 2007, 2012). Additionally, temperature extremes such as heat waves are predicted to become more frequent and severe (IPCC, 2012). Drought events, which are usually associated with high temperature and heat wave events, have increased across continents around the world, including Australia (Dai, 2011). Recent projections suggest that more frequent extreme drought will likely occur accompanied with rising [CO$_2$] and global warming in some regions (IPCC, 2007, 2012), particularly in
Australia, Africa and Latin America (CSIRO, 2007; Meehl et al., 2007; Bates et al., 2008; van der Molen et al., 2011). Thus, there is growing need to understand how Australian ecosystems will respond to global climate change, and in particular rising [CO₂], increasing temperatures and shifting rainfall regimes.

Water availability is a major limiting factor for growth and productivity of plants, thereby shaping vegetation distributions along with global rainfall patterns (Wullschleger et al., 2002; Robredo et al., 2007; Taiz & Zeiger, 2010). Drought in particular leads to reduced hydraulic dysfunction in plants, thereby inducing cascading negative effects on other processes such as growth and photosynthesis (Blackman et al., 2009), with the potential for extreme drought to generate plant mortality (Pockman & Sperry, 2000; Tyree et al., 2002; Breshears et al., 2005; Allen et al., 2010). A recent global survey of forest die-off events suggested that the coincidence of drought periods with higher temperatures and heat waves may already be accelerating the rate of large scale forest die-offs over the past few decades (Allen et al., 2010). Global forest die-off has been observed on all vegetated continents and in a variety of forest biomes (e.g. Hosking & Hutcheson, 1988; Lwanga, 2003; Rice et al., 2004; Chazdon et al., 2005; Wang et al., 2007; Hogg et al., 2008; Phillips et al., 2009, 2010; van Mantgem et al., 2009; Peng et al., 2011). Large scale forest collapse can alter ecosystem structure and biodiversity, ecosystem goods and services, carbon and water budgets, and feedbacks between the biosphere and climate (Dale et al., 2000; Breshears & Allen, 2002; Bonan, 2008; Adams et al., 2010, 2012; Metcalfe et al., 2010; Breshears et al., 2011; Hartmann, 2011; Royer et al., 2011; Anderegg et al., 2013). Despite the importance of forests in the biosphere and the significant potential consequences of forest die-offs, the mechanisms underpinning drought-induced mortality are poorly understood.
Considerable progress has been made in describing the independent effects of drought, rising [CO₂] and temperature on plant physiological responses (e.g. Hsiao, 1973; Chaves & Pereira, 1992; Ceulemans & Mousseau, 1994; Curtis & Wang, 1998; Chaves et al., 2002; Way & Oren, 2010). However, there is only limited understanding of the interactive effects of these three key global change factors on tree responses (but see Wertin et al. 2010, 2012a,b; Ayub et al. 2011; Zeppel et al., 2012; Lewis et al., 2013). To-date, no studies have demonstrated how the interaction of rising [CO₂] and temperature interacts with drought-induced and modifies physiological processes preceding tree mortality. To better understand and predict forest responses to future climate scenarios, we need to conduct experimental, manipulative studies that quantify the main and interactive effects of rising [CO₂] and temperature on tree responses to drought and associated drought-induced mortality.

In the following section, a basic introduction of water relations and carbon dynamics of trees during drought stress is presented, including a review of current knowledge with respect to the main and interactive effects of rising [CO₂] and temperature on tree responses to drought.

1.2 Literature review

1.2.1 Tree response to drought

1.2.1.1 Water relations during drought

*Water transport in the xylem*

Vascular plants extract water from soil and transport this water to leaves via the xylem taking advantage of the unique cohesive forces between water molecules, to
move water along gradients of high water potentials (soil) to low water potentials (atmosphere) (Tyree & Sperry, 1989; Lambers et al., 1998; Brodribb et al., 2010; Taiz & Zeiger, 2010). However, moving water against the forces of gravity and in small diameter xylem conduits creates resistance to flow. As a result, water in the xylem is under tension (i.e. negative pressure) and as the soil dries, or transpiration increases, increases in water tension generate higher risk of cavitation in the xylem, and disruption of water transport (Tyree & Sperry, 1988, 1989). In the soil-plant-atmosphere continuum, the relationship between transpiration \( E \) and hydraulic conductance has been described in the equation according to Darcy’s law (Whitehead, 1998; McDowell et al., 2008):

\[
E = K_l (\Psi_s - \Psi_l - h \rho_w g) \quad \text{(Eqn. 1)}
\]

where \( K_l \) is leaf-specific hydraulic conductance of the soil-plant-atmosphere continuum. \( \Psi_s \) and \( \Psi_l \) are soil and leaf water potentials, respectively. \( h \rho_w g \) is the gravitational pull on a water column of height \( h \) and density \( \rho_w \).

If \( K_l \) remains constant (e.g. when no cavitation occurs), the difference in potential between soil and leaf increases in proportion to \( E \). However, \( K_l \) usually decreases as water potential declines, thus there is a threshold for \( E \) (i.e. \( E_{\text{crit}} \)) and water potential (i.e. \( \Psi_{\text{crit}} \)). As the soil dries or transpiration increases, stomatal regulation acts to maintain leaf water potential above \( \Psi_{\text{crit}} \) to prevent “runaway cavitation” (Sperry, 2000). Nevertheless, if soil drying persists and \( E_{\text{crit}} \) and \( \Psi_{\text{crit}} \) are exceeded, plants may experience a substantial loss of hydraulic conductivity in the xylem (Sperry, 2000; Meinzer, 2002), leading to loss of transport capacity and “hydraulic failure” (McDowell et al., 2008).
During soil drying, sufficient water tension develops in the xylem water column, sucking air bubbles into the xylem and blocking water transport (Zimmermann 1983; Tyree & Sperry, 1989; Choat, 2013). Air bubbles may also spread into adjacent conduits when the pressure difference across the pit membrane between the embolized and functional conduit exceeds the surface tension of the air-water interfaces within pit membranes (Sperry & Tyree, 1988; Tyree & Sperry, 1988, 1989; Tyree & Zimmermann, 2002; Brodersen et al., 2013). The spread of air bubbles leads to reductions in hydraulic conductivity, ultimately causing hydraulic failure and plant death. The air seeding pressure difference \( P_a \) can be predicted from the surface tension of the liquid (\( \sigma \)) and the maximum pore diameter (\( D_p \)) in the pit membrane as

\[
P_a = \frac{4 \sigma \cos \theta}{D_p} \quad \text{(Eqn. 2)}
\]

where \( \theta \) is the contact angle between the pit membrane-air-water interface, normally treated as zero for hydrophilic surfaces (Tyree & Zimmermann, 2002; Choat et al., 2008). The relationship between the air seeding threshold and the pit membrane pore diameter has been demonstrated across a range of species with differing pit membrane structure (Jansen et al., 2009). It has also been reported that large pores responsible for air seeding between conduits are rare, and the chance of having a large pore increases with the surface area of the pit membrane between the connected conduits (Choat et al., 2003; Wheeler et al., 2005; Christman et al., 2009). The size of pores may also be increased by stretching the membrane across the pit chamber as the pressure difference between embolized and functional conduits becomes greater (Choat et al., 2004). In general, the vulnerability to cavitation of a species is determined by a combination of pit and conduit level characteristics, such as the strength and porosity of the pit membranes and the average area of membrane connection conduits (Wheeler et al., 2005; Choat et al., 2008; Lens et al., 2011).
There are some trade-offs between cavitation resistance and hydraulic efficiency. For example, smaller pit membrane pores confer resistance to cavitation, but they may reduce the hydraulic conductivity of the xylem (Choat et al., 2008; Lens et al., 2011).

**Vulnerability to xylem cavitation**

Vulnerability curves, expressed as the percentage loss of hydraulic conductivity (PLC) as a function of xylem pressure, were first described in Sperry et al. (1988), providing a way to quantify common indicators of plant vulnerability to cavitation, for example, the xylem pressure at which 50 % and 88 % loss of conductivity occurs (i.e. $P_{50}$ and $P_{88}$) (Choat et al., 2008, 2012; Brodribb & Cochard, 2009; Brodribb et al., 2010; Meinzer & McCulloh, 2013; Urli et al., 2013). When xylem pressure drops below $P_{50}$ or $P_{88}$, water transport is substantially impaired. The plant is then at high risk of catastrophic xylem cavitation and eventual mortality. Both $P_{50}$ and $P_{88}$ have been observed to be positively correlated with lethal water potential in conifers and angiosperms, respectively (Brodribb & Cochard, 2009; Brodribb et al., 2010; Urli et al., 2013). Despite being an index of cavitation resistance in response to extreme drought conditions, $P_{50}$ and $P_{88}$ do not indicate the magnitude of stomatal regulation in the field required to avoid hydraulic dysfunction in extreme conditions (Meinzer et al., 2009). Thus the concept of hydraulic safety margin was developed to link stomatal regulation to the risk of hydraulic dysfunction during drought stress (Pockman & Sperry, 2000; Brodribb & Holbrook, 2004; Meinzer et al., 2009; Meinzer & McCulloh, 2013). The hydraulic safety margin is defined as the pressure difference between $P_{50}$ or $P_{88}$ and the minimum xylem pressure that plants normally reach during the growing season. Despite there being large variability in cavitation resistance amongst plant species, recent evidence suggests that forests may be globally vulnerable to hydraulic failure if extreme drought events occur, because
most species exhibit a narrow range of safety margins (i.e. < 1 MPa) (Choat et al., 2012). This suggests that drought-induced hydraulic failure could be common across species with increased drought intensities projected to occur under climate change.

1.2.1.2 Carbon dynamics during drought

Carbon dynamics encompass the combination of carbon assimilation (i.e. photosynthesis), growth, carbon utilization (i.e. growth and maintenance respiration) and carbon reserves (i.e. total non-structural carbohydrate (TNC)), while carbon balance reflects the difference between carbon assimilation and carbon utilization. It has been well documented that drought can have negative impacts on plant morphology and physiology (e.g. Hsiao, 1973), leading to growth inhibition (e.g. Hsiao, 1973; Hsiao et al., 1976), physiological limitations (e.g. stomata closure, reduced carbon assimilation and respiration) (e.g. Hsiao, 1973; Chaves, 1991; Atkin et al., 2009) and changes in TNC reserves (e.g. Tissue & Wright, 1995; Körner, 2003; Ayub et al., 2011).

Drought can differentially affect rates of growth, photosynthesis and respiration (Hsiao et al., 1976). In the majority of species, growth is the process most sensitive to drought because cell expansion has higher dependence on turgor pressure than photosynthesis and respiration (e.g. Boyer, 1970; Hsiao, 1973; McCree et al., 1984; Munns, 1988; Bogeat-Triboulot et al., 2007). Photosynthesis appears to be more sensitive to drought than dark respiration (e.g. Boyer, 1970; Hsiao et al., 1976; Wilson et al., 1980; McCree et al., 1984), as photosynthesis often declines earlier than respiration in response to reduced soil water availability. The reduction in growth relative to photosynthesis may explain why TNC reserves often increase during the early phases of drought (e.g. Kozlowski & Pallardy, 2002; Körner, 2003;
Würth et al., 2005; Ayub et al., 2011). However under sustained drought theoretically, the higher sensitivity of photosynthesis to drought than respiration should lead to a decline in the TNC reserves as photosynthesis does not meet respiratory demands (see Hsiao et al. 1976; Amthor & McCree, 1990; McDowell, 2011). If the decline in TNC is significant, “carbon starvation” may occur (McDowell et al., 2008; McDowell, 2011) (see more details in the following section). However, recent experimental studies suggest that there is considerable variability and complexity in responses of TNC to progressive drought, due to differences between species, organs and environmental conditions (e.g. Piper, 2011; Sala et al., 2012; Hartmann et al., 2013a). For instance, TNC concentrations in stem and roots were reduced in relatively drought-sensitive species (Nothofagus nitida (Phil.) Krasser), but increased in relatively drought-resistant species (Nothofagus dombeyi (Mirb.) Oerst) (Piper, 2011). Additionally, Hartmann et al. (2013a) observed that TNC was depleted in roots but not in the canopy, in Norway spruce trees subjected to lethal drought stress. Maintenance of TNC reserves is thought crucial for plants to withstand, survive and recover from environmental stresses (e.g. drought) (Niinemets, 2010). As a consequence, there is increasing need to clearly understand how carbon dynamics respond to future changing climate with more frequent and extreme droughts.

1.2.1.3 Physiological mechanisms of drought-induced mortality

Recent observations of drought-induced forest mortality events worldwide, in combination of increasing confidence in projections from global climate models, have rekindled strong interest in understanding the mechanisms underpinning forest mortality (McDowell et al., 2008; Allen et al., 2010; McDowell & Sevanto, 2010; Sala et al., 2010; McDowell et al., 2013a,b). Two interdependent physiological
mechanisms (i.e. hydraulic failure and carbon starvation) have been proposed to explain water relations and carbon dynamics during drought-induced tree mortality, depending on the intensity and duration of drought and stomatal response strategies of individual species (i.e. relatively isohydric vs. anisohydric) (McDowell et al., 2008, 2011). The hydraulic failure hypothesis states that as the soil dries sufficient cavitation generated in xylem conduits impedes water flow and desiccates plant tissue, eventually leading to cellular death (Brodribb & Cochard, 2009; Urli et al., 2013). Hydraulic failure is expected to occur in a shorter and more intense drought and in trees which sustain carbon assimilation as soil dries but are unable to maintain leaf water status above critical water potential thresholds (i.e. relatively anisohydric species). On the other hand, the carbon starvation hypothesis predicts that long periods of negative carbon balance due to stomatal closure (in order to prevent hydraulic failure) and continued metabolic demands (i.e. respiration) eventually depletes stored carbon reserves leading to plant death. Carbon starvation is more likely to occur in less intense, but longer drought events or in trees which close stomata to maintain leaf water status above water potential thresholds (i.e. relatively isohydric species). However, drought has negative impacts on both water and carbon budgets, and therefore these two mechanisms often are interdependent (McDowell, 2011). In addition, the carbon starvation hypothesis has been modified by Sala et al. (2010) to incorporate drought impacts on carbohydrate mobilization. Sala et al. (2010) argued that failure of carbohydrate transport in the phloem can limit carbohydrate translocation from source to sink, thereby potentially differentially promoting carbon starvation in plant organs. Lastly, drought-induced tree mortality can also be amplified by biotic factors (e.g. insect outbreaks or pathogen infections) (McDowell et al., 2008) and abiotic factors (e.g. rising temperature) (Breshears et al.,...
For instance, recent modeling analyses showed that higher vapour pressure deficit (VPD) due to rising temperature contributed to forest mortality events in the southwestern USA (Williams et al., 2013).

Many studies have tested the hydraulic failure and carbon starvation hypotheses. For most, hydraulic failure was recognized as the primary driver of drought-induced tree mortality (Adams et al., 2009; Anderegg et al., 2012; Anderegg & Anderegg, 2013; Hartmann et al., 2013b; Mitchell et al., 2013; Nardini et al., 2013; Quirk et al., 2013; Sevanto et al., 2013), despite some studies noting significant carbohydrate depletion (Adams et al., 2013; Galiano et al., 2012; Hartmann et al., 2013a,b; Mitchell et al., 2013; Quirk et al., 2013; Sevanto et al., 2013). However, it is hard to separate hydraulic limitations from the carbohydrates effects; thus, it remains unknown whether carbon starvation can independently induce mortality during drought. Recent studies have specifically tested whether trees can die solely of carbon starvation, growing plants under chronic low [CO₂] (Hartmann et al., 2013b) or shading (Sevanto et al., 2013) and showed that carbon starvation may occur under those specific conditions. For instance, in the low [CO₂] conditions (i.e. 75 µl l⁻¹), carbohydrates in organs were depleted even in well-watered Norway spruce saplings. Nonetheless, well-watered saplings grown in low [CO₂] conditions survived c.7 weeks longer than drought saplings (Hartmann et al., 2013b), suggesting that hydraulic failure was the dominant driver of drought-induced tree mortality; thus, they concluded that “thirst beats hunger”.

Stomatal response strategies of tree species are thought to affect the relative contribution of tree hydraulics and carbon metabolism during drought-induced mortality (McDowell et al., 2008). Mitchell et al. (2013) demonstrated that
Eucalyptus smithii R. T. Baker (relatively anisohydric species) had profligate water use and sustained carbon assimilation as water potential declined, but experienced rapid hydraulic failure with small amount of carbohydrates depleted (c. 20 %). In contrast, Pinus radiata D. Don (relatively isohydric species), had conservative water regulation to avoid fast hydraulic dysfunction, but depleted more carbohydrates (c. 50 %). However, it remains interesting to examine how the two species performed if they had controlled the drought duration. Nonetheless, Anderegg & Anderegg (2013) showed that Pinus edulis Engelm. (relatively isohydric species) and Juniperus osteoperma (Torr.) Little (relatively anisohydric species) both experienced catastrophic hydraulic failure, and neither exhibited significant changes in carbohydrates. Clearly, more studies are needed in many other isohydric- anisohydric species to explore the contributions of hydraulic failure and carbon starvation to drought-induced mortality.

1.2.2 Effects of elevated [CO₂] on tree response to drought

Elevated [CO₂] often reduces stomatal conductance (gₛ) under non-water limiting conditions, thereby reducing water loss per unit leaf area (see reviews Ceulemans & Mousseau, 1994; Norby et al., 1999; Medlyn et al., 2001; Ainsworth & Long, 2005; Ainsworth & Rogers, 2007; Franks et al., 2013). For instance, Medlyn et al. (2001) found that elevated [CO₂] led to 21 % declines in gₛ across woody species in 13 long-term studies. If lower gₛ is coupled with reductions in whole-plant water loss, elevated [CO₂] may allow plants to maintain more favourable water status during drought, thereby ameliorating drought stress (Wullschleger et al., 2002; Ainsworth & Rogers, 2007).
Yet, the effects of elevated [CO₂] on tree response to drought vary among studies. Elevated [CO₂] was found to ameliorate drought stress in some studies (Atwell et al., 2007; Wertin et al., 2010, 2012a; Ayub et al., 2011; Franks et al., 2013; Lewis et al., 2013), but not in others (Bobich et al., 2010; Duursma et al., 2011; Warren et al., 2011; Zeppel et al., 2012; Vaz et al., 2012; Perry et al., 2013). In fact, in addition to gs, many other traits also influence whole-plant water loss. First, plant morphology, such as leaf area and root characteristics, can affect water loss from transpiration and water uptake from soil, respectively, thereby having impacts on the whole-plant water budget (Wullschleger et al., 2002; Iversen, 2010). Leaf area is usually increased by elevated [CO₂], which may counteract leaf-level water savings due to elevated [CO₂] (Wullschleger et al., 2002; Norby & Zak, 2011; Way, 2011). Thus, whole-plant water loss depends on the trade-off between reductions in gs and increases in leaf area. For example, despite reduced gs, whole-plant water loss did not differ between ambient and elevated [CO₂] treatments in droughted Prunus avium L. seedlings, largely due to larger leaf area in elevated [CO₂] (Centritto et al., 1999; Wullschleger et al., 2002). Root characteristics, including root biomass, surface area, depth and fine roots, are often increased by elevated [CO₂], thereby potentially ameliorating drought stress by increasing water uptake, particularly in field conditions (Norby et al., 1986; Baxter et al., 1997; Prior & Eamus, 1999; Norby & Jackson, 2000). Conversely, Duursma et al. (2011) found that drought stress was exacerbated in Eucalyptus saligna Sm. exposed to elevated [CO₂] because root depth was much shallower in elevated [CO₂] than ambient [CO₂]. Despite elevated [CO₂] generating root depth results contrary to those normally observed, Duursma et al. (2011) still emphasized the importance of root structure in tree response to drought × [CO₂].
Second, hydraulic traits and xylem anatomy can also affect xylem water transport and whole-tree response to the interaction of elevated \([\text{CO}_2]\) and drought. Elevated \([\text{CO}_2]\) can either lead to increased vessel numbers and diameters, or increased wood density, which are positively correlated with hydraulic conductance and drought resistance, respectively (Conroy et al., 1990; Atkinson & Taylor, 1996; Hacke et al., 2001; Bobich et al., 2010; Kostiainen et al., 2009; Rico et al., 2013). Therefore, tree responses to the interaction of elevated \([\text{CO}_2]\) and drought may depend on the trade-off between water transport efficiency and drought resistance. However, how plant hydraulic and xylem traits respond to the interaction of elevated \([\text{CO}_2]\) and drought remains poorly resolved (Domec et al., 2010). Of the existing studies that have investigated this issue, results are variable (Atwell et al., 2007; Bobich et al., 2010; Domec et al., 2010). For example, Bobich et al. (2010) found that *Populus deltoides* Bartr. trees grown in elevated \([\text{CO}_2]\) had lower wood density than trees in ambient \([\text{CO}_2]\), and therefore these trees were more susceptible to drought. In Domec et al. (2010), higher hydraulic conductance and lower xylem cavitation resistance (indicated by \(P_{50}\)) were observed in *Liquidambar styraciflua* L. and *Cornus floria* L. trees grown in elevated \([\text{CO}_2]\), but not in *Pinus taeda* L. and *Ulmus alata*. Atwell et al. (2007) reported that elevated \([\text{CO}_2]\) ameliorated drought stress, with higher (less negative; -1 MPa) leaf water potentials found in droughted *Eucalyptus tereticornis* Sm. seedlings grown in elevated \([\text{CO}_2]\), despite there being no evidence \(\text{CO}_2\)-induced changes in xylem vessel anatomy (Atwell et al., 2007). Taken together, whole-plant responses to elevated \([\text{CO}_2]\) and drought were associated with alterations in morphological, physiological and hydraulic traits and their feedbacks to whole-plant water loss. Thus, there is growing need to link those traits to clearly understand the effects of elevated \([\text{CO}_2]\) on drought response at the whole-plant level.
Elevated [CO₂] often increases carbon assimilation rates in trees subjected to mild or moderate drought stress compared with those grown in ambient [CO₂] (Wertin et al., 2010; Franks et al., 2013; Lewis et al., 2013). Nevertheless, the positive effects of elevated [CO₂] on carbon assimilation during drought can disappear as drought intensifies and stomata are nearly closed (Warren et al., 2011; Franks et al., 2013; Perry et al., 2013). Elevated [CO₂] has also been found to increase carbohydrate reserves in plants experiencing drought stress (Körner, 2003; Niinemets, 2010; Tissue & Lewis, 2010; Ayub et al., 2011), reflecting the commonly observed increase in starch storage resulting from disparity between greater carbon supply and lower carbon use (e.g. sink limitation) (Körner, 2003). Higher carbohydrate concentrations due to elevated [CO₂] can increase capacity for osmotic adjustment for maintaining turgor even at more negative water potentials (Chaves & Pereira, 1992; Körner et al., 1995; Wullschleger et al., 2002; Lewis et al., 2013). More importantly, higher carbohydrates due to elevated [CO₂] can provide a carbon buffer (i.e. resources for growth and maintenance) to withstand and recover from extreme drought stress (Niinemets, 2010). If the positive effects of elevated [CO₂] on carbohydrate content are maintained as drought progresses, tree species may benefit from elevated [CO₂] during prolonged drought.

However, it still remains unresolved how elevated [CO₂] will affect water relations, carbon dynamics and tree mortality during prolonged, progressive drought. To my knowledge, only one study has addressed this question using Sequoia sempervirens (D.Don.) Endl. seedlings, wherein they found that seedlings grown in elevated [CO₂] (1500 µl l⁻¹) exhibited similar drought mortality as seedlings grown in ambient [CO₂] (Quirk et al., 2013). However, the elevated [CO₂] treatment in that study was nearly four times higher than ambient [CO₂] and far higher than projected [CO₂] conditions.
for this century. Clearly, the effects of projected rising \([\text{CO}_2]\) on vulnerability to drought-induced mortality need to be further examined.

1.2.3 Effects of elevated temperature on tree response to drought

High temperatures have been implicated in many of the observed drought-induced forest mortality events around the globe (Breshears et al., 2005; Van Mantgem et al., 2009; Allen et al., 2010; Williams et al., 2013). Water relations and carbon budgets can both be affected by elevated temperature. On one hand, elevated temperature is usually associated with increased VPD, potentially increasing whole-plant water loss through increased evapotranspiration and thus exacerbating drought stress (Sinclair et al., 2007; Allen et al., 2010; Will et al., 2013). Analysis of heat wave records from nine weather stations in Western Europe over the past 100 years found that VPD was 111% higher in the growing season during heat waves (i.e. more than five consecutive days exceeds the average maximum temperature by 5 °C) compared with normal temperature conditions (De Boeck et al., 2010). Additionally, drought also has positive feedbacks on VPD because reduced evapotranspiration due to stomatal closure can increase the ratio of sensible heat to latent heat, further elevating leaf temperature and VPD (Breshears et al., 2013). Thus, rising temperature associated with higher VPD potentially increases the risk of hydraulic failure. On the other hand, elevated temperature often results in reduced carbon assimilation under drought stress (Chaves, 1991; Zeppel et al., 2012; Lewis et al., 2013; Zhao et al., 2013). Elevated temperature may also amplify drought stress by increasing carbon metabolism (i.e. respiration) because carbon utilization is usually temperature sensitive; respiration increases exponentially with temperature at a constant \(Q_{10}\) value (i.e. rate of change in respiration as a consequence of 10 °C increase in temperature) (Ryan, 1991; Amthor, 2000; Atkin & Tjoelker, 2003; Atkin et al., 2003).
If temperature-induced higher respiration continues as drought intensifies, greater respiratory carbon loss due to higher temperature in combination with lower carbon assimilation may lead to reductions in carbohydrates, thereby generating higher risk of carbon starvation.

Very few studies have experimentally tested to what degree elevated temperature affects drought-induced tree mortality through carbon (Adams et al., 2009, 2013; Zhao et al., 2013) and hydraulic (Will et al., 2013) traits and mechanisms. Adams et al. (2009) was the first experimental study to examine this question, observing that Pinus edulis trees grown in elevated temperature treatment (+ 4.3 °C) exhibited greater cumulative respiration and c. 30 % faster mortality. In a follow up study, Adams et al. (2013) reported that this temperature driven mortality pattern was mainly attributed to different leaf starch-soluble sugar inter-conversions between temperature treatments. However, incomplete assessment of whole-plant carbohydrate pools suggests that their conclusion was speculative rather than conclusive. A recent temperature-drought interaction study found that higher temperatures led to earlier whole-plant negative carbon balance at a given soil water content due to higher respiration rates and earlier stomatal closure (Zhao et al., 2013), which contributes to our knowledge about whole-plant carbon responses to progressive drought. Nevertheless, they did not conduct hydraulic measurements and TNC assays. Consequently, it is difficult to distinguish between temperature effects on hydraulic function and carbon reserves. On the other hand, from the hydraulic perspective, one study reported that higher VPD due to elevated temperature (+ 3 °C) generated more rapid mortality (c. 13 %) in seedlings across ten tree species in a forest-grassland ecotone in the central USA (Will et al., 2013). However, their experiment did not consider the VPD effects on the rate of soil drying. Higher VPD
due to rising temperature can lead to faster soil dry-down, particularly in pot studies, thereby generating earlier onset of drought stress, which could confound the direct effects of temperature treatment (i.e. temperature and associated VPD) on plant function and time-to-mortality. In addition, they did not examine carbon dynamics associated with drought mortality, potentially limiting our deeper understanding of the effects of rising temperature on drought mortality. To sum up, due to the interactive effects of water relations and carbon dynamics during tree mortality, there is substantial benefit in linking hydraulic and carbon attributes to understand how global climate change drivers (e.g. rising temperature and elevated [CO₂]) modify drought-induced tree mortality.

1.2.4 Does elevated [CO₂] offset the negative effects of elevated temperature on tree response to drought?

Under favourable water availability, the combination of elevated [CO₂] and temperature can increase tree growth and carbon assimilation compared to ambient [CO₂] and temperature (Ghannoum et al., 2010a,b; Wertin et al., 2012a). Yet, few studies have investigated the interactive effects of elevated [CO₂] and elevated temperature on tree response to drought (Wertin et al. 2010, 2012a,b; Ayub et al., 2011; Zeppel et al. 2012; Lewis et al., 2013). Elevated [CO₂] has been found to offset the negative effects of elevated temperature on physiological response to drought stress in *Eucalyptus saligna* Sm. and *Eucalyptus sideroxylon* A. Cunn. ex Woolls seedlings (Lewis et al., 2013). Another study, however, observed that negative effects of elevated temperature on *Eucalyptus sideroxylon* A. Cunn. ex Woolls seedlings in response to drought were exacerbated by elevated [CO₂] due to the greater leaf area and higher night-time water loss in elevated [CO₂] (Zeppel et al., 2012). To-date, no studies have examined the interactive effects of elevated [CO₂]
and temperature on drought-induced tree mortality. In the context of global climate change that predicts frequent extreme drought accompanied with rising [CO₂] and temperature, there is increasing interest in investigating the interactive effects on tree responses and mortality, providing valuable data for models to better predict forest response to future climate scenarios. Because elevated [CO₂] and temperature have different impacts on water relations and carbon dynamics, they may differentially affect drought response and mortality of species with contrasting stomatal responses. However, no studies have so far compared tree drought responses and drought-induced mortality in contrasting species within the context of elevated [CO₂] and temperature.

In summary, more experimental studies are required to fill the knowledge gap in how elevated [CO₂] and temperature will independently and/or interactively affect water relations and carbon dynamics during drought-induced tree mortality. Further, studies are also required to examine whether these effects of elevated [CO₂] and temperature differ among species or functional groups.

1.3 Overview of my thesis

1.3.1 Thesis objectives

The overall objective of my PhD thesis was to quantify the impacts of key global climate change factors (i.e. rising [CO₂], increasing temperature, and their interaction) on tree response to drought and drought-induced tree mortality in four tree species, i.e. *Eucalyptus globulus* Labill., *Eucalyptus radiata* Sieber ex DC, *Pinus radiata* D. Don and *Callitris rhomboidea* R. Br, representing different taxa (i.e. angiosperm and gymnosperm) and functional groups (i.e. relatively isohydric and anisohydric).
*Eucalyptus globulus* (relatively isohydric, angiosperm species) (see O’Grady *et al.*, 2008) and *Eucalyptus radiata* (relatively anisohydric, angiosperm species) are ecologically and economically important native trees in Australia. *Pinus radiata* (relatively isohydric, gymnosperm species) (see Brodribb & McAdam, 2013) is native to North America, but widely planted as a commercial timber resource around the world including Australia. *Callitris rhomboidea* (relatively anisohydric, gymnosperm species) (see Brodribb & McAdam, 2013) is an ecologically important native species in Australia. Susceptibility to drought-induced mortality is suggested to be tree size-related and smaller trees tend to be more vulnerable to mortality due to the shallower rooting depth (Ryan *et al.*, 2006; McDowell *et al.*, 2008, 2013b). However, smaller trees allow the multifactor manipulations and the detailed study on physiological mechanisms (McDowell *et al.*, 2013b). Therefore, pot-grown tree seedlings were investigated in this PhD research. This research was conducted in the state-of-the-art glasshouse facility at the University of Western Sydney. The glasshouse was utilized to control [CO₂] (ambient and ambient + 240 µl l⁻¹) and temperature (ambient and ambient + 4 °C) conditions representing current climate and future climate scenarios. The aim was to use seedlings of tree species representing varying taxa and functional attributes to generate improved physiological understanding of tree response to drought as a function of the main and interactive effects of elevated [CO₂] and temperature, which are rising as a function of climate change. Specifically, this thesis aimed to address the following main questions:

1. How do water relations (i.e. plant and soil water status, hydraulic functions) and carbon dynamics (i.e. growth, photosynthesis, respiration and
carbohydrate reserves) vary among species from different taxa or functional groups during drought and drought-induced mortality?

(2) Do elevated [CO₂] and elevated temperature independently or interactively alter tree response to drought and drought-induced tree mortality?

(3) If elevated [CO₂] and elevated temperature have effects on tree response to drought and drought-induced mortality, how do those effects vary among species from different taxa or functional groups?

1.3.2 Outline of my thesis

Chapter 1 presented the general introduction of my PhD research.

Chapter 2 aimed to demonstrate the effects of progressive drought on carbon dynamics in *Eucalyptus globulus* seedlings. It also determined whether the main and interactive effects of elevated [CO₂] and elevated temperature would alter carbon dynamics in response to progressive drought. Seedlings were grown in two [CO₂] concentrations (400 µl l⁻¹ and 640 µl l⁻¹) and two temperatures (28/17 °C and 32/21°C) (day/night) in a sun-lit glasshouse, exposed to well-watered conditions or two progressive drought treatments having undergone different previous water conditions (i.e. rewatered drought and sustained drought). The progressive drought was imposed by replacing 80-90 % of daily gravimetrical water loss, until water loss was minimal, and then watering was discontinued and plants were allowed to desiccate completely. Progressive drought in both drought treatments led to similar limitations in growth, photosynthesis and respiration, but reductions in TNC concentration were not observed. Elevated [CO₂] ameliorated the impact of the drought during the moderate drought phase (i.e. Day 63 to Day 79) by increasing
photosynthesis and enhancing leaf and whole-plant TNC content. In contrast, elevated temperature exacerbated the impact of the drought during the moderate drought phase by reducing photosynthesis, increasing leaf respiration, and decreasing whole-plant TNC content. Extreme drought (i.e. Day 79 to Day 103) eliminated [CO₂] and temperature effects on plant growth, photosynthesis and respiration. The combined effects of elevated [CO₂] and elevated temperature on moderate drought stressed seedlings were reduced with progressive drought, with no sustained effects on growth despite higher whole-plant TNC content. Findings in this chapter suggest that drought intensity may alter the impact of elevated [CO₂] and elevated temperature on carbon dynamics in *E. globulus* seedlings.

**Chapter 3** aimed to investigate the effects of elevated [CO₂] and temperature on drought responses (i.e. water relations and carbon dynamics) and drought-induced mortality in *Eucalyptus radiata* seedlings. Seedlings were grown in two [CO₂] (400 µl l⁻¹ and 640 µl l⁻¹) and two temperature (26/16 °C and 30/20 °C) (day/night) treatments in a sun-lit glasshouse under well-watered conditions. Subsequently half of the seedlings were exposed to two controlled drying and rewatering cycles, and then water was withheld until plants died. In order to minimise the potential confounding effects of differing VPD and plant sizes among [CO₂] and temperature treatments on the rate of soil drying, soil water content was maintained at similar levels in all treatments. Elevated temperature treatments triggered more rapid mortality (30 ~ 40 days faster) than ambient temperature treatments, and were associated with larger water use, increased drought sensitivities of gas exchange traits and earlier occurrence of xylem cavitation. Elevated [CO₂] had a negligible effect on tree seedling response to drought, and most importantly did not ameliorate the negative effects of elevated temperature on drought stress. Drought-induced
mortality of *E. radiata* seedlings was generated primarily by hydraulic failure with limited indication of carbohydrate depletion. Collectively, these findings suggest that elevated temperature and consequent higher VPD, but not elevated [CO₂], may be the primary contributors affecting drought-induced tree seedling mortality in future climates.

**Chapter 4** aimed to examine whether water relations and carbon dynamics during drought-induced mortality would differ between gymnosperms with contrasting stomatal response strategies (i.e. *Pinus radiata* and *Callitris rhomboidea*); and how elevated [CO₂] and temperature would modify mortality in these two gymnosperms. Seedlings of these two gymnosperms were grown in two [CO₂] (400 µl l⁻¹ and 640 µl l⁻¹) and two temperatures (26/16 °C and 30/20 °C) (day/night) in a sun-lit glasshouse under well-watered conditions. Half of the seedlings in each gymnosperm species were then subjected to drought treatment (i.e. irrigation was withheld) until mortality. *Pinus radiata* and *C. rhomboidea* exhibited differing stomatal responses to drought as predicted. Leaf desiccation and catastrophic xylem cavitation eventually occurred in both species at mortality. Although it was found that depletion of leaf starch were offset by increases of leaf soluble sugar in *C. rhomboidea*, carbon starvation was not evident and thereby hydraulic failure played the primary role during drought-induced mortality in both species. Elevated temperature treatments led to faster mortality in *P. radiata*, associated with more rapid declines in leaf water potential, but did not modify the time-to-mortality in *C. rhomboidea*. Furthermore, elevated [CO₂] alone did not generally alter physiological responses (i.e. gas exchange and leaf water potential) to progressive drought or modify time-to-mortality in either species. Elevated [CO₂] did not ameliorate the negative effects of elevated temperature treatments on drought mortality. These findings indicate that elevated temperature
and associated higher VPD were the dominant factors affecting water relations and carbon dynamics during drought-induced mortality and the magnitude of temperature effects may vary depending on species.

**Chapter 5** synthesised the major findings in my PhD research. The study confirmed that hydraulic failure was the dominant mechanism generating tree mortality during severe droughts across functional groups. Increasing temperature (ambient + 4 °C) and consequent higher VPD exacerbated drought stress and led to more rapid mortality through hydraulic failure in most species in this study. Rising [CO$_2$] (ambient + 240 µl l$^{-1}$) ameliorated moderate drought stress in *E. globulus*, but the positive effects of rising [CO$_2$] were eliminated by increasing drought intensity. Further, elevated [CO$_2$] did not ameliorate drought stress in *E. radiata*, *P. radiata* and *C. rhomboidea* or delay the time-to-mortality. These results suggest that elevated [CO$_2$] may not ameliorate drought stress in these tree species, particularly when drought is prolonged and severe. Elevated [CO$_2$] partially offset the negative effects of elevated temperature during moderate drought stress in *E. globulus*, but did not ameliorate drought response to elevated temperature in the other three species in this study, suggesting that rising temperatures and associated higher VPD may be the predominant contributing factors to drought mortality in future climates, independent of atmospheric CO$_2$. 
The following papers from my PhD work have been published or submitted to peer-reviewed journals:

**Chapter 2:** Duan H, Amthor JS, Duursma RA, O’Grady AP, Choat B, Tissue DT. 2013. Carbon dynamics of eucalypt seedlings exposed to progressive drought in elevated [CO$_2$] and elevated temperature. *Tree Physiology*, **33**, 779-792.


Chapter 2

Carbon dynamics of *Eucalypt globulus* seedlings exposed to progressive drought in elevated [CO₂] and elevated temperature

2.1 Introduction

Drought and elevated temperatures have been identified as altering ecosystem structure world-wide (Allen *et al*., 2010), but the potential role of associated increased VPD has been recently indentified (Eamus *et al*., 2013). Progressive drought may influence patterns of tree seedling and sapling establishment and has lasting effects on the composition, dynamics, and carbon balance of forests (Smith, 2011). Loss of seedlings and young trees can profoundly affect ecosystem structure by differentially affecting species demography and community composition, thereby altering patterns of vegetation succession (Jentsch *et al*., 2007). Furthermore, progressive drought may affect reforestation projects, where thousands of newly planted saplings die within the first few years of planting (Harper *et al*., 2009). In a worst-case scenario, extreme drought may generate forest dieback, potentially converting forests from a net carbon sink into a large carbon source (Lewis, 2006; Phillips *et al*., 2009; Choat *et al*., 2012). Despite the importance of understanding the
impact of variable climate on the most drought-susceptible life stages (seedlings and saplings) of trees, we have little information concerning interactive effects of rising atmospheric CO$_2$ concentration ([CO$_2$]) and warming on seedling response to progressive drought.

Drought constrains plant physiology and productivity (Pockman & Sperry, 2000; Ciais et al., 2005; Engelbrecht et al., 2007) through reductions in growth (Hsiao, 1973; Hsiao et al., 1976) and photosynthesis (Hsiao, 1973; Chaves, 1991) and by altering storage and utilization of carbon reserves (e.g. TNC) (Tissue & Wright, 1995; Körner, 2003; Würth et al., 2005). In general, growth is most sensitive to drought, followed by photosynthesis and then respiration (e.g. Boyer, 1970; Hsiao, 1973; Hsiao et al., 1976; Wilson et al., 1980; McCree et al., 1984; Munns, 1988; Bogeat-Triboulot et al., 2007). In the early phases of drought, TNC concentration often increases because growth declines before photosynthesis (e.g. Körner, 2003; Würth et al., 2005; Ayub et al., 2011). With progressive drought, however, photosynthesis may decline before (or to a greater extent than) respiration, resulting in reduced TNC concentration through less favourable balance of carbohydrate production and use (see Hsiao et al., 1976; Amthor & McCree, 1990; McDowell, 2011). However, recent experimental studies suggested that there is considerable variability and complexity in responses of TNC to drought, due to differences between species, organs and environmental conditions (Piper, 2011; Sala et al., 2012; Adams et al., 2013; Galvez et al., 2013; Hartmann et al., 2013a). Overall, plant sensitivity to drought is primarily governed by alterations in plant water balance (i.e. water supply vs. water use), carbon balance (i.e. photosynthesis, respiration, and TNC use), and plant strategies in balancing water loss with carbon gain.

Most climate models predict that the frequency, intensity and duration of drought
events will increase with rising [CO$_2$] and warming (IPCC, 2007; Allison et al., 2011). Yet, the interactive effect of these three climate factors is difficult to predict given that elevated [CO$_2$] and temperature often have complex effects on plant growth, gas exchange and TNC reserves. For example, under well-watered conditions, elevated [CO$_2$] can increase whole-plant leaf area, total productivity, photosynthesis and TNC concentration, but often decreases $g_s$ and does not affect leaf respiration (Ghannoum et al., 2010ab; Ayub et al., 2011; Wertin et al., 2012a). Under drought, elevated [CO$_2$] can increase photosynthesis and total TNC storage (Wertin et al., 2010; Ayub et al., 2011), but could also increase total plant water loss through greater leaf area (Zeppel et al., 2012). On the other hand, warming can increase leaf area, productivity and respiration, but not affect leaf photosynthesis, and reduce TNC concentration under well-watered conditions (Ghannoum et al., 2010ab; Ayub et al., 2011). Under drought, warming can decrease photosynthesis and TNC storage (see Chaves, 1991; Allen et al., 2010), while similarly increasing potential water loss. Overall, elevated [CO$_2$] has usually been observed to ameliorate drought stress on plant physiological traits (i.e. growth, gas exchange and TNC reserves), but warming has often been reported to exacerbate drought stress. However, combined effects of elevated [CO$_2$] and warming on plants under drought may vary depending on the trade-offs between [CO$_2$] and temperature and the interaction among the three factors. To-date, very few studies have addressed these important interactive factors (Wertin et al., 2010; Wertin et al., 2012a; Zeppel et al., 2012) and results are inconclusive. For instance, the combination of elevated [CO$_2$] and warming did not modify drought stress in Pinus taeda L. seedlings (Wertin et al., 2012a), but it exacerbated drought stress in Eucalyptus sideroxylon A. Cunn. ex Woolls seedlings (Zeppel et al., 2012).
This study had two main objectives. The first objective was to demonstrate the effects of progressive drought on carbon dynamics (i.e. growth, photosynthesis, leaf respiration and TNC reserves) in *Eucalyptus globulus* seedlings. The second objective was to determine whether the main and interactive effects of elevated [CO$_2$] and elevated temperature would alter carbon dynamics in response to progressive drought.

This study examined the following hypotheses: (1) Progressive drought would lead to limitations in growth, photosynthesis, and respiration, and organ and whole-plant TNC concentration would be progressively depleted when carbon use exceeded carbon assimilation; (2) Elevated [CO$_2$] would ameliorate drought stress by increasing photosynthesis (carbon gain) and TNC content (carbon storage); (3) Elevated temperature would exacerbate drought stress by increasing leaf respiration (carbon use) and depleting TNC content; and (4) Interactive effects of elevated [CO$_2$] and elevated temperature would generate the same drought response as ambient [CO$_2$] and temperature plants, given the counteractive effects of elevated [CO$_2$] and temperature on plant growth, photosynthesis, respiration and TNC content.

2.2 Materials and Methods

2.2.1 Plant material and previous growth conditions

*Eucalyptus globulus* Labill. (*E. globulus*) seedlings were raised in forestry tube stock under ambient [CO$_2$] and temperature conditions. A month later (in early October 2010), seventy-two seedlings (six-month old, 30 cm tall) were transplanted into 5 L cylindrical pots filled with 9 kg of air-dried loamy-sand soil. Seedlings were then grown in twelve sun-lit and enclosed whole-tree chambers (WTCs) in the field and
maintained at two [CO2] concentrations (400 µl l⁻¹ and 640 µl l⁻¹) and two temperatures (ambient and ambient + 3 °C), with three replicates for each [CO2] and temperature combination. The WTCs are described in Barton et al. (2010). On 15 November 2010 (Day 0), for each of four [CO2] and temperature combinations, six seedlings were randomly selected as well-watered controls (watered daily to field capacity). Six seedlings per treatment were subjected to a sustained drought from 15 November to 3 December 2010 (Day 18), and then maintained as well watered (designated as rewatered drought plants). The remaining six seedlings per treatment were subjected to a sustained drought and designated as sustained drought plants. The sustained drought was achieved by the controlled addition of small amounts of water to maintain leaf gs in the range 0.05-0.10 mol m⁻² s⁻¹, because gs is a good indicator of plant and leaf water stress (Ayub et al., 2011). By controlling gs, similar drought stress was established across [CO2] and temperature treatments, allowing me to assess the direct effects of [CO2] and temperature on plant response at a standardised drought condition. This technique was successfully used in past drought experiments in the glasshouse (see Ayub et al., 2011).

2.2.2 Glasshouse conditions and drought treatment

On 23 December 2010 (Day 38), all seedlings were moved to four adjacent compartments in a nearby sun-lit and fully enclosed glasshouse and exposed to similar [CO2] concentrations (400 µl l⁻¹ and 640 µl l⁻¹) and temperatures (28/17 °C and 32/21°C) (day/night). Seedlings were then grown in the glasshouse until the end of the experiment. In each of the four [CO2] and temperature treatments, there were six well-watered plants, six rewatered drought plants, and six sustained drought plants. Over the course of 24 h, temperatures in the glasshouse compartments were changed five times to simulate temperature variation naturally observed in the field.
The mean temperatures during mid-day and mid-night for the two temperature treatments were 28/17 °C and 32/21°C (day/night), respectively. Within each temperature treatment, the two compartments were automatically regulated to maintain ambient (400 µl l⁻¹) and elevated (640 µl l⁻¹) [CO₂], respectively. These treatments are abbreviated as: \( C_aT_a \) (400 µl l⁻¹, 28 °C), \( C_aT_e \) (400 µl l⁻¹, 32 °C), \( C_eT_a \) (640 µl l⁻¹, 28 °C) and \( C_eT_e \) (640 µl l⁻¹, 32 °C). Detailed descriptions of the glasshouse and growth conditions are in Ghannoum *et al.* (2010a). Only one glasshouse compartment was available for each of the four [CO₂] and temperature treatments, which lacked true replication of [CO₂] and temperature treatment. To minimise the potential effects of glasshouse compartments, plants were moved among the four compartments weekly. Meanwhile, plants were also rotated within each treatment to minimise possible impacts of varying environmental effects within compartments on plant performance.

During the period 23 December 2010 (Day 38) to 16 January 2011 (Day 62), well-watered and rewatered drought plants were kept well-watered, while sustained drought plants were subjected to the same watering regime outlined above. From 17 January 2011 (Day 63), a new watering regime was imposed (Figure 2-1). Well-watered plants were kept well-watered as before, while rewatered drought plants and sustained drought plants were subjected to a progressive drought with increasing intensity, ranging from the early, moderate drought phase (i.e. Day 63 to Day 79) to the final, extreme drought phase (i.e. Day 79 to Day 103). Because rewatered drought and sustained drought plants experienced different previous watering regimes, whether antecedent conditions affected the response of plants to progressive drought was examined (Figure 2-1). The progressive drought was imposed in rewatered drought and sustained drought treatments by replacing 80-90 % of total
Gravimetric water loss over the course of one full day. Watering of drought plants was discontinued when the daily water additions were lower than 20 ml (from Day 90 to Day 95). In summary, the three water treatments in this study were simply defined as follows: (1) **well-watered**: watered to field capacity on Day 0- Day 103; (2) **rewatered drought**: small amount of water added causing drought (Day 0- Day 18), watered to field capacity (Day 19- Day 62), and then subjected to progressive drought (Day 63- Day 103); (3) **sustained drought**: small amount of water added causing drought (Day 0- Day 62), and then subjected to progressive drought (Day 63- Day 103) (Figure 2-1). Soil volumetric water content (VWC) (m$^3$ m$^{-3}$) at a depth of 30 cm was measured by time domain reflectometer (TDR) probes (CS616, Campbell Scientific, Logan, UT). All measurements were logged every hour, and data were stored in a digital data logger (CR3000, Campbell Scientific, Logan, UT).
Figure 2-1 The watering regime across [CO₂] and temperature treatments during the previous growth period (Day 0- Day 62) and experimental period (Day 63- Day 103). Rewatered drought and sustained drought plants were subjected to different previous water conditions. The experimental period was separated into “moderate drought stage” (Day 63- Day 79) and “extreme drought stage” (Day 79- Day 103). The three water treatments in this study were simply defined as follows: (1) well-watered: watered to field capacity on Day 0- Day 103; (2) rewatered drought: small amount of water added causing drought (Day 0- Day 18), watered to field capacity (Day 19- Day 62), and then subjected to progressive drought (Day 63- Day 103); (3) sustained drought: small amount of water added causing drought (Day 0- Day 62), and then subjected to progressive drought (Day 63- Day 103). The “progressive drought” was achieved by replacing 80-90 % of total gravimetric water loss over the course of one full day. The two drought treatments received no water after Day 95.
2.2.3 Gas exchange measurements (leaf photosynthesis and respiration)

Leaf net photosynthesis at saturating light (PPFD of 2000 μmol m$^{-2}$ s$^{-1}$) ($A_{\text{sat}}$, μmol m$^{-2}$ s$^{-1}$) was measured using a portable open path gas exchange system (Licor-6400, Li-Cor, Lincoln, NE, USA) supplying photosynthetic photon flux density (PPFD) with a red-blue light source (6400-02B). Measurements were taken at mid-day (between 1000 h and 1400 h) on Days 63, 70, 79, 95 and 103. One recent, fully expanded leaf on each plant was measured at growth [CO$_2$] (400 μl l$^{-1}$ or 640 μl l$^{-1}$) and mid-day growth temperature (28 °C or 32 °C). Leaf night respiration rate ($R_n$) was measured at night-time (2 hours after sunset), following the daytime measurement of $A_{\text{sat}}$ on the same leaf at zero PPFD, growth [CO$_2$] (400 μl l$^{-1}$ or 640 μl l$^{-1}$) and night-time growth temperature (17 °C or 21°C). Replicates (plants) for $A_{\text{sat}}$ and $R_n$ varied from six to two due to sequential harvests during the experimental period, i.e. Day 63 n = 6; Day 70 n = 5; Day 79 n = 4; Day 95 n = 3; Day 103 n = 2.

2.2.4 Plant dry mass production

Following each day of leaf gas exchange measurements, one seedling from each of the three water treatments (well-watered; rewatered drought; sustained drought) in all four [CO$_2$] and temperature treatments was randomly selected for harvest; overall, twelve seedlings were harvested each time. Height ($H$, cm) was measured from the stem base to the highest shoot tip, and basal diameter ($D$, cm) was measured at 1cm height, in all seedlings. At each harvest, seedlings were separated into leaves, stems and roots. Roots were washed free of soil. Subsequently, all harvested plant organs were dried in an oven at 70°C for 72 h and weighed.

From the harvested plants, linear regressions were developed to estimate dry mass ($W$) of all plant organs. There was no treatment effect on any of the regressions,
therefore the data was pooled. The linear regressions were:

\[ \ln (W_{\text{leaf}}) = 0.615 \times \ln (D^2H) + 0.360 \quad (R^2 = 0.912, P < 0.001, n = 44); \]

\[ \ln (W_{\text{stem}}) = 0.758 \times \ln (D^2H) + 0.735 \quad (R^2 = 0.931, P < 0.001, n = 47); \]

\[ \ln (W_{\text{root}}) = 0.456 \times \ln (D^2H) + 0.774 \quad (R^2 = 0.905, P < 0.001, n = 27). \]

Replicates (plants) for estimated dry mass varied from six to two during the experimental period, i.e. Day 63 n = 6; Day 70 n = 5; Day 79 n = 4; Day 95 n = 3; Day 103 n = 2.

2.2.5 TNC assay

Oven-dried plant organ samples were ground to fine powder in a ball mill. Organ samples (20 mg) were weighed and then extracted with 5 ml of 80% aqueous ethanol (v/v) in a polyethylene tube. The mixture was boiled in a water bath at 95°C for 30 min, and then centrifuged at 3000 rpm for 5 min. The supernatant was collected and the pellet re-extracted once with 5 ml of 80% aqueous ethanol (v/v) and once with 5 ml of distilled water, then boiled and centrifuged as before. The supernatants were reserved and evaporated to the last 1-3 ml in a Rotational Vacuum Concentrator at 40°C. Total starch was determined on the pellets remaining after the ethanol and water extractions, and assayed enzymatically using a total starch assay kit (Megazyme, Australia). Total soluble sugar was determined on the supernatants by anthrone method (Ebell, 1969). TNC concentration was calculated as the sum of starch (mg g\(^{-1}\)) and soluble sugar (mg g\(^{-1}\)) concentrations. Whole-plant TNC content (g) was calculated as the sum of the TNC content of the three plant organs (leaf, stem and root). Whole-plant starch or soluble sugar concentration (mg g\(^{-1}\)) was calculated as whole-plant starch or soluble sugar content (mg) divided by plant dry mass (g).
2.2.6 Statistical analyses

Leaf gas exchange, plant dry mass and whole-plant TNC content were analysed using a repeated measures three-way ANOVA to test temporal effects of the water, [CO₂] and temperature treatments on these factors. Subsequently, those parameters were analysed by a repeated measures two-way ANOVA to test temporal effects of [CO₂] and temperature treatments within each water treatment. Data were tested for normality and homogeneity of variance prior to the analysis and all data passed the tests. Subsequently, a three-way ANOVA was used to test factor (water, [CO₂] and temperature) effects on each measurement day (i.e. Days 63, 70, 79, 95 and 103). When the water treatment was significant, a two-way ANOVA was conducted to test [CO₂] and temperature treatment effects on previous parameters within each water treatment. For organ TNC concentration, which was determined during each harvest period on one plant in each water treatment, [CO₂] and temperature treatment combination (without replicates), a two-way ANOVA without replication was used to test main factor effects on organ TNC concentration. The Duncan post-hoc test was used to compare [CO₂] and temperature treatment means. All results were considered to be significant if $P \leq 0.05$. Statistical analyses were conducted using SPSS (version 18.0; SPSS, Chicago, IL, USA) except for analyses of two-way ANOVA without replication using Microsoft Excel 2007.

2.3 Results

2.3.1 Leaf photosynthesis and respiration

At the beginning of the experiment, leaf $A_{sat}$ and $R_n$ of well-watered plants were similar to rewatered drought plants across [CO₂] and temperature treatments, but
were 260 % and 30 % higher than sustained drought plants, respectively ($P < 0.001$ for $A_{sat}$ and $R_n$) (Figure 2-2). Leaf $A_{sat}$ and $R_n$ in both drought treatments declined gradually to zero with progressive drought (Figure 2-2), but leaf $R_n$ was less sensitive to drought than $A_{sat}$ (Figure 2-3).

In the well-watered treatment (Figures 2-2a and d), elevated [CO$_2$] significantly increased $A_{sat}$ during the experiment (Two-way ANOVA repeated measures: $P = 0.03$), while elevated temperature did not decrease $A_{sat}$ except for Day 63 ($P = 0.001$; Day 63). By contrast, elevated [CO$_2$] and elevated temperature did not have consistent impacts on leaf $R_n$ (Two-way ANOVA repeated measures: $P > 0.373$ for both cases).

During the moderate drought phase (i.e. Day 63 to Day 79), elevated [CO$_2$] increased $A_{sat}$ of rewated drought plants at elevated temperature (i.e. $C_eT_e > C_aT_a$; $P < 0.05$), but it did not affect $A_{sat}$ of sustained drought plants (Figures 2-2b and c). Meanwhile, elevated temperature reduced $A_{sat}$ in the two drought treatments at both [CO$_2$] levels ($P < 0.002$ for all cases). In the sustained drought plants, $A_{sat}$ was lower in $C_eT_e$ and $C_aT_a$ ($P < 0.05$). During the moderate drought phase, elevated temperature increased leaf $R_n$ of rewated drought and sustained drought plants (Figures 2-2e and f; $P < 0.001$ for both on Day 70). In both drought treatments, leaf $R_n$ was higher in $C_eT_e$ than $C_aT_a$ ($P < 0.05$). During the extreme drought phase (i.e. Day 79 to Day 103), when soil VWC was very low (i.e. soil VWC $< 0.05$ m$^3$ m$^{-3}$), the impacts of elevated [CO$_2$] and elevated temperature on leaf $A_{sat}$ and $R_n$ diminished when $A_{sat}$ and $R_n$ approached zero (Figures 2-2 and 2-3). These results demonstrated that leaf $A_{sat}$ and $R_n$ in elevated [CO$_2$] and elevated temperature were modified by the intensity of the drought, essentially disappearing in the extreme drought phase.
Figure 2-2 Effects of atmospheric [CO₂] (400 µl l⁻¹ and 640 µl l⁻¹) and growth temperature (28 °C and 32 °C) on (a, b, c) leaf net photosynthesis at saturating light ($A_{sat}$) and (d, e, f) leaf night respiration ($R_n$) of *Eucalyptus globulus* seedlings in different watering regimes (“well-watered”, “rewatered drought” and “sustained drought”) during the glasshouse experimental period (Day 63 to Day 103). Values are Means ± SE (n = 2-6). More details about watering regime refer to Figure 2-1.
Figure 2-3 (a) Leaf net photosynthesis at saturating light ($A_{sat}$) and (b) leaf night respiration ($R_n$) of *Eucalyptus globulus* seedlings as a function of soil volumetric water content (at a depth of 30 cm) in four [CO$_2$] and temperature combinations during the glasshouse experimental period (Day 63 to Day 103). Each curve is a non-linear fit with all data across watering regimes (“well-watered”, “rewatered drought” and “sustained drought”) in each [CO$_2$] and temperature combination. Data points are the means of the measured variables. More details about watering regime refer to Figure 2-1.
2.3.2 Plant dry mass

At the beginning, plant dry mass in well-watered treatment was 20 % larger than that in rewatered drought treatments and 50 % larger than that in sustained drought treatment across [CO₂] and temperature treatments ($P < 0.001$) (Figures 2-4a, b and c). Dry mass accumulation in the well-watered treatment increased throughout the experimental period, while dry mass accumulation in the two drought treatments was limited by drought (Three-way ANOVA repeated measures: $P < 0.001$) (Figures 2-4a, b and c).

In the well-watered treatment, elevated [CO₂] did not significantly affect plant dry mass over the experimental period, while elevated temperature increased dry mass on Day 63 and Day 70 ($P < 0.031$ for both dates) (Figure 2-4a). During the moderate drought phase, elevated [CO₂] significantly increased plant dry mass accumulation in the two drought treatments on Day 63 and Day 70 ($P < 0.035$ for all cases), but the impacts diminished in the extreme drought phase (Figures 2-4b and c). Elevated temperature did not significantly affect dry mass accumulation in drought treatments over the experimental period. In both drought treatments, plant dry mass was larger in $C_eT_e$ than $C_aT_a$ ($P < 0.05$; Day 63 and Day 70).
Figure 2-4 Effects of atmospheric [CO₂] (400 µl l⁻¹ and 640 µl l⁻¹) and growth temperature (28 °C and 32 °C) on (a, b, c) plant dry mass and (d, e, f) whole-plant TNC content of *Eucalyptus globulus* seedlings in different watering regimes (“well-watered”, “rewatered drought” and “sustained drought”) during the glasshouse experimental period (Day 63 to Day 103). Values are Means ± SE (n = 2-6). In (d), the value of treatment (640 µl l⁻¹, 28 °C) on Day 79 was missed. More details about watering regime refer to Figure 2-1.
2.3.3 Whole-plant TNC content

At the start of the experiment, whole-plant TNC content in the well-watered treatment was similar to that in the rewatered drought treatment, but was 60 \% higher than that in the sustained drought treatment across [CO₂] and temperature treatments \((P < 0.001)\) (Figures 2-4d, e and f). Whole-plant TNC content accumulation in the well-watered treatment increased over time, but TNC content accumulation in both drought treatments was limited by drought, similar with the trend of dry mass (Three-way ANOVA repeated measures: \(P < 0.001\)).

In the well-watered treatment, elevated [CO₂] did not have consistent effects on whole-plant TNC content over the experimental period, but elevated temperature generally increased whole-plant TNC content \((P < 0.001\) for Day 70 and Day 79) (Figure 2-4d). In the rewatered drought treatment, elevated [CO₂] increased whole-plant TNC content in elevated temperature (i.e. \(C_eT_e > C_aT_e; P < 0.05\)) throughout the experimental period (Figure 2-4e). Elevated temperature reduced whole-plant TNC content of rewatered drought plants in ambient [CO₂] (i.e. \(C_aT_e < C_aT_a; P < 0.05\)). Additionally, whole-plant TNC content of rewatered drought plants was similar in \(C_eT_e\) and \(C_aT_a\) \((P > 0.05)\). In the sustained drought treatment, elevated [CO₂] increased whole-plant TNC content at both temperature treatments \((P < 0.05\) for all cases), but elevated temperature did not significantly affect TNC content (Figure 2-4f). Whole-plant TNC content of sustained drought plants was greater in \(C_eT_e\) than \(C_aT_a\) \((P < 0.05;\) except for Day 95).

2.3.4 Organ and whole-plant starch and soluble sugar concentration

Across [CO₂] and temperature treatments, leaf starch concentration in the well-watered treatments increased over time. Conversely, leaf starch concentration was
significantly depleted by drought at the end of the experiment ($P < 0.001$; Day 103) (Table A-1). For rewatered drought plants, leaf starch concentration accumulated initially (130 % higher than well-watered plants; Day 63) and then declined dramatically during the drought period to $< 10$ % of well-watered plants at the end of the drought (Figure 2-5a). For sustained drought plants, leaf starch concentration was lower than well-watered plants throughout the experimental period, but declined to $< 10$ % of well-watered plants at the end of the experiment. Leaf soluble sugar concentration in both drought treatments increased in absolute value and in relative ratio with progressive drought, compared with well-watered plants ($P = 0.03$; Day 103) (Table A-1; Figure 2-5e). Stem starch concentration was generally similar for well-watered and both drought treatment plants, but soluble sugar concentration was approximately 20-50 % higher in drought plants compared to well-watered plants during the experimental period (Table A-1; Figures 2-5b and f). Root starch concentration was highly variable, but also similar for well-watered and drought treatment plants (Figure 2-5c), while root soluble sugar concentration was up to 100 % higher in drought plants (Figure 2-5g). Relative ratios of whole-plant TNC concentration showed similar trends with leaf TNC concentration (Figures 2-5d and h) because leaf TNC concentration accounted for about 75 % of plant TNC (Table A-3). Further, whole-plant TNC concentration in well-watered and drought plants increased similarly (i.e. not different from each other) over time (Table 2-1). In contrast, despite the similar whole-plant TNC concentration, whole-plant TNC content was lower in drought plants compared with well-watered plants because plant dry mass was lower in drought plants relative to well-watered plants (Figure 2-4 and Table 2-1).
Elevated [CO₂] did not affect leaf starch concentration in the *well-watered* treatment, but increased leaf starch concentration in *rewatered drought* treatment (Two-way ANOVA without replication: \( P = 0.034 \); Day 95). Elevated temperature decreased leaf starch concentration in *rewatered drought* treatment (Two-way ANOVA without replication: \( P = 0.042 \) and \( P = 0.021 \); Day 70 and Day 95, respectively). However, elevated [CO₂] and elevated temperature did not affect leaf soluble sugar concentration.
Figure 2-5 (a) Leaf starch concentration, (b) stem starch concentration, (c) root starch concentration, (d) whole-plant starch concentration, (e) leaf soluble sugar concentration, (f) stem soluble sugar concentration, (g) root soluble sugar concentration and (h) whole-plant soluble sugar concentration across four [CO₂] and
temperature combinations of *Eucalyptus globulus* seedlings expressed as relative ratios of drought treated plants (i.e. rewatered drought plants and sustained drought plants) to well-watered plants (control) during the glasshouse experimental period (Day 63 to Day 103). Parameters (% of well-watered control) = (drought treated parameters /well-watered parameters) \times 100\%. Values are Means ± SE (n = 4). The 4 seedling replicates were from [CO2] and temperature combination treatments (i.e. $C_aT_a$, $C_aT_e$, $C_eT_a$ and $C_eT_e$). The dash lines represent the relative values of well-watered control, indicating no effect of drought on measured parameters. More details about watering regime refer to Figure 2-1.
Table 2-1 Temporal changes of measured parameters in different watering regimes ("well-watered", "rewatered drought" and "sustained drought") during the glasshouse experimental period (Day 63 to Day 103). “▲” means “increased with time” and “▼” means “decreased with time”. “----” means “no significant temporal changes”. Data were pooled across [CO₂] and temperature treatments.

<table>
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<th>Sustained drought</th>
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<td>Whole-plant TNC content</td>
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Note More details about watering regime refer to Figure 2-1.
2.4 Discussion

Progressive drought performed similarly in rewatered drought and sustained drought treatments, leading to limitations in growth, photosynthesis and respiration (Table 2-1; Figures 2-2 and 2-4). However, drought treatments did not generally decrease organ and whole-plant TNC concentration compared with the well-watered control (Tables 2-1 and A-1; Figure 2-5). This led to the partial rejection of the first hypothesis that TNC concentration would be progressively depleted by drought, although significant conversion of starch into soluble sugar occurred at the leaf and whole-plant levels. In partial agreement with the hypotheses, elevated [CO₂] ameliorated the moderate drought stress imposed on E. globulus seedlings by increasing photosynthesis and enhancing leaf and whole-plant TNC reserves (Figures 2-6b and c; Table A-1). In contrast, elevated temperature exacerbated moderate drought stress by reducing photosynthesis, increasing leaf respiration, and decreasing leaf and whole-plant TNC reserves (Figures 2-6b and c). However, extreme drought eliminated [CO₂] and temperature effects on plant growth, photosynthesis and respiration (Figures 2-6d and e). The combined effects of elevated [CO₂] and elevated temperature on moderate drought stressed seedlings were reduced with progressive drought, with no sustained effects on growth despite greater whole-plant TNC content (Figure 2-6).
Figure 2-6 Mean percentage changes of key measured parameters ($A_{\text{sat}}$, $R_n$, plant dry mass and whole-plant TNC content) of *Eucalyptus globulus* seedlings in growth [CO$_2$] and temperature (i.e. 400 µl l$^{-1}$, 32 °C; 640 µl l$^{-1}$, 28 °C; 640 µl l$^{-1}$, 32°C) relative to ambient [CO$_2$] and temperature (i.e. 400 µl l$^{-1}$, 28 °C control) during the experimental period. (a) In the well-watered treatment, mean percentage changes of
the four key measured parameters were averaged from percentage changes on each measured date during the whole experimental period (i.e. Day 63 to Day 103). In *rewatered drought* and *sustained drought* treatments, the intensity of drought was separated into “moderated drought phase” (i.e. Day 63 to Day 79) and “extreme drought phase” (i.e. Day 79 to Day 103), respectively. Thus, mean percentage changes of the four key measured parameters were averaged from percentage changes on each measured date during (b, c) moderate drought phase and (d, e) extreme drought phase, respectively. Percentage changes were expressed as relative ratios of growth [CO₂] and temperature combinations to ambient [CO₂] and temperature. Parameters (% of 400 μl l⁻¹, 28 °C control) = (parameters in growth [CO₂] and temperature combinations / parameters in ambient [CO₂] and temperature) ×100%. The dash lines represent \( C_a T_a \) (400 μl l⁻¹, 28 °C). \( C_a \) and \( C_e \) indicate 400 μl l⁻¹ and 640 μl l⁻¹, respectively. \( T_a \) and \( T_e \) indicate 28 °C and 32 °C, respectively. “↑” means “increased” and “↓” means “decreased”. For instance, “\( C_e \uparrow \)” means elevated [CO₂] increased the measured parameters. The four treatments were \( C_a T_a \) (400 μl l⁻¹, 28 °C), \( C_a T_e \) (400 μl l⁻¹, 32 °C), \( C_e T_a \) (640 μl l⁻¹, 28 °C) and \( C_e T_e \) (640 μl l⁻¹, 32 °C). More details about watering regime refer to Figure 2-1.
2.4.1 TNC concentration was not reduced during drought stress

In contrast to the first hypothesis, I did not observe declines in organ or whole-plant TNC concentration of drought plants, regardless of previous water conditions (i.e. rewatered drought and sustained drought) (Tables 2-1 and A-3; Figure 2-5). Several factors may explain this disparity in TNC between the expectation and experimental results. First, the direct effect of drought stress on maintenance respiration varies, which may be reduced (Wilson et al., 1980; Amthor & McCree, 1990) or increased (Flexas et al., 2005; Callister & Adams, 2006; Adams et al., 2009) by drought. In E. globulus, the measured leaf $R_n$ declined slowly with progressive drought, dropping to zero in the last 7 days, indicating that the minimal impact of drought stress on respiration greatly reduced TNC consumption, leading to a TNC surplus. Second, soluble sugar may serve functions beyond that of a respiratory carbon source during drought (Sala et al., 2010; McDowell, 2011; McDowell et al., 2011; Sala et al., 2012), such as osmotic adjustment (Hsiao et al., 1976; Morgan, 1984; Munns, 1988) and hydraulic transport (Salleo et al., 2009; McDowell, 2011; Sala et al., 2012). In drought stress, soluble sugar accumulates in the vacuole and cytosol leading to osmotic adjustment to maintain cell turgor. However, soluble sugar utilised in osmotic adjustment may not be available to meet metabolic demand, which may lead to reductions in consumption of TNC. Sala et al. (2012) also argued that distinguishing the passive vs. active roles of TNC can improve our understanding of trees in responses to drought stress, although these issues remain unsolved.

2.4.2 Elevated [CO$_2$] ameliorated moderate drought stress

Elevated [CO$_2$] ameliorated drought stress by increasing photosynthesis and plant dry mass when drought stress was moderate (Day 63 to Day 79) (Figures 2-6b and c).
This is consistent with several studies showing that elevated [CO$_2$] mitigates the negative effects of drought on plant growth, carbon exchange and leaf water status (Atwell et al., 2007; Robredo et al., 2007; Wertin et al., 2010; Albert et al., 2011; Wertin et al., 2012a; Perry et al., 2013). Carbon storage reserves in the form of TNC (starch and soluble sugar) provide some evidence for the capacity of plants to withstand drought (Picon et al., 1997; Körner, 2003), although data exploring the impacts of elevated [CO$_2$] on the accumulation of TNC during drought are generally lacking (Allen et al., 2010; Niinemets, 2010). The results showed that elevated [CO$_2$] resulted in higher leaf TNC concentration and whole-plant TNC content in drought-treated seedlings (Table A-1; Figures 2-6b and c), which has been observed in other studies (Körner, 2003; Niinemets, 2010; Ayub et al., 2011). For example, in *Eucalyptus saligna* Sm. seedlings exposed to a sustained 30-day drought, leaf starch accumulation was substantially higher in elevated [CO$_2$] compared with ambient [CO$_2$] (Ayub et al., 2011), reflecting the commonly observed increase in starch storage resulting from disparity between carbon supply and carbon use (e.g. sink limitation) (Körner, 2003). A potential advantage to increased starch accumulation, and subsequent conversion to soluble sugar during progressive drought, is that the accumulation of soluble sugar may have an active role in osmotic adjustment and therefore be beneficial to plant growth and photosynthesis under drought (Chaves & Pereira, 1992; Körner et al., 1995; Wullschleger et al., 2002).

2.4.3 **Elevated temperature exacerbated moderate drought stress**

Elevated temperature and drought commonly interact to negatively affect plant functioning (Machado & Paulsen, 2001; Vile et al., 2011). Higher transpiration rates due to elevated temperature often increase the negative impacts of drought on plants.
(Sinclair et al., 2007; Allen et al., 2010; Vile et al., 2011). Similarly, I observed that
drought plants grown under elevated temperature had lower photosynthesis and
higher leaf respiration during the moderate drought phase (Figures 2-6b and c),
indicating that elevated temperature exacerbated moderate drought stress by
decreasing carbon assimilation and increasing carbon loss. In *E. globulus*, elevated
temperature also reduced leaf TNC concentration and whole-plant TNC content in
drought plants, particularly in *rewatered drought* plants (Figure 2-6b), compared
with ambient temperature plants. These effects of elevated temperature on plant traits
(i.e. photosynthesis, respiration and TNC storage) together could reduce plant
capacity to withstand drought. Although it has been proposed that warming
accelerates the rate of carbon depletion due to higher metabolic requirements (Adams
*et al.*, 2009; Tjoelker *et al.*, 2009; Allen *et al.*, 2010), I did not find that elevated
temperature affected the rate of TNC depletion during drought.

2.4.4 **Interactive effects of elevated [CO$_2$] and elevated temperature during
drought**

The interactive effect of elevated [CO$_2$] and elevated temperature on plant growth
and photosynthesis during drought can be complex (Wertin *et al.*, 2010; Albert *et al.*, 2011; Wertin *et al.*, 2012a; Zeppel *et al.*, 2012), because the combination of the two
factors can exacerbate drought stress or have no impacts on drought stress compared
with ambient [CO$_2$] and temperature. In this study, I found only moderate benefits to
plants grown in elevated [CO$_2$] and temperature, in that $C_cT_e$ plants were larger than
$C_aT_a$ plants, but had similar/lower carbon uptake ($A_{sat}$) and greater carbon loss (leaf
$R_n$) during the moderate drought phase (Figure 2-6). Whole-plant TNC in *sustained
drought* seedlings was higher in $C_cT_e$ than $C_aT_a$, suggesting that drought seedlings
may have greater carbon storage to withstand sustained drought stress when grown in elevated [CO₂] and elevated temperature (Figures 2-6c and e). The effects of elevated [CO₂] and temperature were eliminated as drought became more extreme (Figures 2-6d and e). For example, when soil VWC dropped below a threshold value of 0.05 m³ m⁻³, [CO₂] and temperature treatments became irrelevant in determining plant response to drought (Figure 2-3). Similarly, Albert et al. (2011) observed that the pronounced positive effects of elevated [CO₂] on carbon uptake in Calluna vulgaris L. diminished when plants experienced a severe drought. Perry et al. (2013) also found that positive responses of riparian seedling growth and water-use-efficiency (WUE) to elevated [CO₂] were counteracted by the large negative effects of increasing soil aridity.

2.4.5 Conclusions

Progressive drought performed similarly in rewatered drought and sustained drought treatments, reducing leaf gas exchange and limiting growth. These findings suggest that previous water conditions may have little impacts on responses of plant to progressive drought. Yet, in contrast to the expectation, progressive drought did not significantly decrease organ and whole-plant TNC concentration, indicating that it is difficult to predict TNC responses to drought (given variation in intensity and duration) and that additional factors (e.g. drought impacts on respiration and/or functions such as osmotic adjustment and hydraulic transport) may need to be considered. The data indicated that a significant pool of non-structural carbohydrates is maintained for a significant period of drought.

In summary, elevated [CO₂] partially ameliorated the moderate drought stress by increasing photosynthesis, dry mass accumulation and TNC content. Elevated
temperature exacerbated the moderate drought stress by reducing photosynthesis, increasing leaf respiration and decreasing TNC content. The combination of elevated [CO₂] and elevated temperature ameliorated the moderate drought stress with greater dry mass and TNC content. Plants exposed to drought may partially benefit from the combination of elevated [CO₂] and elevated temperature during moderate drought, but these effects on growth vanish when drought becomes extreme.
Chapter 3

Elevated $[\text{CO}_2]$ does not ameliorate the negative effects of elevated temperature on drought-induced mortality in *Eucalyptus radiata* seedlings

3.1 Introduction

Recent evidence suggests that all forest biomes may be vulnerable to drought-induced mortality if the duration or severity of droughts increases with rising global temperatures (Choat *et al.*, 2012). Widespread forest die-offs can alter forest structure and function (Breshears *et al.*, 2005; Fensham *et al.*, 2009; Anderegg *et al.*, 2013), and affect budgets of energy, carbon and water (Breshears & Allen, 2002; Phillips *et al.*, 2009). Drought-induced tree mortality is an important and complex process, yet the underlying mechanisms are not clearly understood. In the context of climate change, physiological processes preceding drought induced mortality could be influenced by both increasing atmospheric $[\text{CO}_2]$ and temperatures (Breshears *et al.*, 2005; Allen *et al.*, 2010; McDowell *et al.*, 2011; Zeppel *et al.*, 2012; Chapter 2), thereby leading to increased complexity in assessing key mortality processes. Recent predictions suggest that the frequency of extreme drought will increase in the next
few decades along with rising \([\text{CO}_2]\) and temperature (IPCC, 2012). In combination, these factors will modify drought-induced mortality in tree seedlings.

Despite numerous potential pathways to mortality, two interdependent mechanisms explaining the physiological basis for drought-induced mortality are most currently assessed (McDowell et al., 2008, 2011). The hydraulic failure hypothesis states that reduced soil water supply and high evaporative demand generate extensive cavitation in xylem conduits impeding water flow, desiccating plant tissue and leading to cellular death (Brodribb & Cochard, 2009; Urli et al., 2013; Choat, 2013). Alternatively, the carbon starvation hypothesis predicts that stomatal closure (in order to prevent hydraulic failure) reduces photosynthetic carbon uptake substantially such that metabolic consumption of carbohydrates (i.e. respiration) eventually depletes stored carbon reserves leading to plant death. Interestingly, this is likely to be further amplified by phloem transport failure among organs (Sala et al., 2010) or increased respiratory carbon losses due to elevated temperature (Atkin & Tjoelker, 2003). Recent evidence has supported the hypothesis that hydraulic failure plays the dominant role during drought-induced tree mortality (Anderegg et al., 2012; Adams et al., 2013; Anderegg & Anderegg, 2013; Hartmann et al., 2013b; Mitchell et al., 2013; Nardini et al., 2013; Quirk et al., 2013; Sevanto et al., 2013), although significant carbohydrate depletion has also been observed (Hartmann et al., 2013a,b; Mitchell et al., 2013; Quirk et al., 2013; Sevanto et al., 2013). However, it remains to be definitively demonstrated that carbon starvation can independently and solely generate mortality during drought. To-date, few studies have addressed the interactive impacts of elevated \([\text{CO}_2]\) and elevated temperature on tree response to drought (Wertin et al., 2010, 2012a,b; Ayub et al., 2011; Zeppel et al., 2012; Lewis
et al., 2013), and even fewer have addressed the interactive effects of these factors on trees during lethal drought (Chapter 2).

It is well established that elevated [CO$_2$] leads to reduced $g_s$ (Medlyn et al., 2001; Wullschleger et al., 2002) and could therefore theoretically reduce plant water loss and delay drought-induced mortality by allowing plants to maintain more favourable water status during drought. However, existing studies suggest that plant responses to elevated [CO$_2$] and drought stress are highly variable (Atwell et al., 2007; Domec et al., 2010; Wertin et al., 2010, 2012; Ayub et al., 2011; Duursma et al., 2011; Warren et al., 2011; Zeppel et al., 2012; Franks et al., 2013; Lewis et al., 2013; Perry et al., 2013). For example, in some studies, elevated [CO$_2$] can partially moderate the impact of drought stress by increasing photosynthesis (Wertin et al., 2010; Lewis et al., 2013) and carbohydrate reserves (Niinemets, 2010; Tissue & Lewis, 2010; Ayub et al., 2011), thereby providing increased availability of carbon for growth and metabolic activity during mild or moderate drought stress. Yet, this may not occur due to the confounding effects of alterations in plant architecture, including changes in leaf area and root distribution (Duursma et al., 2011; Warren et al., 2011; Zeppel et al., 2012). Therefore, it is difficult to determine whether elevated [CO$_2$] would delay tree mortality unless drought mortality studies are designed to test this hypothesis.

In contrast to elevated [CO$_2$], elevated temperature frequently exacerbates drought stress due to higher rates of water loss and greater respiratory carbon loss (Breshears et al., 2005; Adams et al., 2009; Allen et al., 2010; Williams et al., 2013; Zhao et al., 2013), potentially generating more rapid tree mortality (Adams et al., 2009). Furthermore, the interactive effects of elevated [CO$_2$] and temperature on tree
response to drought and drought-induced mortality remain unresolved (Way, 2013). Elevated [CO₂] and temperature can interactively affect physiological responses of tree seedlings to non-lethal drought stress, synergistically (Zeppel et al., 2012) or additively (Lewis et al., 2013), but these interactive effects may be diminished under lethal drought stress (Chapter 2). In the context of future climate scenarios, experimental, manipulative studies are necessary to quantify the main and interactive effects of elevated [CO₂] and temperature on drought-induced mortality.

Consistent with observations of mortality around the globe (Allen et al., 2010), drought-induced die-off in eucalypt communities has been frequently observed in the past few decades (e.g. Fensham & Fairfax, 2007; Cunningham et al., 2010; Poot & Veneklaas, 2013; Matusick et al., 2013). For instance, extreme drought and heat in 2010/2011 resulted in a 70 % reduction in canopy cover in a Mediterranean-type forest dominated by Eucalyptus marginata Donn ex Sm. in southwestern Australia (Matusick et al., 2013). Given the ecological and economic importance of eucalypts in Australia and world-wide, we require a better understanding of the potential impact of all climate factors (particularly elevated [CO₂], temperature and drought) associated with climate change in eucalypt trees. The primary objective of this study was to investigate the main and interactive effects of elevated [CO₂] and temperature on drought responses and mortality of Eucalyptus radiata Sieber ex DC. Eucalyptus radiata is an ecologically important species widely distributed along the east coast of Australia from south Queensland to Tasmania, and an economically important source of essential eucalyptus oils. In this study, [CO₂], temperature and soil water content were experimentally manipulated. Pot-grown E. radiata seedlings were exposed to two controlled drying and rewatering cycles, and then water was withheld until
plants died. The following hypotheses were tested: (1) Mortality of *E. radiata* seedlings would be primarily driven by hydraulic failure due to the dominant role of hydraulic limitation during drought; (2) At a given soil water content, elevated [CO₂] would ameliorate drought stress and delay seedling mortality, by increasing carbon assimilation, leading to greater carbohydrate accumulation, and improving plant water status through reduced water loss at leaf and whole-plant levels; (3) At a given soil water content, elevated temperature would exacerbate drought stress and accelerate seedling mortality, by reducing carbon assimilation, decreasing carbohydrate storage due to greater carbon metabolism, and increasing water loss due to higher VPD; and (4) At a given soil water content, elevated [CO₂] would ameliorate the negative effects of elevated temperature on seedling response to drought stress because they have opposing effects.

### 3.2 Materials and Methods

#### 3.2.1 Plant material and growth conditions

*Eucalyptus radiata* Sieber ex DC (narrow-leaved peppermint) seedlings were obtained from Greening Australia (Sydney, NSW) and raised in forestry tube stock in a sunlit poly-tunnel under ambient environmental conditions for one month. Thereafter, eighty seedlings (about three months old) were transplanted from trays into PVC pots (15 cm diameter × 40 cm length) containing about 10 kg of air-dried loamy sand soil (86.5 % sand and 9.5 % clay) in summer 2012 (13 January 2012). One seedling was planted into every pot. Twenty pots with seedlings were then randomly placed in each of the four adjacent glasshouse compartments (each 3 m (width) × 5 m (length) × 3.5 m (height)) with natural sunlight (direct light attenuated
by 10-15 %) and [CO₂]/temperature control on the campus of the University of Western Sydney (Richmond, NSW, Australia).

Four glasshouse compartments were used in a factorial [CO₂] and temperature design, with two [CO₂] (400 µl l⁻¹ and 640 µl l⁻¹) and two temperature (ambient and ambient +4 °C/elevated) treatments. Two glasshouse compartments for ambient temperature treatments were set to simulate the 30- year daily average temperature in the growing season (November to May) experienced in Richmond, NSW. Over the course of 24 h, temperature was changed five times to simulate a daily temperature cycle in the field. The other two glasshouse compartments for elevated temperature treatments were designed to simulate a constant 4 °C increase in temperature relative to the ambient daily temperature cycle. The mean growing temperatures for ambient and elevated temperature treatments were 26/16 °C and 30/20 °C (day/night), respectively. For the two compartments within each temperature treatment, [CO₂] treatments were automatically regulated to maintain ambient (target 400 µl l⁻¹) and elevated (target 640 µl l⁻¹) [CO₂], respectively. The target temperature and [CO₂] were adjusted and constantly monitored by the computer control system. Details of glasshouse design can be found in Ghannoum et al. (2010a). The four treatments were termed as follows: CₐTₐ (400 µl l⁻¹, 26 °C), CₐTₑ (400 µl l⁻¹, 30 °C), CₑTₐ (640 µl l⁻¹, 26 °C) and CₑTₑ (640 µl l⁻¹, 30 °C). According to the primary objective, it was not attempted to separate temperature and VPD effects in this study. Therefore, during the experimental period, VPD was not maintained constant among [CO₂] and temperature treatments. VPD in the glasshouse compartments varied between 0.1 and 2.9 kPa in the ambient temperature treatments (mean value: 0.86 ± 0.01 kPa) and between 0.2 and 4.3 kPa in the elevated temperature treatments (mean value: 1.50 ±
0.01 kPa). VPD did not vary between [CO₂] treatments. Seedlings were irrigated daily to field capacity and fertilized on three occasions (17 February, 19 March and 5 May) with a commercial fertilizer (All Purpose, Brunnings, N:P:K- 27:2:10). Seedlings were rotated within and among glasshouse compartments every two weeks to minimise potential effects of position on plant performance.

### 3.2.2 Watering regime

Following two months of additional growth in the four [CO₂] and temperature treatments (i.e. seedlings were five months old), half of the seedlings (ten seedlings) were randomly allocated to the *well-watered* treatment and half of the seedlings to the *drought* treatment within each of the four [CO₂] and temperature treatments. Every pot was weighed in the morning (between 0900 h and 1000 h) to determine water loss every second or third day. *Well-watered* seedlings were maintained at field capacity throughout the experiment by replacing the amount of water loss during the preceding measurement interval. For *drought* seedlings, two drought cycles were imposed to more realistically mimic natural drought events in the field, which often occur as multiple wet-dry cycles (see Figure 3-1). The first cycle of drought (hereafter referred as “first drought phase”) was imposed on 26 March 2012 (Day 1). During the first drought phase, water was withheld in *drought* seedlings until physiological stress was evident based on leaf $g_s$ (defined as $g_s = 0.05$- 0.1 mol m⁻² s⁻¹; see Ayub *et al.*, 2011; Chapter 2). By controlling $g_s$ in this range, drought stress was standardised across [CO₂] and temperature treatments, ensuring that seedlings in all treatments were acclimated to moderate drought before imposing a subsequent more extreme drought. It is assumed here that the relationship between $g_s$ and xylem water potential is identical for all treatments. Subsequently, the progression of soil drying
was slowed down in treatments that used water more rapidly (e.g. elevated temperature) to match the rate of soil drying in less profligate water using treatments (e.g. ambient temperature) by adding small amounts of water (i.e. 150 ml every second or third day) to the soil of these higher water using seedlings (Day 20 to Day 31). Once all drought seedlings were stressed, on 25 April 2012 (Day 31), these drought seedlings were rewatered to field capacity and kept well-watered for ten days until gs of drought seedlings was fully recovered (hereafter referred as “rewatered phase”). The second cycle of drought (hereafter referred as “second drought phase”) was then imposed on 6 May 2012 (Day 42) using a controlled dry-down approach, which was implemented to slow the progression of the onset of drought, thereby making it similar to field conditions. Meanwhile, in order to minimise the potential confounding effects of differing VPD and plant sizes among [CO₂] and temperature treatments on soil drying, similar soil water content was maintained in all treatments at a 2-3 day period. Thus, each pot in the drought treatment was weighed every second or third day to determine the water loss of each pot during that period and the minimum water loss among all pots. Then, each pot was rewatered with an individually calculated amount of water (i.e. rewatering amount = water loss for each pot - minimum water loss among all pots). Watering was discontinued on 4 June 2012 (Day 71) when minimum water loss among [CO₂] and temperature treatments was less than 20 ml during the 2-3 day period. Drought seedlings were then allowed to desiccate completely. Four blank pots filled with soil, but without seedlings, in each of the four glasshouse compartments (i.e. two for well-watered and two for drought) were used to monitor soil water evaporation. Hence, seedling water use could be determined independent of soil water evaporation.
Figure 3-1 Estimated soil volumetric water content (VWC) (m$^3$ m$^{-3}$) in (a) well-watered and (b) drought treatments exposed to two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments over the course of the experiment. Soil VWC was estimated by linear regressions between pot weight and monitored TDR values. Drought seedlings sequentially experienced the first drought phase (Day 1-30), the rewatered phase (Day 31-41), and the second drought phase (from Day 42). The first drought phase was achieved by withholding water mostly, while the second drought phase was conducted by adding water to pots to maintain the same rate of water loss across the [CO$_2$] and temperature treatments. Watering for the second drought phase was discontinued on Day 71 when minimum water loss across [CO$_2$] and temperature treatments was less than 20 ml. The grey area represents the rewatered phase.
In this study, seedlings were considered ‘dead’ when there was no photosynthesis, no leaf respiration, 100% loss of hydraulic conductivity and all leaves were brown, brittle and necrotic. Leaf browning was considered as the visual indicator of drought seedling mortality (see Anderegg & Anderegg, 2013), assessing necrosis by estimating the percentage of brown leaves compared with all leaves on the plant. Mortality tracking was conducted in drought seedlings (n = 7) starting on Day 82 when leaf water potential was more negative than -10 MPa.

### 3.2.3 Soil water monitoring

Soil VWC (m³ m⁻³) was assessed using TDR probes (Time Domain Reflectometer; 30 cm) (CS616, Campbell Scientific, Logan, UT) in two randomly selected seedlings (one for well-watered and drought, respectively) in each of the four [CO₂] and temperature treatments. All measurements were logged every hour, and data were stored on a data logger (CR3000, Campbell Scientific, Logan, UT). According to pot weight data from seedlings with TDRs, a linear regression between changes in pot weight (i.e. pot weight during drought progress compared with those when pots were well-watered) and soil VWC was developed with pooled data and soil VWC could be estimated for all seedlings using pot weight. Because biomass accumulation of seedlings was relatively inconsequential to the pot weight, the biomass accumulation was ignored when the regression was obtained.

### 3.2.4 Leaf gas exchange measurements

Leaf gas exchange measurements were taken on recent, fully expanded leaves from four seedlings per treatment (i.e. 2 water treatments × 2 [CO₂] × 2 temperatures) (n = 4 for well-watered and drought) using a portable open path gas exchange system.
(Licor-6400, Li-Cor, Lincoln, NE, USA) supplying PPFD by red-blue light source (6400-02B). Leaf photosynthesis under saturating light \( A_{\text{sat}}, \mu\text{mol m}^{-2}\text{s}^{-1} \) and stomatal conductance \( g_s, \text{mol m}^{-2}\text{s}^{-1} \) were measured at mid-day (between 0930 h and 1400 h) on a weekly basis throughout the experiment, at PPFD of 1500 \( \mu\text{mol m}^{-2}\text{s}^{-1} \), growth \([\text{CO}_2]\) (400 \( \mu\text{l l}^{-1} \) or 640 \( \mu\text{l l}^{-1} \)) and mid-day growth temperature (26 °C or 30 °C). Through the entire measurement period, measured leaf to air VPD in Licor-6400 cuvettes ranged from 1.1 to 2.4 kPa (mean value: 1.76 ± 0.02 kPa) in the ambient temperature treatments and from 1.4 to 3.5 kPa (mean value: 2.25 ± 0.03 kPa) in the elevated temperature treatments \( (n = 183) \) (Figure A-1). Leaf night respiration \( R_n, \mu\text{mol m}^{-2}\text{s}^{-1} \) was measured at night-time (2 hours after sunset) following the daytime measurement of \( A_{\text{sat}} \) on the same leaf at zero PPFD, growth \([\text{CO}_2]\) (400 \( \mu\text{l l}^{-1} \) or 640 \( \mu\text{l l}^{-1} \)) and night time growth temperature (16 °C or 20°C).

### 3.2.5 Plant dry mass production

Three seedlings assigned to well-watered \( (n = 3) \) treatments, from each of the four \([\text{CO}_2]\) and temperature treatments, were randomly selected for destructive harvest as a pre-drought baseline at onset of the experiment (Day 1; pre-drought). Thereafter, a second harvest was conducted on Day 73, with three seedlings per treatment (i.e. 2 water treatments × 2 \([\text{CO}_2]\) × 2 temperatures) \( (n = 3 \text{ for well-watered and drought}) \). At the end of the experiment, when all drought seedlings were considered ‘dead’ (Day 198; at mortality), the remaining seedlings were harvested \( (n = 4 \text{ for well-watered and } n = 7 \text{ for drought}) \).

At each harvest, seedlings were separated into leaves, stem and roots. Roots were washed free of soil. Leaf area was determined using a portable leaf area meter (LI-
3100A, Li-Cor, Lincoln, NE, USA). At the final harvest (at mortality), because leaves of drought seedlings were dry and brittle, leaf area was estimated from leaf dry mass using linear regression. All harvested organs were oven-dried at 70°C for 72 h and dry mass was determined.

### 3.2.6 Leaf water relations and stem hydraulic measurements

Pre-dawn ($\Psi_{pd}$, MPa) and mid-day ($\Psi_{md}$, MPa) leaf water potentials were measured on a bi-weekly basis during the second drought phase (Day 40, Day 53, Day 68 and Day 82) using a Scholander-type pressure chamber (PMS instruments, Corvalis, Oregon USA). On the evening prior to measurements, three seedlings from each treatment (i.e. 2 water treatments $\times$ 2 [CO$_2$] $\times$ 2 temperatures) were randomly selected. Overnight, each seedling was covered in a large black plastic bag to ensure that there was no nocturnal transpiration and to ensure equilibration of the water potential between the soil and the seedling. Approximately one hour before sunrise, the black plastic bags covering seedlings were removed. $\Psi_{pd}$ of one small twig from each seedling was measured. At mid-day (1200 h to 1400 h), another small twig from each seedling was sampled for the determination of $\Psi_{md}$. Given limitations of the pressure bomb used in this study, leaf water potential was considered to be -10 MPa when it was more negative than -10 MPa.

Stem hydraulic measurements were conducted at each harvest ($n = 3$ for well-watered/drought). On the night prior to each harvest, to maximize the equilibrium between leaf and xylem and to estimate xylem pressure by leaf water potential, two twigs from each seedling were wrapped with cling tape and covered with aluminium foil. Meanwhile, seedlings were covered by black plastic bags and sealed at the
bottom of the pots to prevent nocturnal transpiration. All harvests were conducted at predawn because xylem water potential was highest (i.e. least negative) and this could minimize problems associated with air entry into the cut surface. Therefore, approximately one hour before sunrise on the harvest day, aboveground sections of seedlings were cut at the bottom of the stem and the stem was recut under water immediately to prevent air entry into the xylem. The cut surface was sealed with parafilm and the seedling was sealed in a black plastic bag, humidified with wet paper towelling, and returned immediately to the laboratory.

Water potentials of leaves attached to the wrapped twigs were measured to estimate xylem water potential (Ψxylem) using the Scholander-type pressure chamber. Stem segments of 10-15 cm in length were then cut under perfusing solution (i.e. 2 mM KCl solution) for hydraulic measurements. Hydraulic conductivity measurements were conducted by pressure-flow method described by Sperry et al. (1988), and modified by Choat et al. (2010). With a pressure head of 1.8 kPa, the initial/pre-flush hydraulic conductivity (K-initial) was directly measured from the segment. Then, the same segment was attached to a captive water tank with a pressure of 150 kPa and flushed for 30 min to remove any embolism that may have been present before a hydraulic measurement was taken to establish Kmax. The percentage loss of conductivity (PLC) of the stem segment was determined by:

\[ \text{PLC} = 100 \times \frac{K_{\text{max}} - K_{\text{initial}}}{K_{\text{max}}} \]

### 3.2.7 TNC assay

Oven-dried plant organ samples were ground to a fine powder in a ball mill. TNC assay was described in Chapter 2. TNC concentration was calculated as the sum of
starch (mg g\(^{-1}\)) and soluble sugar (mg g\(^{-1}\)) concentrations. Whole-plant TNC concentration (mg g\(^{-1}\)) was calculated as the sum of the weighed concentrations (concentration multiplied by the proportion of organ dry mass to total dry mass) of the different organs (leaf, stem and root). Whole-plant TNC content (g) was calculated as whole-plant TNC concentration multiplied by the total dry mass and a coefficient 1/1000.

3.2.8 Statistical analyses

Time-series measurements (i.e. soil VWC, \(A_{\text{sat}}\), \(g_s\), \(R_n\) and leaf water potential) were analysed using repeated measures ANOVA to test temporal effects of the factors (water, [CO\(_2\)] and temperature). Three-way ANOVAs were then used to test main and interactive effects of factors (water, [CO\(_2\)] and temperature) on all parameters for each time step, after checking for the assumption of normality and homogeneity of variances. Thereafter, two-way ANOVAs were assessed to test the main and interactive effects of [CO\(_2\)] and temperature within each water treatment. Duncan post-hoc tests were assessed to compare means among [CO\(_2\)] and temperature treatments when interactions of [CO\(_2\)] and temperature were significant. The effects of [CO\(_2\)] and temperature on the time-to-mortality were analysed based on the overlap of 95 % confidence intervals. In all cases, results were considered significant if \(P \leq 0.05\). All analyses were performed in SPSS (version 20.0; SPSS, Chicago, IL, USA).

3.3 Results

3.3.1 Soil water conditions
In the *well-watered* treatment, mean soil VWC was 0.23 ± 0.01 m$^3$ m$^{-3}$ across [CO$_2$] and temperature treatments during the experimental period. On average, soil VWC of *well-watered* seedlings was lower in elevated temperature treatments than ambient temperature treatments (Two-way repeated measures ANOVA: $P < 0.001$) (Figure 3-1a). During the first drought phase, soil VWC declined sharply from 0.25 to 0.08 m$^3$ m$^{-3}$ (Figure 3-1b), declining faster in elevated temperature treatments than ambient temperature treatments during the first 20 days (Two-way ANOVA: $P < 0.05$ for all cases). Soil VWC then rose to field capacity after drought seedlings were rewatered. During the second drought phase, soil VWC generally declined at a slower rate, but eventually declined to < 0.06 m$^3$ m$^{-3}$. The decline in soil VWC during this drought phase was faster in elevated temperature treatments than in ambient temperature treatments prior to Day 60 (i.e. when soil VWC was > 0.12 m$^3$ m$^{-3}$) (Two-way ANOVA: $P < 0.05$ for most cases), but soil VWC was not affected by temperature after this date. During the entire experimental period, soil VWC was not impacted by [CO$_2$] treatments.

### 3.3.2 Leaf gas exchange

Prior to onset of the first drought phase (Day 1; pre-drought), leaf gas exchange parameters (i.e. leaf $A_{sat}$, $g_s$ and $R_n$) were similar for seedlings allocated to *well-watered* and *drought* treatments (Three-way ANOVA: $P \geq 0.800$ for all cases) (Figure 3-2). However, $A_{sat}$ was 42 % higher in seedlings grown in elevated [CO$_2$] treatments (Three-way ANOVA: $P < 0.001$) and 12 % lower in seedlings grown in elevated temperature treatments (Three-way ANOVA: $P = 0.032$). $g_s$ and $R_n$ were not affected by [CO$_2$] or temperature (Three-way ANOVA: $P > 0.05$ for all cases).
Throughout the experimental period, $A_{sat}$ and $g_s$ of well-watered seedlings in elevated $[CO_2]$ treatments declined by 27 % and 40 %, respectively, but those in ambient $[CO_2]$ treatments were relatively stable (Figures 3-2a and c). Leaf $R_n$ of well-watered seedlings declined by 50 % (on average) across $[CO_2]$ and temperature treatments (Figure 3-2e). In the well-watered treatment, $A_{sat}$ was higher in the elevated $[CO_2]$ treatments (Two-way repeated measures ANOVA: $P < 0.001$), but lower in elevated temperature treatments (Two-way repeated measures ANOVA: $P = 0.008$). $g_s$ and $R_n$ were not generally affected by $[CO_2]$ (Two-way repeated measures ANOVA: $P \geq 0.100$ for both) and temperature (Two-way repeated measures ANOVA: $P \geq 0.110$ for both). During the first and second drought phases, $A_{sat}$ and $g_s$ of drought seedlings declined to near-zero more rapidly in elevated temperature treatments compared with ambient temperature treatments (Two-way ANOVA: $P < 0.05$ for most cases) (Figures 3-2b and d). During the second drought phase, leaf $R_n$ of drought seedlings did not substantially decline compared with well-watered seedlings until about Day 80 (i.e. when soil VWC $\approx 0.06$ m$^3$ m$^{-3}$) (Three-way ANOVA: $P = 0.001$) and reached zero earlier in the elevated temperature treatments ( Two-way ANOVA: $P < 0.05$ for Day 86 and Day 93) (Figure 3-2f). However, $[CO_2]$ did not affect the decline in leaf gas exchange parameters of drought seedlings (Two-way repeated measures ANOVA: $P > 0.05$ for all cases).
Figure 3-2  (a, b) Photosynthesis at saturating light ($A_{sat}$), (c, d) stomatal conductance ($g_s$) and (e, f) leaf night respiration ($R_n$) of *Eucalyptus radiata* seedlings in well-watered (closed symbols) and drought (open symbols) treatments exposed to two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments over the experimental period. Values are Means ± SE (n = 4). The grey areas represent the rewatered phase. More details about watering regime refer to Figure 3-1.
Figure 3-3 (a) Photosynthesis at saturating light ($A_{sat}$), (b) stomatal conductance ($g_s$) and (c) leaf night respiration ($R_n$) of *Eucalyptus radiata* seedlings in the drought treatment as a function of soil VWC exposed to two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments. All data points are raw data from the rewatered phase and the second drought phase (from Day 39). Data are fitted with three-parameter sigmoid relationships for each of the four [CO$_2$] and temperature treatments: \[ y = a/ (1 + e^{-(x-c)/b}) \] \( P < 0.001, R^2 = 0.73-0.96, n = 26-33 \).
Leaf gas exchange parameters exhibited sigmoidal functions with soil VWC (Figure 3-3). $A_{sat}$ and $g_s$ in all [CO$_2$] and temperature treatments were generally more sensitive to drought than $R_n$. Responses of $A_{sat}$ and $g_s$ to soil VWC were not different between [CO$_2$] treatments, while they shifted between temperature treatments indicating that sensitivities of $A_{sat}$ and $g_s$ to drought were altered by temperature. For instance, $A_{sat}$ in elevated temperature treatments declined and reached zero at higher soil VWC (i.e. about 0.02 m$^3$ m$^{-3}$ higher) than in ambient temperature treatments (Figure 3-3a). Thus, as drought progressed, at a given soil VWC, elevated temperature induced earlier declines in $A_{sat}$ and $g_s$. However, the response of $R_n$ to soil VWC was not significantly impacted by [CO$_2$] or temperature treatments.

### 3.3.3 Plant growth

Prior to initiation of the drought treatment, plant leaf area and dry mass were similar between seedlings assigned to well-watered and drought treatments (Table 3-1); thus, I used the harvested well-watered seedlings as the baselines for leaf area and dry mass. Leaf area prior to the drought was increased by elevated [CO$_2$] (Two-way ANOVA: $P < 0.001$) and elevated temperature (Two-way ANOVA: $P = 0.051$; marginally) treatments, while plant dry mass increased only in elevated [CO$_2$] treatments (Two-way ANOVA: $P = 0.035$).

Leaf area and dry mass of well-watered seedlings increased substantially in all [CO$_2$] and temperature treatments throughout the experiment (on average, 9-fold and 20-fold increase in leaf area and dry mass, respectively) (Table 3-1). At the final harvest (Day 198), leaf area of well-watered seedlings was higher in elevated [CO$_2$] treatments than in ambient [CO$_2$] treatments (Two-way ANOVA: $P = 0.007$). Dry mass of well-watered seedlings was increased by elevated [CO$_2$] treatments (Two-way ANOVA: $P = 0.035$).
way ANOVA: $P = 0.005$) and elevated temperature treatments (Two-way ANOVA: $P = 0.032$), with a significant interaction between [CO$_2$] and temperature (Two-way ANOVA: $P = 0.046$). 

*Drought* limited leaf area production and dry mass accumulation. In contrast to *well-watered* seedlings, *drought* seedlings only exhibited a 2-fold and 3-fold increase (on average) in leaf area and dry mass, respectively, throughout the experiment (Table 3-1). Leaf area of *drought* seedlings did not differ among [CO$_2$] and temperature treatments (Two-way ANOVA: $P > 0.05$), but dry mass of *drought* seedlings were higher in elevated [CO$_2$] treatments compared with ambient [CO$_2$] treatments throughout the experiment (Two-way ANOVA: $P \leq 0.027$ for Day 73 and Day 198).
Table 3-1 Leaf area and plant dry mass of *Eucalyptus radiata* seedlings in *well-watered* and *drought* treatments exposed to two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperatures (26 °C and 30 °C) on Day 1(pre-drought), Day 73 and Day 198 (at mortality). Values are Means ± SE. Replicates: Day 1 and 73: n = 3; Day 198: n = 4 in *well-watered* treatment, n = 7 in *drought* treatment. Only *well-watered* seedlings were harvested as the baseline at pre-drought conditions. More details about watering regime refer to Figure 3-1.

<table>
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<th>Parameters</th>
<th>CO$_2$</th>
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<th>Day1</th>
<th>Day73</th>
<th>Day198</th>
</tr>
</thead>
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<tr>
<td><strong>Leaf area (cm$^2$)</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>400 µl l$^{-1}$</td>
<td>26 °C</td>
<td><em>Well-watered</em></td>
<td>397 ± 33</td>
<td>1846 ± 76</td>
<td>3938 ± 458</td>
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<tr>
<td></td>
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<td>1170 ± 18</td>
<td>1138 ± 82</td>
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<tr>
<td></td>
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<td>2401 ± 222</td>
<td>4754 ± 685</td>
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<tr>
<td></td>
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<td>1091 ± 88</td>
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<tr>
<td></td>
<td>640 µl l$^{-1}$</td>
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<td><em>Well-watered</em></td>
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<td>1769 ± 163</td>
<td>5604 ± 560</td>
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<td>1194 ± 82</td>
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<td><strong>Dry mass (g)</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>400 µl l$^{-1}$</td>
<td>26 °C</td>
<td><em>Well-watered</em></td>
<td>6 ± 1</td>
<td>33 ± 2</td>
<td>136 ± 17</td>
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<tr>
<td></td>
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<td></td>
<td>Drought</td>
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<td>25 ± 1</td>
<td>22 ± 1</td>
</tr>
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<td>400 µl l$^{-1}$</td>
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<td><em>Well-watered</em></td>
<td>7 ± 1</td>
<td>38 ± 2</td>
<td>139 ± 11</td>
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<td>25 ± 2</td>
<td>21 ± 1</td>
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<td>640 µl l$^{-1}$</td>
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<td>38 ± 4</td>
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<td>----</td>
<td>28 ± 1</td>
<td>24 ± 2</td>
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<tr>
<td></td>
<td>640 µl l$^{-1}$</td>
<td>30 °C</td>
<td><em>Well-watered</em></td>
<td>9 ± 1</td>
<td>39 ± 5</td>
<td>229 ± 18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Drought</td>
<td>----</td>
<td>30 ± 2</td>
<td>26 ± 2</td>
</tr>
</tbody>
</table>
### 3.3.4 Leaf water relations and PLC

Leaf $\Psi_{pd}$ (mean = - 0.21 ± 0.01 MPa) and $\Psi_{md}$ (mean = - 0.76 ± 0.13 MPa) of well-watered seedlings were relatively stable during the measured period (i.e. Day 40 to Day 82) and were not affected by [CO₂] and temperature treatments (Two-way repeated measures ANOVA: $P > 0.05$ for all cases) (Figure A-2). Leaf $\Psi_{pd}$ and $\Psi_{md}$ of drought seedlings dropped to below -10 MPa by Day 82 (i.e. soil VWC ≈ 0.06 m³ m⁻³), exhibiting faster declines in elevated temperature treatments than in ambient temperature treatments during the second drought phase (Two-way repeated measures ANOVA: $P < 0.05$ for $\Psi_{pd}$ and $\Psi_{md}$).

PLC as a function of xylem pressure was measured on Day 73 (i.e. soil VWC ≈ 0.08 m³ m⁻³), when $A_{sat}$ of drought seedlings declined to near-zero (Figure 3-4). Well-watered seedlings remained at higher xylem pressure (- 0.29 ± 0.04 MPa) with smaller PLC (19 ± 3 %), while drought seedlings experienced more negative xylem pressure (ranging from - 1.88 to - 5.03 MPa) with larger PLC (ranging from 27 to 91 %) across [CO₂] and temperature treatments. PLC of drought seedlings was not different between [CO₂] treatments, but it was larger in elevated temperature treatments than in ambient temperature treatments, associated with lower xylem pressure (Two-way ANOVA: $P = 0.002$ for PLC and $P < 0.001$ for xylem pressure). Thus, xylem cavitation occurred earlier in elevated temperature treatments than ambient temperature treatments. At mortality, all drought seedlings experienced 100 % PLC (data not shown).
Figure 3-4 Percentage loss of stem hydraulic conductivity (PLC) as a function of xylem pressure in *Eucalyptus radiata* seedlings in well-watered (closed symbols) and drought (open symbols) treatments exposed to two [CO₂] (400 µl l⁻¹ and 640 µl l⁻¹) and two temperature (26 °C and 30 °C) treatments on Day 73 when $A_{sat}$ was near-zero. Values are Means ± SE (n = 3). The dashed line shows 50 % loss of hydraulic conductivity.
3.3.5 Organ and whole-plant TNC

Pre-drought, starch and soluble sugar concentrations were not different among [CO₂] and temperature treatments (Figure 3-5). Starch concentrations of all organs were lower than 10 mg g⁻¹. Mean soluble sugar concentrations were 47.5 ± 2.2 mg g⁻¹, 21.5 ± 2.6 mg g⁻¹ and 18.8 ± 2.1 mg g⁻¹ in the leaves, stem and roots, respectively.

At the final harvest, well-watered seedlings in elevated temperature treatments exhibited significant accumulation of starch concentration in leaves (Figure 3-5a) (Three-way ANOVA: \( P < 0.001 \)). In contrast, there were no changes in soluble sugar concentration in any plant organ (Figure 3-5). Whole-plant TNC concentrations did not differ through time in any treatment except for the \( C_{e}T_{e} \) treatment, but whole-plant TNC content accumulated in all treatments (on average 24-fold higher than pre-drought values) (Three-way ANOVA: \( P < 0.001 \)) (Table 3-2). Whole-plant TNC concentration and content were dominated by leaf contributions. For drought seedlings, there were no significant differences in starch or soluble sugar concentrations of organs at mortality compared with pre-drought values (Figure 3-5). Whole-plant TNC concentration of drought seedlings at mortality were not different from pre-drought values, but whole-plant TNC content of drought seedlings was slightly greater than pre-drought values (on average 2-fold higher) (Three-way ANOVA: \( P < 0.05 \)) (Table 3-2). Overall, TNC of drought seedlings was not affected by [CO₂] or temperature treatments.
Figure 3-5 TNC concentration (mg g⁻¹) expressed as starch concentration and soluble sugar concentration for different organs (leaf, stem and root) of Eucalyptus radiata seedlings in well-watered and drought conditions exposed to two [CO₂] (400 µl l⁻¹ and 640 µl l⁻¹) and two temperature (26 °C and 30 °C) treatments on Day 1(pre-drought) and Day 198 (mortality). Values are Means ± SE (n = 3 at pre-drought and n = 4 at mortality). Only well-watered seedlings were harvested as the baseline at pre-drought conditions. More details about watering regime refer to Figure 3-1.
Table 3-2 Whole-plant TNC concentration (TNC conc, mg g⁻¹) and whole-plant TNC content (g) of *Eucalyptus radiata* seedlings in well-watered and drought treatments exposed to two [CO₂] (400 µl l⁻¹ and 640 µl l⁻¹) and two temperatures (26 °C and 30 °C) on Day 1 (pre-drought) and Day 198 (at mortality). Values are Means ± SE. Replicates: n = 3 on Day 1; n = 4 on Day 198. Only well-watered seedlings were harvested as the baseline at pre-drought conditions. More details about watering regime refer to Figure 3-1.

<table>
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<th>Parameters</th>
<th>CO₂</th>
<th>Temp</th>
<th>Water</th>
<th>Day1</th>
<th>Day198</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole-plant TNC conc (mg g⁻¹)</td>
<td>400 µl l⁻¹</td>
<td>26 °C</td>
<td>Well-watered</td>
<td>41.2 ± 1.8</td>
<td>39.0 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>400 µl l⁻¹</td>
<td>30 °C</td>
<td>Well-watered</td>
<td>40.7 ± 2.8</td>
<td>47.2 ± 1.3</td>
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<tr>
<td></td>
<td>640 µl l⁻¹</td>
<td>26 °C</td>
<td>Well-watered</td>
<td>43.7 ± 3.5</td>
<td>41.1 ± 2.6</td>
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<td>640 µl l⁻¹</td>
<td>30 °C</td>
<td>Well-watered</td>
<td>42.8 ± 5.4</td>
<td>58.3 ± 3.0</td>
</tr>
<tr>
<td>Whole-plant TNC content (g)</td>
<td>400 µl l⁻¹</td>
<td>26 °C</td>
<td>Well-watered</td>
<td>0.2 ± 0.0</td>
<td>4.7 ± 0.9</td>
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<tr>
<td></td>
<td>400 µl l⁻¹</td>
<td>30 °C</td>
<td>Well-watered</td>
<td>0.3 ± 0.0</td>
<td>7.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>640 µl l⁻¹</td>
<td>26 °C</td>
<td>Well-watered</td>
<td>0.3 ± 0.0</td>
<td>5.7 ± 0.4</td>
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<tr>
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<td>640 µl l⁻¹</td>
<td>30 °C</td>
<td>Well-watered</td>
<td>0.4 ± 0.1</td>
<td>13.4 ± 1.8</td>
</tr>
</tbody>
</table>

Drought...
3.3.6 Seedling mortality

The mean time-to-mortality (100 % leaf browning), was shorter in $C_eT_e$ (125 days) and $C_aT_e$ (131 days) compared with $C_aT_a$ (164 days) and $C_eT_a$ (169 days) (Figure 3-6). Based on examination of the 95 % confidence intervals (CI) analysis, the mortality pattern suggested that elevated temperature induced more rapid seedling mortality, but [CO$_2$] did not have a significant impact on time-to-mortality.

3.3.7 Water use and WUE

Water addition and water use were higher in seedlings grown in elevated temperature compared to ambient temperature for both the well-watered and drought treatments. In contrast, water addition and use were not different between ambient and elevated [CO$_2$] treatments (Figures 3-7 and A-3). Nonetheless, leaf instantaneous WUE of both well-watered and drought seedlings was both higher in elevated [CO$_2$] treatments (Two-way ANOVA: $P < 0.001$ for both) (Figure 3-8a). Whole-plant WUE of drought seedlings was lowest in the $C_aT_e$ treatment (significant interaction of [CO$_2$] and temperature; Duncan post-hoc test: $P < 0.05$) and did not differ among other [CO$_2$] and temperature treatments (Figure 3-8b); hence, elevated [CO$_2$] increased whole-plant WUE of drought seedlings only under elevated temperature. For example, drought seedlings in $C_eT_e$ gained greater dry mass (i.e. 20 %) than those in $C_aT_e$, but had similar water use. Additionally, drought seedlings in $C_aT_e$ gained similar dry mass compared with $C_aT_a$, but used more water (i.e. 150 % greater than $C_aT_a$) (Figure 3-8c).
Figure 3-6 Percentage of leaf browning of *Eucalyptus radiata* seedlings in the *drought* treatment exposed to two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments. Values are Means ± SE (n = 7). The grey area represents the rewatered phase. Tracking of leaf browning began on Day 82 when leaf water potential was more negative than -10 MPa. Lines indicate the 95% confidence intervals (CI) of time-to-mortality when all leaves were brown for individual seedlings among [CO$_2$] (dashed for 400 µl l$^{-1}$ and solid for 640 µl l$^{-1}$) and temperature (blue for 26 °C and red for 30 °C) treatments. The mean time to complete (100%) leaf browning was 125 days (640 µl l$^{-1}$, 30 °C), 131 days (400 µl l$^{-1}$, 30 °C), 164 days (400 µl l$^{-1}$, 26 °C) and 169 days (640 µl l$^{-1}$, 26 °C). The mean time was significantly different between ambient and elevated temperature treatments, but not between [CO$_2$] treatments.
Figure 3-7 (a,b) Plant weekly water use and (c,d) cumulative water use of *Eucalyptus radiata* seedlings in well-watered (closed symbols) and drought (open symbols) treatments exposed to two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments over the experimental period. Water additions to drought seedlings were discontinued on Day 71 (Week 11) when minimum water loss across [CO$_2$] and temperature treatments was less than 20 ml. Values are Means ± SE (n = 7-10). Data for well-watered seedlings was not collected on Week 11. The grey areas represent the rewatered phase.
Figure 3-8 (a) Mean leaf instantaneous WUE and (b) total whole-plant WUE of *Eucalyptus radiata* seedlings in well-watered and drought treatments exposed to two [CO₂] (400 µl l⁻¹ and 640 µl l⁻¹) and two temperature (26 °C and 30 °C) treatments over the period with positive photosynthesis (Day 1 to Day 73). Values are Means ±
SE (n = 40 for instantaneous WUE and n = 3 for total plant WUE). Instantaneous WUE (µmol CO₂ mol⁻¹ H₂O) = \( A_{\text{sat}} \) (µmol m⁻² s⁻¹) / \( g_s \) (mol m⁻² s⁻¹). Total plant WUE = total plant dry mass (g)/ total water use (kg). (c) Total water use per plant as a function of total plant dry mass of *Eucalyptus radiata* seedlings in well-watered (closed symbols) and drought (open symbols) treatments exposed to two [CO₂] (400 µl l⁻¹ and 640 µl l⁻¹) and two temperature (26 °C and 30 °C) treatments at the harvest on Day 73. Values are Means ± SE (n = 3).
3.4 Discussion

Consistent with the first hypothesis, drought-induced mortality of *E. radiata* seedlings in all [CO₂] and temperature treatments was primarily driven by hydraulic failure as evidenced by catastrophic hydraulic dysfunction (PLC = 100 %) and subsequent cell desiccation (leaf water potentials < -10 MPa). I found no evidence of significant carbohydrate depletion in individual organs or at the whole-plant level compared with pre-drought conditions. In contrast, the second hypothesis was not supported. Elevated [CO₂] did not ameliorate drought stress in *E. radiata* seedlings or delay the time-to-mortality because there was no difference observed in water loss, physiological responses, and hydraulic functions between ambient and elevated [CO₂] treatments, despite an observed increase in leaf instantaneous WUE due to elevated [CO₂]. In partial support of the third hypothesis, elevated temperature treatments exacerbated drought stress in *E. radiata* seedlings of both [CO₂] treatments and promoted more rapid mortality through hydraulic failure, driven by higher water use, increased drought sensitivities of gas exchange traits (at given soil VWC), and earlier substantial xylem cavitation. The fourth hypothesis was rejected because I found that elevated [CO₂] did not ameliorate the negative effects of elevated temperature on drought stress. Taken together, the findings suggest that elevated temperature and consequent increases in VPD, but not elevated [CO₂], may be the primary contributors affecting drought-induced tree seedling mortality in future climates, which are predicted to exhibit rising [CO₂] and temperature, as well as more frequent and intense droughts.

3.4.1 Hydraulic failure primarily determined mortality of *E. radiata* seedlings
In the present study, I found complete stomatal closure, leaf dehydration and catastrophic xylem cavitation of stems during *E. radiata* seedling mortality. However, I did not observe significant changes in organ and whole-plant TNC concentrations relative to pre-drought values. These findings indicate that hydraulic failure played the primary role in promoting mortality during lethal drought, which was consistent with recent studies in other species, such as *Eucalyptus globulus* Labill., *Eucalyptus smithii* R.T. Baker (Mitchell *et al*., 2013), *Populus tremuloides* Michx. (Anderegg *et al*., 2012), *Quercus pubescens* Willd. and *Acer campestre* L. (Nardini *et al*., 2013).

For instance, *E. globulus* seedlings exhibited high rates of water use and rapid hydraulic dysfunction with < 5 % depletion of carbohydrates (Mitchell *et al*., 2013).

In the present study, $A_{\text{sat}}$ and $g_s$ of *E. radiata* seedlings declined steeply once soil moisture fell below a 0.05 m$^3$ m$^{-3}$ threshold (Figure 3-3). However, the rapid drop in leaf water potential (Figure A-2) suggested that *E. radiata* seedlings could not regulate water status, eventually leading to catastrophic hydraulic dysfunction.

### 3.4.2 Elevated [CO$_2$] did not ameliorate drought stress or delay mortality

Elevated [CO$_2$] may reduce leaf $g_s$ and leaf-level water loss in many tree species when water is not limiting and may therefore mitigate drought stress (Medlyn *et al*., 2001; Wullschleger *et al*., 2002; Ainsworth & Rogers, 2007). However, studies of [CO$_2$] × drought interactions are inconclusive in that elevated [CO$_2$] has been reported to ameliorate drought stress in some instances (Atwell *et al*., 2007; Domec *et al*., 2010; Wertin *et al*., 2010; Lewis *et al*., 2013), but not in others (Bobich *et al*., 2010; Duursma *et al*., 2011; Warren *et al*., 2011; Zeppel *et al*., 2012). For example, in a recent short-term drought study, elevated [CO$_2$] delayed onset of drought stress by slowing the decline of $A_{\text{sat}}$ in the faster growing species (*Eucalyptus saligna* Sm.), but not in the slower growing species (*Eucalyptus sideroxylon* A. Cunn. ex Woolls).
(Lewis et al., 2013). Further, the positive effects of elevated [CO₂] on drought vanish when stomata are nearly closed as drought intensifies (Warren et al., 2011; Franks et al., 2013; Perry et al., 2013; Chapter 2). In this study, *E. radiata* seedlings exhibited similar drought sensitivities of gas exchange traits in ambient and elevated [CO₂] treatments, indicating that elevated [CO₂] did not delay the onset of drought stress on leaf gas exchange parameters.

Reductions in leaf-level water loss in elevated [CO₂] under non-limiting water conditions could be offset by changes in plant architecture, including increased leaf area and reduced rooting depth (Wullschleger et al., 2002; Duursma et al., 2011; Warren et al., 2011; Way, 2011), and differences between day and night *gₛ* (Zeppel et al., 2012). For example, elevated [CO₂] did not reduce the water loss of *Eucalyptus sideroxylon* A. Cunn. ex Woolls seedlings exposed to drought compared with ambient [CO₂], because greater leaf area and higher nocturnal water fluxes in elevated [CO₂] counteracted the reduction of daytime *gₛ* (Zeppel et al., 2012).

However, in this study, leaf area of *drought* seedlings did not differ between ambient and elevated [CO₂] treatments. The observed higher leaf instantaneous WUE in elevated [CO₂] treatments mainly resulted from increases in *Aₙₐₑ* due to elevated [CO₂] rather than reductions in *gₛ*. Elevated [CO₂] did not reduce leaf *gₛ* of *drought* seedlings at a given soil VWC and did not affect whole-plant water use, suggesting that elevated [CO₂] did not lead to water savings at leaf and whole-plant levels in our study. These findings suggest that leaf instantaneous WUE may be a less useful trait than leaf and whole plant water use in accurately defining key characteristics of water use in [CO₂] × drought studies. Furthermore, elevated [CO₂] did not delay xylem cavitation and mortality in *drought* seedlings of *E. radiata*. Taken together, those findings indicate that elevated [CO₂] did not ameliorate drought stress because
it did not reduce water use at the leaf and whole-plant levels, and therefore did not modify plant water status and the progression to drought-induced mortality. It is noted that carbohydrates in individual organs and whole-plant of drought seedlings were not increased by elevated [CO₂], which is contrary to other observations (e.g. Niinemets et al., 2010; Ayub et al., 2011), probably reflecting the balance between source and sink activity. This suggests that carbohydrate reserves in *E. radiata* may not benefit from elevated [CO₂] during drought stress.

### 3.4.3 Elevated temperature and consequent increases in VPD exacerbated drought stress and accelerated hydraulic-driven mortality

Elevated temperature is considered a key driver that exacerbates drought stress, leading to increased forest mortality (Breshears et al., 2005; Adams et al., 2009; Allen et al., 2010; Williams et al., 2013). Consistent with these observations, this study confirmed that elevated temperature treatments triggered more rapid drought-induced mortality of *E. radiata* seedlings, in association with increased drought sensitivity of gas exchange and earlier hydraulic dysfunction (i.e. lower leaf water potential and earlier occurrence of substantial xylem cavitation). Elevated temperature treatments reduced *A*ₘₐₓ and *g*s at higher soil VWC than ambient temperature, indicating that sensitivities of *A*ₘₐₓ and *g*s to declining soil moisture were increased in elevated temperature treatments, which is consistent with the recent study in *Thuja occidentalis* L. (Zhao et al., 2013).

In this study, I did not separate the effects of elevated temperature and higher VPD on drought responses, because it was beyond the primary objective. Given that elevated temperature will be associated with higher VPD under natural conditions (Allen et al., 2010; Way et al., 2013; Eamus et al., 2013), the results of the elevated
temperature treatment presented here provide a realistic representation of conditions that seedlings will be exposed to under future climate warming. In this study, leaf area of seedlings was not different between temperature treatments, but VPD was c. 70 % higher in elevated temperature compared with ambient temperature. In addition, elevated temperature has been suggested to decrease water viscosity, thereby increasing water transport capacity (Roderick & Berry, 2001; Way et al., 2013). Consequently, higher whole-plant water use was observed in elevated temperature when soil VWC was not limiting (i.e. weeks 6-8). However, when soil water availability could no longer meet the larger transpiration demand in elevated temperature treatments, *E. radiata* seedlings were subjected to drought stress (i.e. onset of stomatal closure) at higher soil VWC than in ambient temperature seedlings. The earlier stomatal closure observed in elevated temperature treatments is likely to play a protective role in drought response of eucalypt seedlings, as the plant attempts to delay the formation of drought induced embolism in the stem (Martorell et al., 2013). However, despite tighter stomatal regulation I found that *E. radiata* seedlings in elevated temperature treatments experienced a more negative xylem pressure (< -2.7 MPa) and a higher loss of xylem conductance (> 50 %) when watering was discontinued, suggesting that elevated temperature treatments induced earlier occurrence of xylem cavitation and impedance of water transport. Therefore, elevated temperature treatments triggered more rapid mortality (30 ~ 40 days faster) than ambient temperature treatments. I did not examine temperature effects on vulnerability to cavitation (e.g. *P*50) in this study. Way et al. (2013) found that elevated temperature (+ 5 °C) led to a greater vulnerability to cavitation in leaves, but not in stems, of *Populus tremuloides* Michx. seedlings, suggesting that temperature effects on drought vulnerability may vary among plant organs. The
extent to which elevated temperature alters plant vulnerability to drought remains to be explored.

Elevated temperature may also amplify lethal drought stress via mechanisms that affect carbon metabolism because carbon utilization is usually temperature sensitive (Amthor, 2000; Allen et al., 2010). It was observed that elevated temperature (+ 4 °C) led to higher cumulative respiration and 30 % faster mortality in Pinus edulis Engelm. trees (Adams et al., 2009), but had minimal effects on foliar carbohydrates (Adams et al., 2013). By contrast, in this study, leaf $R_n$ in the drought treatment was not altered by temperature treatments. Additionally, organ and whole-plant TNC of drought seedlings were not different between temperature treatments either pre-drought or at mortality. More rapid E. radiata seedling mortality in elevated temperature compared with ambient temperature was not attributable to carbon depletion, but predominantly to hydraulic failure. Collectively, E. radiata seedling mortality was accelerated by elevated temperature and consequent higher VPD through hydraulic failure, in association with negative impacts of elevated temperature on physiological and hydraulic function.

3.4.4 Elevated [CO$_2$] did not ameliorate the negative effects of elevated temperature on drought stress

Under well-watered conditions, the combination of elevated [CO$_2$] and temperature can increase tree growth and carbon assimilation compared to ambient [CO$_2$] and temperature (Ghannoum et al., 2010a; Wertin et al., 2012a); however, these positive effects may be negated by drought stress (Wertin et al., 2012a; Chapter 2). In this study, elevated temperature treatments had large negative effects on gas exchange, leaf water potential, PLC and time-to-mortality during progressive drought, while
elevated [CO2] did not modify seedling responses to drought. Importantly, there were no significant interactive effects of elevated [CO2] and temperature on measured traits in response to drought, which was evident in that $C_e T_e$ had similar effects as $C_a T_e$. Therefore, elevated [CO2] did not ameliorate the negative effects of elevated temperature on seedling response to drought stress. It should be noted that leaf area of drought seedlings was not different between ambient and elevated [CO2] treatments in this study, which may affect the magnitude of elevated [CO2] on drought response to elevated temperature. If elevated [CO2] increased leaf area, the CO2 enrichment effect on drought vulnerability might be larger than was detected here. It has been observed that elevated [CO2] exacerbated the negative effects of elevated temperature on drought responses, leading to greater drought stress and faster mortality in *E. sideroxylon* seedlings (Zeppel *et al.*, 2012; Chaszar *et al.* unpublished data). In summary, the finding in this study suggests that drought-induced mortality in *E. radiata* seedlings may be primarily contributed by rising temperatures (and associated higher VPD), independent of rising atmospheric [CO2], in future climates with expectations of more frequent and intense droughts.
Chapter 4

Elevated temperature but not elevated [CO$_2$] plays a dominant role in modifying drought-induced mortality in two contrasting gymnosperms

4.1 Introduction

Extreme drought, rising atmospheric [CO$_2$], increasing global temperature and associated increased VPD are likely to be the major issues in the context of global change (Breshears et al., 2013; IPCC, 2012). Worldwide, forest die-offs have been observed in a number of forest biomes over the past few decades as a result of extreme drought and rising temperature (Breshears et al., 2005; Van Mantgem et al., 2009; Allen et al., 2010; Peng et al., 2011; Matusick et al., 2013). Recent work also demonstrates that woody plant species from wet and dry forest biomes maintain narrow hydraulic safety margins and are vulnerable to future global change-type droughts (Choat et al., 2012). Responses of trees to drought may vary among species or functional groups, thereby possibly leading to different mortality patterns within and among forest types (McDowell et al., 2008; Mitchell et al., 2013) and shifts in forest composition. Further, rising [CO$_2$] and temperature may differentially modify drought responses of tree species among functional groups (Lewis et al., 2013), and
hence may generate uncertainty in predictions of drought-induced mortality under future climates. Consequently, there is a growing need to investigate how rising [CO₂] and temperature will differentially impact drought-induced tree mortality among functional groups.

Drought may differentially affect survival of tree species with contrasting stomatal response strategies (i.e. relatively isohydric vs. anisohydric) through differing regulations of water and carbon balance (McDowell et al., 2008; McDowell, 2011). As they hypothesized, during progressive drought, relatively isohydric species tend to maintain midday leaf water potentials in a narrow range to avoid hydraulic failure, but long periods of negative carbon balance due to stomatal closure can eventually deplete carbon reserves. Thus, relatively isohydric species have been hypothesized to be more vulnerable to carbon starvation (McDowell et al., 2008). Alternatively, relatively anisohydric species maintain transpiration to sustain carbon assimilation, allowing midday leaf water potentials to decline as soil dries. Once transpiration exceeds the critical transpiration rates, relatively anisohydric species may have the risk of extensive xylem cavitation and ultimately die of hydraulic failure (Sperry, 2000; McDowell et al., 2008). However, hydraulic failure and carbon starvation are interrelated through the trade-off between water loss and carbon uptake (McDowell, 2011). Despite the differing stomatal strategies, very few studies have experimentally determined the relative role of plant hydraulics and carbon metabolism during drought-induced mortality by comparing relatively isohydric and anisohydric species (but see Anderegg & Anderegg, 2013; Mitchell et al., 2013). Glasshouse research focusing on two gymnosperms (Pinus edulis Engelm. and Juniperus osteosperma (Torr.) Little) in the US found that both species exhibited catastrophic xylem cavitation and that neither experienced carbohydrate depletion (Anderegg &
Anderegg, 2013). In another study, Mitchell et al. (2013) examined this issue for the first time using Australian native angiosperms (Eucalyptus smithii R. T. Baker; relatively anisohydric) and an economically important gymnosperm (Pinus radiata D. Don; relatively isohydric), demonstrating that the relatively anisohydric E. smithii were water spenders and experienced rapid hydraulic failure with less carbohydrate depletion (c. 20%), while the relatively isohydric P. radiata tightly regulated water use at the expense of carbohydrate depletion (c. 50%). However, given the large difference in morphology, physiology and hydraulic architecture, it is not surprising that the studied angiosperms and gymnosperm performed differentially during drought-induced mortality and thus difficult to draw any conclusions on the importance of isohydry in modulating plant response to rising temperatures, [CO₂] and drought. It is important to extend this research in how contrasting stomatal strategies among phylogenetically close species perform during mortality and under potential future climate scenarios.

Drought response of tree species can be modified by elevated [CO₂] and temperature. Elevated [CO₂] has been found to favour droughted plants by higher leaf water status (Atwell et al., 2007), greater carbon assimilation (Wertin et al., 2010; Franks et al., 2013; Lewis et al., 2013; Chapter 2) and increased carbohydrate reserves (Körner, 2003; Niinemets, 2010; Ayub et al., 2011; Chapter 2). However, elevated [CO₂] has also been found to have minimal effects on leaf and whole-plant water loss and therefore could not ameliorate drought stress (Chapter 3). Conversely, elevated temperature has often been observed to exacerbate drought stress (e.g. earlier onset of stomatal closure and negative carbon balance) through greater evapotranspiration demand (Will et al., 2013) and higher respiration rates (Adams et al., 2009). Studies of the interactive effects of elevated [CO₂] and temperature on tree response to
drought are limited (Way, 2013) and inconclusive, largely depending on species and
drought intensity. Current literatures suggest that elevated [CO₂] can ameliorate
(Lewis et al., 2013) or exacerbate (Zeppel et al., 2012) the negative effects of
elevated temperature on plant response to drought, but the combined effects of
elevated [CO₂] and temperature may be not evident as drought intensifies (Wertin et
al., 2012a; Chapter 2). Evidence also shows that elevated [CO₂] does not ameliorate
the effects of elevated temperature on drought-induced mortality (Chapter 3). More
importantly, no studies have explicitly examined the interactive effects of elevated
[CO₂] and temperature on tree mortality in species with contrasting stomatal
response strategies. This knowledge gap is a shortcoming to predict how changes of
forest composition will cope with rising [CO₂] and temperature in the future.

In this study, the water relations and carbon dynamics during tree seedling mortality
were examined under two [CO₂] (400 µl l⁻¹ and 640 µl l⁻¹) and temperature (ambient
and ambient +4 °C) treatments in a full-factorial experiment, with two drought
tolerant gymnosperms Pinus radiata D. Don (Radiata Pine) and Callitris rhomboidea
R. Br (Oyster Bay Pine) with contrasting stomatal response strategies (Brodribb &
McAdam, 2013). Pinus radiata (relatively isohydric) has a very constrained natural
distribution, occurring in three isolated populations in California and Mexico (Moran
et al., 1988). Despite this it is widely planted as a commercial timber resource around
the world (> 7 million ha). In contrast, C. rhomboidea (relatively anisohydric) is only
found in Australia but is thought to be more drought-tolerant with more cavitation-
resistant xylem than P. radiata (Brodribb & Cochard, 2009; Brodribb & McAdam,
2013). This study aimed to investigate the main and interactive effects of elevated
[CO₂], elevated temperature and drought on water relations and carbon dynamics in
these two contrasting gymnosperms towards mortality. Specifically, it addressed the
following hypotheses: (1) The mechanisms associated with mortality would differ between species, i.e. *P. radiata* would die of carbon starvation, while *C. rhomboidea* would die of hydraulic failure; (2) At a given soil water content, elevated temperature would exacerbate drought stress associated with earlier onset of stomatal closure than ambient temperature in both species. Elevated temperature would accelerate mortality in *P. radiata* through carbon starvation and in *C. rhomboidea* through hydraulic failure; (3) At a given soil water content, elevated [CO₂] would ameliorate drought stress and delay onset of stomatal closure in both species than ambient [CO₂]. Elevated [CO₂] would delay mortality in *P. radiata* due to increased carbon reserves and in *C. rhomboidea* because of more favorable water status; and (4) Elevated [CO₂] would ameliorate the negative effects of elevated temperature on plant response to drought and mortality in both species.

4.2 Materials and Methods

4.2.1 Plant material and growth conditions

Three months old seedlings of *Pinus radiata* D. Don (Pinaceae family) and *Callitris rhomboidea* R. Br (Cupressaceae family) previously raised in forestry tube stock were obtained from Greening Australia (Sydney) in summer 2012 (13 January 2012). Thereafter, eighty seedlings of each species were transplanted from tube stocks into eighty PVC pots (15 cm diameter × 40 cm length) containing about 10 kg air-dried loamy sand soil (86.5 % sand and 9.5 % clay). Pots contained small drainage holes at the bottom to prevent excessive soil waterlogging. Twenty seedlings of each species were then randomly placed in each of the four adjacent, naturally lit and [CO₂]/temperature-controlled glasshouse compartments (each 3 m (width) × 5 m (length) ×
3.5 m (height)), located at the University of Western Sydney (Richmond, NSW, Australia). Seedlings were irrigated daily to field capacity and fertilized at four occasions (17 February, 19 March, 5 May and 10 July) with a commercial fertilizer (All Purpose, Brunnings. N:P:K- 27:2:10). Seedlings were rotated regularly within and between glasshouse compartments regularly to minimise potential effects of position on plant performance.

The experiment was conducted using a factorial design for [CO2] and temperature, exposed to two [CO2] (400 µl l⁻¹ and 640 µl l⁻¹) and two temperature (ambient and ambient +4 °C/elevated) treatments. The target [CO2] and temperature were adjusted and constantly monitored through the control system. Details of glasshouse design and [CO2]/ temperature controlling can be found in Ghannoum et al. (2010a). In summary, two glasshouse compartments for ambient temperature treatment were set to simulate the 30- year daily average temperature in the growing season (November to May) experienced in Richmond, NSW. Over the course of 24 h, temperature was changed five times to simulate diurnal cycles. The other two glasshouse compartments for elevated temperature treatments were designed to simulate a constant 4 °C increase in temperature relative to ambient daily temperature cycle. The mean growing temperature for ambient and elevated temperature was 26/16 °C and 30/20°C (day/night), respectively. For the two compartments within each temperature treatment, [CO2] treatments were automatically regulated to maintain ambient (target 400 µl l⁻¹) and elevated (target 640 µl l⁻¹) [CO2], respectively. The four treatments were termed as follows: $C_aT_a$ (400 µl l⁻¹, 26 °C), $C_aT_e$ (400 µl l⁻¹, 30 °C), $C_eT_a$ (640 µl l⁻¹, 26 °C) and $C_eT_e$ (640 µl l⁻¹, 30 °C). According to the primary objective, it was not intended to separate temperature and VPD effects in this study. Therefore, during the experimental period, VPD was not maintained constant among
[CO₂] and temperature treatments. VPD varied between 0.1 and 2.9 kPa in ambient temperature treatments (mean value: 0.86 ± 0.01 kPa) and between 0.2 and 4.3 kPa in elevated temperature treatments (mean value: 1.50 ± 0.01 kPa). VPD did not vary between [CO₂] treatments.

4.2.2 Watering regime

After two months of growth in the [CO₂] and temperature treatments (when seedlings were five months old), half of the seedlings (i.e. ten seedlings) within each species were randomly assigned to one of the two water treatments (i.e. well-watered or drought) in each of the four [CO₂] and temperature treatments. The drought treatment commenced on 26 March 2012 (defined as Day 1). Pots were weighed in the morning (between 0900 h and 1000 h) to determine water loss of the proceeding 2-3 day period. Well-watered seedlings were maintained at field capacity through the experiment by replacing the amount of water loss during the preceding measurement interval, while drought seedlings received no water after the onset of the drought treatment.

Soil VWC (m³ m⁻³) was measured using TDR probes (30 cm length; CS616, Campbell Scientific, Logan, UT) in two random seedlings (one each for well-watered and drought, respectively) for each species in each of the four [CO₂] and temperature treatments. All measurements were logged every hour, and data were stored in a data logger (CR3000, Campbell Scientific, Logan, UT). Using pot weight data from seedlings with TDRs, a linear regression between changes in pot weight (i.e. pot weight during drought progress compared with those when pots were well-watered) and soil VWC for each species was obtained. Therefore, soil VWC could be estimated for all seedlings by pot weight.
Seedlings were defined as “dead” in this study when there was no photosynthesis, no leaf respiration, 100 % loss of hydraulic conductivity, and all needles were brown, brittle and necrotic. Needle browning was considered to be an indicator of seedling mortality, and was assessed this visually by estimating the percentage of brown needles compared with whole plant needles. Leaf mortality observations commenced on drought seedlings (n = 4-7) on Day 82.

4.2.3 Leaf gas exchange measurements

Leaf gas exchange measurements were taken on recent fascicles (P. radiata) or a small branch (C. rhomboidea) from four seedlings within each species per treatment (i.e. 2 water treatments × 2 [CO₂] × 2 temperature) (n = 4 for well-watered and drought) using a portable open path gas exchange system (Licor-6400, Li-Cor, Lincoln, NE, USA) supplying PPFD by red-blue light source (6400-02B). Leaf photosynthesis under saturating light (A_sat, µmol m⁻² s⁻¹) and stomatal conductance (g_s, mol m⁻² s⁻¹) were measured at mid-day (between 0930 h and 1400 h) on a weekly basis throughout the experiment, at PPFD of 1500 µmol m⁻² s⁻¹, growth [CO₂] (400 µl l⁻¹ or 640 µl l⁻¹) and mid-day growth temperature (26 °C or 30 °C). Leaf night respiration (R_n, µmol m⁻² s⁻¹) was measured at night-time (2 hours after sunset) following the daytime measurement of A_sat on the same leaf at zero PPFD, growth [CO₂] (400 µl l⁻¹ or 640 µl l⁻¹) and night time growth temperature (16 °C or 20°C).

4.2.4 Plant dry mass

Three seedlings within each species assigned to well-watered (n = 3) in each of the four [CO₂] and temperature treatments were randomly selected for destructive harvest as the pre-drought baseline at predawn at onset of the experiment (Day 1). Harvests were then conducted on Day 58 and Day 120 (n = 3 for well-watered and
drought). At the end of the experiment, when all drought seedlings were considered ‘dead’ (at mortality, Day 206), all the remaining seedlings were harvested (n = 4 for well-watered and drought). At each harvest, seedlings were separated into leaves, stem and roots. Roots were washed free of soil. All harvested organs were placed into the oven to dry at 70°C for 72 h and dry mass was determined.

4.2.5 Leaf water relations and stem hydraulic measurements

Pre-dawn (Ψpd, MPa) and mid-day (Ψmd, MPa) leaf water potentials were measured on Day 46, Day 81, Day 102 and Day 118 using a Scholander-type pressure chamber (PMS instruments, Corvalis, Oregon USA). On the evening prior to measurements, three seedlings from each treatment for each species (i.e. 2 water treatments × 2 [CO2] × 2 temperature) (n = 3 for well-watered and drought) were randomly selected. Overnight, each seedling was covered in a large black plastic bag to ensure that there was no nocturnal transpiration and to maximize the water equilibrium between the soil and the seedling. Approximately one hour before sunrise, the black plastic bags covering seedlings were removed. Ψpd of one small fascicle or branch from each seedling was measured. At mid-day (1200 h to 1400 h), Ψmd was determined on same seedlings with Ψpd measurements. Leaf water potential was recorded as -10 MPa when it was more negative than -10 MPa (i.e. beyond the operating range of the instrument).

Stem hydraulic measurements were conducted at each harvest (n = 3 for well-watered and drought). On the night prior to each harvest, to maximize the equilibrium between leaf and xylem and estimate xylem pressure by leaf water potential, two fascicles or small branches from each seedling were selected to be wrapped with cling tape and covered by aluminium foil. Meanwhile, seedlings were
covered by black plastic bags and sealed at the bottom of the pots to prevent nocturnal transpiration. All harvests were conducted at predawn because xylem water potential was least negative at this time, and this minimizes problems associated with air entry into the cut surface. Approximately one hour before sunrise on the harvest day, aboveground sections of seedlings were cut at the bottom of the stem and the stem was recut under water immediately to prevent air entry into the xylem. Then, the cut surface was sealed with parafilm and the seedling was sealed in a black plastic bag humidified with wet paper towelling and returned to the laboratory immediately.

Water potentials of wrapped fascicles or branches were measured to estimate xylem water potential ($\Psi_{\text{xylem}}$) using Scholander-type pressure chamber (PMS instruments, Corvalis, Oregon USA). Stem segments of 5 cm in length were cut under perfusing solution (i.e. 2 mM KCl solution) for hydraulic measurements. Hydraulic conductivity measurements were conducted by pressure-flow method described by Sperry et al. (1988). With a pressure head of 3.4-4.4 kPa, the initial/pre-flush hydraulic conductivity ($K_{\text{initial}}$) was directly measured from the segment. Because high pressure could cause pit blocking by the torus in gymnosperms, the segment was vacuum infiltrated with water for 48-60 h to remove any embolism that may have been present before a hydraulic measurement was taken to establish $K_{\text{max}}$. The percentage loss of conductivity (PLC) of the stem segment was determined by:

$$\text{PLC} = 100 \times \left( \frac{K_{\text{max}} - K_{\text{initial}}}{K_{\text{max}}} \right)$$

### 4.2.6 TNC assay
Oven-dried plant organ samples were ground to a fine powder in a ball mill. TNC assay was described in Chapter 2. TNC concentration (mg g\(^{-1}\)) was sum of starch concentration and soluble sugar concentration.

### 4.2.7 Statistical analyses

Time-series measurements (i.e. soil VWC, \(A_{sat}\), \(g_s\), \(R_n\), leaf water potential and water use) were analysed using repeated measures ANOVA to test temporal effects of the factors (species, water, [CO\(_2\]) and temperature). Four-way ANOVAs were then used to test main and interactive effects of factors (species, water, [CO\(_2\]) and temperature) on all parameters for each time step, after checking for the assumption of normality and homogeneity of variances. Thereafter, two-way ANOVAs were assessed to test the main and interactive effects of [CO\(_2\]) and temperature for each species within each water treatment. Duncan post-hoc tests were assessed to compare means among [CO\(_2\]) and temperature treatments when interactions of [CO\(_2\]) and temperature were significant. Temporal changes in dry mass and TNC from pre-drought harvest to final harvest for each species within each water treatment were conducted with three-way ANOVAs (time, [CO\(_2\]) and temperature) followed by Duncan post-hoc tests when time effects were significant. The effects of species, [CO\(_2\]) and temperature on the time-to-mortality were analysed using 95 % confidence intervals. In all cases, results were considered significant if \(P \leq 0.05\). All analyses were performed in SPSS (version 20.0; SPSS, Chicago, IL, USA).

### 4.3 Results

#### 4.3.1 Soil water conditions
In the *well-watered* treatment, soil VWC was stable in *P. radiata* (0.257 ± 0.010 m\(^3\) m\(^{-3}\)) and *C. rhomboidea* (0.262 ± 0.015 m\(^3\) m\(^{-3}\)) during the experimental period, and did not differ significantly between species or among [CO\(_2\)] and temperature treatments (Three-way repeated measures ANOVA: *P* > 0.05 for all cases) (Figure 4-1). In the *drought* treatment, soil VWC declined in both species as drought progressed (Figure 4-1), but it was differentially affected by [CO\(_2\)] and temperature treatments between the two species. In *P. radiata*, declines in soil VWC did not differ among [CO\(_2\)] and temperature treatments (Two-way ANOVA: *P* > 0.05 for both cases). By contrast, in *C. rhomboidea*, declines in soil VWC were more rapid in elevated temperature treatments compared with ambient temperature treatments prior to Day 40 (Two-way ANOVA: *P* < 0.05 for all cases) (Figure 4-1), after which reductions in soil VWC slowed down in *C\(_e\)T\(_e\).*
Figure 4-1 Estimated soil volumetric water content (m$^3$ m$^{-3}$) in (a, b) *Pinus radiata* and (c, d) *Callitris rhomboidea* seedlings subjected to *well-watered* and *drought* under two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments over the course of the experiment. Soil volumetric water content was estimated by relationships of pot weight and monitored TDR values. Water was withheld in *drought* seedlings.
4.3.2 Leaf gas exchange

Pre-drought, $A_{\text{sat}}$, $g_s$ and $R_n$ were similar for seedlings assigned to well-watered and drought treatments in both species (Four-way ANOVA: $P \geq 0.485$ for all cases) (Figures 4-2 and 4-3). $A_{\text{sat}}$ and $g_s$ were higher in *P. radiata* than in *C. rhomboidea* (Four-way ANOVA: $P < 0.001$ for both cases). Pre-drought $A_{\text{sat}}$ in both species was increased by elevated [CO$_2$] treatments (Three-way ANOVA: $P < 0.001$ for both species) and decreased by elevated temperature treatments (Three-way ANOVA: $P < 0.001$ for both species). However, pre-drought $g_s$ in the two species was reduced by both elevated [CO$_2$] and temperature treatments (Three-way ANOVA: $P < 0.001$ for all cases). Pre-drought leaf $R_n$ did not differ between the two species or [CO$_2$] treatments within species ($P > 0.05$ for all cases), but $R_n$ in *P. radiata* was higher in elevated temperature treatments (Three-way ANOVA: $P < 0.001$).

Over the duration of the experiment, $A_{\text{sat}}$ of well-watered seedlings in *P. radiata* and *C. rhomboidea* was higher (c. 50% and 44%, respectively) in elevated [CO$_2$] treatments compared with ambient [CO$_2$] treatments (Two-way repeated measures ANOVA: $P < 0.05$ for both species), but $A_{\text{sat}}$ of well-watered seedlings in *C. rhomboidea* was lower (c. 30%) in elevated temperature treatments than in ambient temperature treatments (Two-way repeated measures ANOVA: $P < 0.05$) (Figure 4-2). The pre-drought effects of elevated [CO$_2$] and elevated temperature on $g_s$ of well-watered seedlings in *P. radiata* vanished over time, but $g_s$ of well-watered seedlings in *C. rhomboidea* was consistently lower in elevated temperature treatments (Two-way repeated measures ANOVA: $P < 0.05$). However, $R_n$ of well-watered seedlings in both species did not vary among [CO$_2$] and temperature treatments through time (Two-way repeated measures ANOVA: $P > 0.05$ for both species).
Figure 4-2 Photosynthesis at saturating light ($A_{sat}$), stomatal conductance ($g_s$) and leaf night respiration ($R_n$) of (a, b, c) *Pinus radiata* and (d, e, f) *Callitris rhomboidea* seedlings in well-watered treatments exposed to two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments over the course of the experiment. Values are Means ± SE (n = 4).
Figure 4-3 Photosynthesis at saturating light ($A_{sat}$), stomatal conductance ($g_s$) and leaf night respiration ($R_n$) of (a, b, c) *Pinus radiata* and (d, e, f) *Callitris rhomboidea* seedlings in drought treatments exposed to two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments over the course of the experiment. Values are Means ± SE (n = 4).
For the drought seedlings, $A_{sat}$ and $g_{s}$ in both species decreased as the soil dried, approaching zero 20 days earlier (on average) in $P. radiata$ than in $C. rhomboidea$ (Figure 4-3), suggesting that $A_{sat}$ and $g_{s}$ exhibited sharper declines in $P. radiata$ than in $C. rhomboidea$ as drought intensified (Figure 4-4). In $P. radiata$, the declines in $A_{sat}$ and $g_{s}$ converged among [CO$_2$] and temperature treatments as drought progressed, thereby promoting stomatal closure at similar soil VWCs across [CO$_2$] and temperature treatments (Figures 4-3 and 4-4). In $C. rhomboidea$, however, $A_{sat}$ and $g_{s}$ declined more rapidly in elevated temperature treatments compared with ambient temperature treatments through time (Two-way ANOVA: $P < 0.05$ for most cases) (Figure 4-3). Nevertheless, once soil VWC was considered (Figure 4-4), $A_{sat}$ and $g_{s}$ in $C. rhomboidea$ started to decline and reached zero at higher soil VWCs in $C_eT_e$ than in other [CO$_2$] and temperature treatments, indicating that $C_eT_e$ induced earlier onset of stomatal closure at a given soil VWC (Figure 4-4). For instance, $g_{s}$ in $C. rhomboidea$ reached zero at soil VWC of c. 0.10 m$^3$ m$^{-3}$ in $C_eT_e$ but at c. 0.05 m$^3$ m$^{-3}$ in other [CO$_2$] and temperature treatments (Figure 4-4d). The declining pattern of $R_{n}$ was different in the two species, although $R_{n}$ decreased much slower than $A_{sat}$ and $g_{s}$ in both species (Figures 4-3c and f). For example, $R_{n}$ in $P. radiata$ did not exhibit substantial declines until about Day 100, followed by a rapid decline to zero, while $R_{n}$ in $C. rhomboidea$ decreased gradually to zero through time. In both species, elevated temperature treatments resulted in more rapid decreases of $R_{n}$ (Two-way ANOVA: $P < 0.05$ for both species) (Figure 4-3).
Figure 4-4 Photosynthesis at saturating light ($A_{sat}$) and stomatal conductance ($g_s$) of (a, b) *Pinus radiata* and (c, d) *Callitris rhomboidea* seedlings in the drought treatment as a function of soil volumetric water content (VWC) exposed to two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments. All data points are raw data from the entire experiment. Data are fitted with the three-parameter sigmoid relationships for each of the four [CO$_2$] and temperature treatments: $y = a'/ (1 + e^{-(x-c)/b})$ ($P < 0.001$, $R^2 = 0.85-0.96$, $n = 40$)
4.3.3 Plant dry mass

Pre-drought, plant dry mass did not differ between the two species or among [CO₂] and temperature treatments (Three-way ANOVA: P > 0.05 for all cases) (Figure 4-5). In the well-watered treatment, dry mass in *P. radiata* rose similarly in all [CO₂] and temperature treatments throughout the experiment (on average 19-fold) (Figure 4-5a), while temporal increases (i.e. relative to pre-drought values) in dry mass in *C. rhomboidea* were higher in elevated [CO₂] treatments (on average 61-fold) than in ambient [CO₂] treatments (on average 52-fold) (Two-way ANOVA: P = 0.005) (Figure 4-5b). *Drought* limited dry mass accumulation in both species (Figure 4-5). The dry mass of drought seedlings in *P. radiata* and *C. rhomboidea* increased by 2-fold and 3-fold, respectively throughout the experiment, but it did not differ among [CO₂] and temperature treatments.
Figure 4-5 Plant dry mass in (a, b) *Pinus radiata* and (c, d) *Callitris rhomboidea* seedlings applied with well-watered (closed symbols) and drought (open symbols) under two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments over the course of the experiment. Values are Means ± SE. Pre-drought (Day 1), only well-watered seedlings were harvested as the baseline. Replicates: n = 3 for the first two/three harvests and n = 4 for the final harvest.
4.3.4 Leaf water potential and PLC

In the well-watered treatment, leaf $\Psi_{pd}$ and $\Psi_{md}$ in *P. radiata* (mean values: $\Psi_{pd} = -0.32 \pm 0.01$ MPa and $\Psi_{md} = -0.95 \pm 0.02$ MPa) and *C. rhomboidea* (mean values: $\Psi_{pd} = -0.35 \pm 0.02$ MPa and $\Psi_{md} = -0.91 \pm 0.03$ MPa) were relatively stable during the measured period (i.e. Day 40 to Day 118) and did not vary among [CO$_2$] and temperature treatments (Two-way repeated measures ANOVA: $P > 0.05$ for all cases; data not shown). In the drought treatment, $\Psi_{pd}$ and $\Psi_{md}$ in *P. radiata* exhibited gradual declines to $c. -2.5$ MPa as drought progressed (Figures 4-6a and b), but had sharp decreases after that point. By contrast, $\Psi_{pd}$ and $\Psi_{md}$ in *C. rhomboidea* decreased continuously to $< -10$ MPa and was generally more negative than those in *P. radiata* (Three-way repeated measures ANOVA: $P < 0.001$ for both cases) (Figure 4-6). Elevated temperature treatments led to more rapid declines in $\Psi_{pd}$ and $\Psi_{md}$ in *P. radiata* compared with ambient temperature treatments (Two-way repeated measures ANOVA: $P < 0.001$ for both cases), but elevated [CO$_2$] treatments did not alter the declines in $\Psi_{pd}$ and $\Psi_{md}$ in both species (Two-way repeated measures ANOVA: $P > 0.05$ for all cases) (Figure 4-6). As drought progressed, PLC reached 100 % in both species and in all [CO$_2$] and temperature treatments, suggesting that catastrophic hydraulic failure occurred in both gymnosperm species during the progressive drought (Figure A-5).
Figure 4-6 Pre-dawn ($\Psi_{pd}$) and mid-day ($\Psi_{md}$) leaf water potentials of (a, b) *Pinus radiata* and (c, d) *Callitris rhomboidea* seedlings subjected to drought (open symbols) under two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments over the course of the experiment. Values are Means ± SE (n=3). In leaf water potential measurements, values were considered as -10 MPa when leaf water potential was more negative than -10 MPa. $\Psi_{md}$ of both species on Day 81 was not reliable and thus was not used.
4.3.5 Organ TNC

Pre-drought, starch concentrations and soluble sugar concentrations of all organs in *P. radiata* and *C. rhomboidea* did not differ among [CO2] and temperature treatments (Figures 4-7 and 4-8). At the final harvest (i.e. at mortality), leaf starch concentrations of well-watered seedlings in *P. radiata* and *C. rhomboidea* were higher in elevated [CO2] treatments (Two-way ANOVA: *P* < 0.05 for both species), but starch concentrations of all organs of drought seedlings in both species were not affected by [CO2] and temperature treatments (Figure 4-7). In the well-watered treatment, leaf soluble sugar concentrations in *P. radiata* and root soluble sugar concentrations in *C. rhomboidea* were greater in elevated [CO2] treatments compared with ambient [CO2] treatments (Two-way ANOVA: *P* < 0.05 for both species) (Figure 4-8). In the drought treatment, however, root soluble sugar concentrations in *P. radiata* and stem soluble sugar concentrations in *C. rhomboidea* were increased by elevated [CO2] treatments (Two-way ANOVA: *P* < 0.05 for both species).

Over the duration of the experiment, in *P. radiata*, leaf starch concentrations of well-watered seedlings were reduced by c. 50 % in ambient [CO2] treatments compared with pre-drought values, but those of drought seedlings were reduced by a similar extent in all [CO2] and temperature treatments (*P* < 0.05 for all cases) (Figure 4-7a). However, there were no significant temporal changes in starch concentrations of stem and roots in *P. radiata* in all water, [CO2] and temperature treatments (*P* > 0.05 for all cases) (Figures 4-7b and c). In *C. rhomboidea*, well-watered seedlings exhibited significant accumulation in starch concentrations of leaves (i.e. in *C*<sub>c</sub>*T*<sub>a</sub>) and roots (i.e. in elevated temperature treatments), while drought seedlings had notable reductions (c. 70 %) in leaf starch concentrations in all [CO2] and temperature treatments (*P* < 0.05 for all cases) (Figure 4-7). There was significant
accumulation in root soluble sugar concentrations of well-watered and drought seedlings in *P. radiata* in all [CO₂] and temperature treatments compared with pre-drought values (*P* < 0.05 for all cases) (Figure 4-8c), however, there were no significant temporal changes in soluble sugar concentrations of leaves and stems in *P. radiata* (*P* > 0.05 for all cases) (Figures 4-8a and b). By contrast, drought seedlings of *C. rhomboidea* showed significant accumulation in leaf and stem soluble sugar concentrations compared with pre-drought values and accumulations were greater in elevated [CO₂] treatments, while well-watered seedlings only showed accumulations in leaf soluble sugar concentrations (*P* < 0.05 for all cases) (Figure 4-8). Overall, well-watered and drought seedlings in *P. radiata* exhibited similar temporal trends in starch and soluble sugar concentrations of all organs among [CO₂] and temperature treatments. In contrast to *P. radiata*, *C. rhomboidea* displayed opposite temporal changes in leaf starch concentrations between well-watered and drought treatments, but showed greater accumulation in leaf and stem soluble sugar concentrations in drought treatments than in well-watered treatments. Additionally, elevated [CO₂] treatments resulted in larger accumulation in soluble sugar concentrations of drought seedlings in *C. rhomboidea* compared with ambient [CO₂] treatments (*P* < 0.05 for all cases).
Figure 4-7 Starch concentrations in different organs (leaf, stem and root) of (a, b, c) *Pinus radiata* and (d, e, f) *Callitris rhomboidea* seedlings subjected to well-watered and drought under two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments pre-drought and at mortality. Pre-drought, only well-watered seedlings were harvested as the baseline. Values are Means ± SE (n=3 for pre-drought and n = 4 for mortality).
Figure 4-8 Soluble sugar concentrations in different organs (leaf, stem and root) of (a, b, c) *Pinus radiata* and (d, e, f) *Callitris rhomboidea* seedlings subjected to well-watered and drought under two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments pre-drought and at mortality. Pre-drought, only well-watered seedlings were harvested as the baseline. Values are Means ± SE (n=3 for pre-drought and n = 4 for mortality).
4.3.6 Seedling mortality

In *P. radiata*, seedlings were dead c. 40 days earlier in elevated temperature treatments compared with ambient temperature treatments, which was related to higher air VPD (Figure 4-9), but the time-to-mortality of seedlings did not differ between [CO₂] treatments. In *C. rhomboidea*, however, the mortality pattern was not significantly different among [CO₂] and temperature treatments (Figure 4-9).

4.3.7 Water use and WUE

In the *well-watered* treatment, more water was used in seedlings grown in elevated temperature treatments compared with ambient temperature treatments in both species (Two-way repeated measures ANOVA: *P* < 0.05) (Figure 4-10). By contrast, in the *drought* treatment, water use declined continuously to zero in both species. Water use of *drought* seedlings in *P. radiata* was not different among [CO₂] and temperature treatments (Two-way repeated measures ANOVA: *P* > 0.05) (Figure 4-10), but water use of *drought* seedlings in *C. rhomboidea* was greater in elevated temperature treatments in the first three weeks (Two-way ANOVA: *P* < 0.05 for those cases).

Leaf WUE of *well-watered* and *drought* seedlings was higher in elevated [CO₂] treatments than ambient [CO₂] treatments in both species (Two-way ANOVA: *P* < 0.001 for all cases) (Figure 4-11). However, whole-plant WUE did not differ among [CO₂] and temperature treatments within each species (Two-way ANOVA: *P* > 0.05 for all cases) (Figure 4-11), suggesting that greater leaf WUE as a result of elevated [CO₂] did not lead to higher whole-plant WUE in this study.
Figure 4-9 Leaf browning, mean time-to-mortality and time-to-mortality as function of air VPD in (a, b, c) *Pinus radiata* and (d, e, f) *Callitris rhomboidea* seedlings in the *drought* treatment exposed to two [CO2] (400 µl l⁻¹ and 640 µl l⁻¹) and two temperature (26 °C and 30 °C) treatments. Seedlings with no brown leaf are 0% browning and seedlings full of brown leaves are 100% browning. In (a) and (d), values are Means ± SE (n = 4). In (b) and (e), values are Means ± 95% confidence intervals (n = 4). The values of air VPD were Means ± SE (n = 10000) during the experimental period. Leaf browning tracking started on Day 82.
Figure 4-10 Plant weekly water use of (a, b) *Pinus radiata* and (c, d) *Callitris rhomboidea* seedlings subjected to well-watered (closed symbols) and drought (open symbols) under two [CO₂] (400 μl l⁻¹ and 640 μl l⁻¹) and two temperature (26 °C and 30 °C) treatments over the course of the experiment. Values are Means ± SE (n = 4-10). The data of well-watered seedlings was not applicable in week 11 because of missing pot weights for some days.
Figure 4-11 Mean leaf instantaneous WUE and total plant WUE of (a, b) *Pinus radiata* and (c, d) *Callitris rhomboidea* seedlings subjected to well-watered and drought under two [CO₂] (400 µl l⁻¹ and 640 µl l⁻¹) and two temperature (26 °C and 30 °C) treatments over the period with positive photosynthesis (Day 1 to Day 58). Values are Means ± SE (n = 40 for instantaneous WUE and n = 3 for total plant WUE). Instantaneous WUE (µmol CO₂ mol⁻¹ H₂O) = $A_{sat}$ (µmol m⁻² s⁻¹)/$g_s$ (mol m⁻² s⁻¹). Total plant WUE = total plant dry mass (g)/ total water use (kg).
4.4 Discussion

In this study, the water relations and carbon dynamics during drought-induced mortality were examined by comparing two gymnosperms *P. radiata* and *C. rhomboidea* with contrasting stomatal strategies (relatively isohydric vs. anisohydric). The first hypothesis predicted that mortality would result from carbon starvation in *P. radiata* and hydraulic failure in *C. rhomboidea*. However, leaf desiccation and catastrophic xylem cavitation eventually occurred in both species prior to mortality. Although depletion of leaf starch was offset by increased leaf soluble sugar in *C. rhomboidea*, carbon starvation was not evident and therefore hydraulic failure was primarily responsible for drought-induced mortality in both species. Additionally, the main and interactive effects of elevated [CO2] and temperature on drought mortality were addressed. In partial agreement with the second hypothesis that elevated temperature would exacerbate drought stress and accelerate mortality in *P. radiata* through carbon starvation and in *C. rhomboidea* through hydraulic failure, the data showed elevated temperature treatments led to faster mortality in *P. radiata*, but primarily through hydraulic failure associated with more rapid decline in pre-dawn and mid-day leaf water potentials. However, elevated temperature treatments did not modify the time-to-mortality in *C. rhomboidea* or promoted earlier onset of stomatal closure independent of soil VWC in either species. The third hypothesis predicted that elevated [CO2] would ameliorate drought stress and delay mortality in both species. However, elevated [CO2] did not alter physiological responses (i.e. gas exchange and leaf water potential) to progressive drought nor did it modify time-to-mortality in either species, thus leading to the rejection of the third hypothesis. Finally, the fourth hypothesis that elevated [CO2] would offset the negative effects of elevated temperature on drought response and mortality in both species was not
supported. In contrast, the data showed that although \( C_3T_o \) induced earlier declines in \( A_{sat} \) and \( g_s \) in \( C. rhomboidea \) independent of soil VWC compared with other [CO\(_2\)] and temperature treatments, elevated [CO\(_2\)] did not alter the effects of elevated temperature on drought-induced mortality in either species.

4.4.1 Water relations and carbon dynamics during drought-induced mortality: 

\textit{P. radiata vs. C. rhomboidea}

\textit{P. radiata} and \textit{C. rhomboidea} exhibited differing responses of water relations during soil drying. \textit{P. radiata} displayed complete stomatal closure with only small changes in soil VWC (c. range of 0.02 m\(^3\) m\(^{-3}\)) and leaf water potentials (c. range of 2 MPa) regardless of [CO\(_2\)] and temperature treatments. Leaf water potentials in \textit{P. radiata} remained > -2.5 MPa for c. 80 days and c. 100 days in elevated and ambient temperature treatments, respectively, despite continuous declines in soil VWC, suggesting that \textit{P. radiata} seedlings were hydraulically isolated from the soil (Plaut \textit{et al.}, 2012; Mitchell \textit{et al.}, 2013; Sevanto \textit{et al.}, 2013). In contrast, \textit{C. rhomboidea} gradually shut stomata down across a wider range of soil VWC (c. range of 0.05 m\(^3\) m\(^{-3}\)) and tolerated a much broader range of leaf water potentials (c. range of 6 MPa) across [CO\(_2\)] and temperature treatments. Leaf water potentials in \textit{C. rhomboidea} continued to decline to < -10 MPa during the progressive drought in all [CO\(_2\)] and temperature treatments. Despite these differences, both species eventually incurred substantial leaf desiccation and xylem cavitation during the mortality event.

Carbon dynamics in response to drought may be divergent among species with contrasting stomatal responses (Hartmann \textit{et al.}, 2013b; Mitchell \textit{et al.}, 2013). Relatively isohydric species are expected to have increased carbohydrate depletion due to longer periods of stomatal closure and negative carbon balance, while
relatively anisohydric species may exhibit minimal changes in carbohydrates (McDowell et al., 2008). However the findings here indicate that progressive drought did not induce significant carbohydrate depletion in relatively isohydric *P. radiata*. In relatively anisohydric *C. rhomboidea*, the significant depletion of leaf starch concentration was offset by the accumulation of soluble sugar concentration. In fact, carbohydrate depletion has not always been found in relatively isohydric gymnosperms during drought-induced mortality. For instance, *P. radiata* was shown to have depleted c. 50 \% carbohydrate reserves in a less intense but longer drought (i.e. progressive reduced rewatering for 120 days in the 200-day pot study) (Mitchell et al., 2013), while *Pinus edulis* did not exhibit significant temporal changes in carbohydrates during severe droughts (i.e. withholding water all the time in the 80 to100-day pot studies) (Anderegg & Anderegg, 2013; Sevanto et al., 2013). These divergent results suggest that the intensity and duration of drought can affect the relative roles of plant hydraulics and carbon metabolism. In Mitchell et al. (2013), the less intense drought maintained photosynthesis and respiration of *P. radiata* for 46 \% and 93 \% time of the entire experimental period, respectively. In this study (i.e. withholding water for the entire 150-day experimental period), however, the more intense drought in the present study restricted the period of photosynthesis and respiration of *P. radiata* to a shorter time period (i.e. 38 \% and 60 \%, respectively). Further, dry mass of *P. radiata* in Mitchell et al. (2013) was c. 6 times higher than that in this study, potentially leading to greater maintenance respiration. Thus, relatively longer periods of negative carbon balance plus larger respiration in Mitchell et al. study was more likely to lead to depletion of carbohydrates. By contrast, in this study, hydraulic failure played a greater role due to rapid soil drying, thereby preventing carbon utilization that would take place in the longer duration. In
contrast to *P. radiata*, the temporal conversion between starch and soluble sugar in *C. rhomboidea* in this study was consistent with other studies using angiosperms, such as *Eucalyptus* (Mitchell *et al.*, 2013; Chapter 2), but was different from the gymnosperm *Juniperus osteosperma* of which carbohydrates did not change during drought (Anderegg & Anderegg, 2013). Soluble sugar accumulation may be beneficial to plants during drought stress, by maintaining osmotic adjustment or hydraulic function (Hsiao *et al.*, 1976; Zwieniecki & Holbrook, 2009; McDowell, 2011; Sala *et al.*, 2012). Collectively, consistent with other gymnosperm studies (Plaut *et al.*, 2012; Anderegg & Anderegg, 2013; Hartmann *et al.*, 2013b; Sevanto *et al.*, 2013), hydraulic failure appears to be the primary factor in determining seedling death of gymnosperm species in this study, leading to the conclusion that “thirst beats hunger” (Hartmann *et al.*, 2013b).

### 4.4.2 Elevated temperature treatments resulted in more rapid mortality in *P. radiata* but not in *C. rhomboidea*

Experimental studies indicate that elevated temperature hastens drought-induced mortality of seedlings in environmentally controlled conditions (Adams *et al.*, 2009; Will *et al.*, 2013; Zhao *et al.*, 2013; Chapter 3), mainly due to two factors: increased evapotranspiration and increased metabolic respiration. In this study, *P. radiata* died ~ 40 days sooner in elevated temperature treatments than in ambient temperature treatments. Whole-plant water use in *P. radiata* did not significantly differ between temperature treatments, although VPD in elevated temperature treatments was c. 70 % higher than in ambient temperature treatments. However, with closed stomata, *P. radiata* in elevated temperature treatments exhibited faster declines in leaf water potentials and earlier substantial xylem cavitation than in ambient temperature treatments. Reduced evapotranspiration due to stomatal closure during drought can
increase the ratio of sensible heat to latent heat, thereby elevating plant surface
temperature and potentially increasing water loss from plant surface (e.g. through
cuticular tissues) after stomatal closure (Breshears et al., 2013; O’Grady et al., 2013).
Consequently, *P. radiata* seedlings desiccated faster in elevated temperature
treatments than in ambient temperature treatments. This finding highlights that
elevated temperature (and associated higher VPD) also have significant effects on
plant dehydration post-stomatal closure. On the other hand, there was no evidence
that carbohydrates at mortality were significantly different between ambient and
elevated temperature treatments in *P. radiata*. The difference between carbohydrates
pre-drought and at mortality did not vary between temperature treatments. Leaf
respiration in droughted *P. radiata* seedlings was not higher in elevated temperature
treatments than in ambient temperature treatments, reflecting the acclimation of
respiration to elevated temperatures (Atkin & Tjoelker, 2003; Wythers et al., 2005).
The results were thus inconsistent with Adams et al. (2009, 2013) whereby leaf
respiration and starch-sugar conversion were highly temperature dependent. Clearly,
more rapid mortality in *P. radiata* observed in this study was temperature driven and
promoted by hydraulic failure. In contrast to *P. radiata*, there were no significant
differences in time-to-mortality in *C. rhomboidea* between temperature treatments.

The higher VPD due to elevated temperature can generate higher evapotranspiration
demand, thereby generating more rapid stomatal closure with progressive drought
(Eamus et al., 2013; Will et al., 2013; Zhao et al., 2013; Chapter 3). For instance, *g*s
declines with stomatal closure at higher soil VWC in elevated temperature treatments
than in ambient temperature treatments in *Eucalyptus radiata* Sieber ex DC (Chapter
3) and *Thuja occidentalis* L. (Zhao et al., 2013). However, in this study, *P. radiata*
exhibited similar soil VWC, similar evapotranspiration and similar stomatal closure
among [CO₂] and temperature treatments, suggesting that stomatal responses of *P. radiata* to progressive drought were not modified by temperature treatments. In contrast to *P. radiata*, *C. rhomboidea* exhibited faster soil drying in *Cₜₑ* than *Cₜₐ* prior to Day 40, but did not display differences in stomatal response to drought, suggesting that elevated temperature alone did not increase the drought sensitivity of stomata but did accelerate soil drying.

### 4.4.3 Elevated [CO₂] did not delay mortality in both gymnosperms and did not offset the negative effects of elevated temperature on drought-induced mortality

There was no evidence that elevated [CO₂] ameliorated drought stress or delayed drought-induced mortality in either species. These results are consistent with the finding in *Eucalyptus radiata* (Chapter 3). The findings presented here suggest that elevated [CO₂] may not delay drought-induced tree mortality, at least in these two gymnosperms.

Although drought-induced mortality in *P. radiata* was more rapid in elevated temperature treatments than in ambient temperature treatments, there was no evidence that elevated [CO₂] offset the negative effects of elevated temperature on water relations and carbon dynamics (i.e. *Cₜₑ* = *Cₜₐ*), again consistent with findings from the *Eucalyptus radiata* study (Chapter 3). By contrast, the onset of stomatal closure in *C. rhomboidea* was earlier and at a higher soil VWC in *Cₜₑ* than other [CO₂] and temperature treatments. However, this did not lead to different mortality patterns in *C. rhomboidea*, which is a very drought tolerant species (i.e. *P₅₀* at about -9 MPa) (Brodirribb & Cochard, 2009; Brodirribb *et al.*, 2010). Despite differential stomatal responses among [CO₂] and temperature treatments under early drought
stress, xylem water transport and water status in *C. rhomboidea* seedlings were not altered by [CO₂] and temperature under late severe drought stress, thereby leading to similar mortality patterns.

### 4.4.4 Conclusions

This study indicates that hydraulic failure was the primary driver of drought-induced mortality in *P. radiata* and *C. rhomboidea* seedlings during severe drought conditions regardless of stomatal response strategies, highlighting that drought characteristics (e.g. drought intensity and duration) can affect the relative roles of plant hydraulics and carbon metabolism more so than physiological characteristics. Further studies are needed to examine whether carbon starvation independently occurs in less intense but longer droughts. Elevated temperature treatments generated more rapid mortality in *P. radiata* seedlings primarily through hydraulic dysfunction, but did not alter mortality patterns in *C. rhomboidea* seedlings. Elevated [CO₂] did not affect drought response in both gymnosperms or offset the effects of elevated temperature on drought. These findings suggest that rising temperatures (and associated higher VPD) appear to play the dominant role in affecting tree seedling survivals in future climates with more frequent severe droughts, rising [CO₂] and increasing air temperatures. Whether rising temperatures differentially affect seedling survivals among species requires more studies in other species. If so, the differing effects of rising temperatures on tree seedling response to drought may thus potentially influence forest succession and shifts in ecosystem composition.
Chapter 5

Synthesis and conclusions

5.1 Synthesis

The research presented in this thesis was designed to investigate the main and interactive effects of elevated [CO₂] and temperature on tree response to drought and subsequent mortality in four species representing different taxa and functional groups: *Eucalyptus globulus* Labill. (relatively isohydric, angiosperm; Chapter 2), *Eucalyptus radiata* Sieber ex DC (relatively anisohydric, angiosperm; Chapter 3), *Pinus radiata* D. Don (relatively isohydric, gymnosperm; Chapter 4) and *Callitris rhomboidea* R. Br (relatively anisohydric, gymnosperm; Chapter 4). In particular, the impact of these climate factors on water relations and carbon dynamics were assessed, in an effort to improve understanding of the drivers of forest mortality in future climate scenarios. This research sought to answer the following questions:

(1) How do water relations (i.e. plant and soil water status, hydraulic functions) and carbon dynamics (i.e. growth, photosynthesis, respiration and carbohydrate reserves) vary among species from different taxa or functional groups during drought and drought-induced mortality?
(2) Do elevated [CO$_2$] and elevated temperature independently or interactively alter tree response to drought and drought-induced tree mortality?

(3) If elevated [CO$_2$] and elevated temperature have effects on tree response to drought and drought-induced mortality, how do those effects vary among species from different taxa or functional groups?

5.1.1 Water relations and carbon dynamics during drought-induced tree mortality

Hydraulic failure and carbon starvation were proposed by McDowell et al. (2008) as a framework for interpreting interaction between water and carbon budgets during drought-induced tree mortality. The hydraulic failure hypothesis predicts cell desiccation and death due to extensive xylem cavitation, while the carbon starvation hypothesis predicts carbohydrate depletion due to prolonged periods of negative carbon balance. Despite experimental studies indicating that hydraulic failure appears to be the primary mechanism associated with drought-induced tree mortality (e.g. Adams et al., 2009, 2013; Anderegg et al., 2012; Anderegg & Anderegg, 2013; Hartmann et al., 2013b; Mitchell et al., 2013; Quirk et al., 2013; Sevanto et al., 2013), relative contributions of tree hydraulics and carbon metabolism may differ depending on water use and stomatal response strategies (McDowell et al., 2008; Mitchell et al., 2013), drought duration and intensity (McDowell et al., 2008), and interactions with abiotic factors such as [CO$_2$] and temperature (Adams et al., 2009, 2013; Quirk et al., 2013). The objectives of this thesis were to provide insights into tree response to drought and assess potential mechanisms associated with drought-induced mortality through a range of tree species with varying stomatal response strategies under potential future scenarios.
In this study, progressive drought generated negative effects on water and carbon budgets in all four studied species, leading to growth limitations, stomatal closure, sequential cessation of photosynthesis and leaf respiration, leaf desiccation, and catastrophic xylem cavitation. However, the timing of processes in response to drought varied among species depending on water use and stomatal response strategies. *E. globulus* and *E. radiata* seedlings had profligate water use, exhibited steep declines in $A_{sat}$ and $g_s$ in response small changes in soil VWC (range of c. 0.03 m$^3$ m$^{-3}$) and experienced rapid leaf desiccation (Chapters 2 and 3). *Pinus radiata* seedlings displayed rapid stomatal closure over a narrow soil VWC range (c. 0.02 m$^3$ m$^{-3}$), but maintained higher leaf water potential for longer periods prior to rapid declines in leaf water potentials (Chapter 4). In contrast, *C. rhomboidea* seedlings displayed gradual stomatal closure and tolerated a wider range of soil VWC (c. 0.05 m$^3$ m$^{-3}$) and leaf water potentials (Chapter 4). Despite these differences, hydraulic failure eventually occurred in all tree species.

Carbon starvation was not evident in either individual plant organs or at the whole-plant level in any of the studied species. Conversely, whole-plant TNC concentration increased over the experimental period in both well-watered and drought treatments compared with pre-drought values (Figure 5-1). Increases in whole-plant TNC concentration ranged from < 10 % in *E. globulus* and c. 20 % in *E. radiata* and *P. radiata*, to c. 50 % in *C. rhomboidea*. Nevertheless, the contribution of starch and soluble sugar to increased TNC concentration differed between well-watered and drought treatments. In the well-watered treatment, increased TNC was largely attributed to starch accumulation (e.g. *E. globulus*, *E. radiata* and *C. rhomboidea*). However, in the drought treatment, increased TNC was mainly due to accumulation
of soluble sugar (e.g. *E. globulus*, *P. radiata* and *C. rhomboidea*). Progressive
drought altered the ratio of starch and soluble sugar by depleting starch and
accumulating soluble sugar, in *E. globulus* and *C. rhomboidea*, but not in *E. radiata*
and *P. radiata* (Figure 5-1), indicating substantially different carbohydrate strategies
among species. During progressive drought, carbohydrates may contribute to osmotic
adjustment (Hsiao *et al.*, 1976) or maintainence of hydraulic transport (Salleo *et al.*, 2009; McDowell, 2011), among many potential coordinated processes (Sala *et al.*, 2010, 2012). Overall, this remains an understudied aspect of plant response to
drought.

Collectively, hydraulic failure played the predominant role during drought-induced
tree mortality in the severe droughts in this study, regardless of species of varying
taxa and functional groups. This study is consistent with those previous studies and
supports the conclusion of “thirst beats hunger” (Hartmann *et al.*, 2013b; O’Grady *et al.*, 2013). Nonetheless, whether carbon starvation plays a greater role in drought
mortality needs to be investigated in longer (multi-year), but less intense droughts.
**Figure 5-1** Temporal changes of whole-plant TNC concentration (mg g⁻¹) in seedlings exposed to *well-watered* and *drought* conditions in all four species studied over the experimental period. All the values are means among the four [CO₂] and temperature treatments. Values are Means (n = 4). Numbers represent the relative changes of final harvest values compared with pre-drought values. For *E. globulus*, sustained drought and rewatered drought treatments performed similarly, so I combined them as the “*drought*” treatment here. “*” indicates that the relative change is significantly different (P ≤ 0.05).
5.1.2 Effects of elevated temperature on drought stress and drought-induced tree mortality varied among species

Elevated temperature has been experimentally demonstrated to exacerbate drought stress and accelerate drought-induced tree mortality through higher evapotranspiration demand (Will et al., 2013) or a temperature driven imbalance of starch and soluble sugar (Adams et al., 2009, 2013). Nevertheless, these studies did not simultaneously address hydraulic and carbohydrate traits during prolonged drought. In contrast, my thesis addressed the impact of elevated temperature treatments on tree response to drought and associated drought-induced mortality by linking both hydraulic and carbohydrate traits and examining drought sensitivities of $A_{sat}$ and $g_s$ at given soil VWCs (i.e. $A_{sat}$ and $g_s$ as functions of soil VWC) (Chapters 2 to 4).

Elevated temperature and associated higher VPD increased drought sensitivities of $A_{sat}$ and $g_s$ in _E. radiata_ compared with ambient temperature treatments, thereby leading to earlier onset of stomatal closure at higher soil VWC (Chapter 3 and Table 5-1). Elevated temperature treatments also exacerbated drought stress in _E. radiata_ by inducing larger evapotranspiration demands, more rapid declines in leaf water potential and earlier catastrophic xylem cavitation, thereby generating faster mortality (Table 5-1). Despite elevated temperature treatments not altering drought sensitivities of $A_{sat}$ and $g_s$ in _P. radiata_, they still generated more rapid declines in leaf water potential and led to more rapid mortality in _P. radiata_ through hydraulic limitation (Chapter 4 and Table 5-1), suggesting that elevated temperature treatments accelerated plant dehydration after stomatal closure. Elevated temperature treatments exacerbated moderate drought stress in _E. globulus_ (Chapter 2), but negative effects
of elevated temperature treatments on $A_{sat}$ diminished as drought intensified. In $C. rhomboidea$ (Chapter 4 and Table 5-1), however, elevated temperature treatments did not modify drought stress at a given soil VWC or alter the mortality pattern.

Leaf night respiration was increased by elevated temperature under moderate drought conditions (e.g. $E. globulus$). However, effects of elevated temperature on leaf night respiration were not evident in any species as drought intensified. Conversely, elevated temperature led to more rapid declines in leaf respiration during progressive drought (e.g. $P. radiata$ and $C. rhomboidea$). Accordingly, in contrast to current expectations (see Allen et al., 2010), these observations suggest that elevated temperature may not increase leaf metabolism as drought becomes severe.

Furthermore, effects of elevated temperature on carbohydrates varied among species. Elevated temperature reduced carbohydrate reserves in $E. globulus$ and $C. rhomboidea$ seedlings exposed to drought treatment at the final harvest, but did not have impacts in $E. radiata$ and $P. radiata$ seedlings (Chapters 2 to 4; Table 5-1). But clearly, differences in carbohydrates due to temperature treatments did not alter the effects of elevated temperature on drought response in $E. globulus$ and $C. rhomboidea$. Overall, this study highlights that, as drought progresses, elevated temperature may have minimal effects on carbon metabolism and does not appear to affect drought responses through carbon reserves.

This study indicates that elevated temperature and consequent higher VPD can exacerbate drought stress and lead to more rapid tree mortality through hydraulic failure in $E. radiata$ and $P. radiata$, but not in $C. rhomboidea$ (note: mortality rate was not quantified in $E. globulus$). Although drought sensitivities of gas exchange traits were examined in this study, they were not sufficient to interrogate variation in
tree response to drought \(\times\) elevated temperature among species, particularly during the post-stomatal closure phase. Future investigations into traits indicative of drought vulnerability (e.g. xylem anatomy, \(P_{50}\), safety margins and whole-plant capacitance), will further improve our mechanistic understandings of how elevated temperature would alter tree response to drought among species. Furthermore, elevated temperature and higher VPD are usually confounding factors with respect to tree hydraulics under the interaction of elevated temperature and drought. More studies are needed to separate the direct effects of elevated temperature and VPD on tree response to drought given their importance for modelling forest response to future climate scenarios.

5.1.3 Elevated [CO\(_2\)] did not ameliorate drought stress or offset negative effects of elevated temperature in severe drought

Elevated [CO\(_2\)] often reduces leaf \(g_s\) and leaf level water loss under well-watered conditions, thus it is usually thought to ameliorate drought stress by maintaining more favourable water status (Wullschleger et al., 2002). However, the positive effects of elevated [CO\(_2\)] on drought responses were not always observed, mainly due to trade-offs between leaf-level water savings and increased whole-plant water loss (Warren et al., 2011; Zeppel et al., 2012). More importantly, the impact of elevated [CO\(_2\)] on drought-induced tree mortality remains to be explored. This study contributes to the current understanding of the effects of elevated [CO\(_2\)] on tree response to drought and drought-induced mortality by linking water relations and carbon dynamics at leaf and whole-plant levels.

In this study, leaf \(g_s\) and whole-plant water loss of the four species studied were not reduced by elevated [CO\(_2\)] treatments regardless of water treatments (Chapters 2 to 4
and Table 5-1), suggesting that leaf and whole-plant water savings were not evident in those four species. Elevated $[\text{CO}_2]$ was observed to ameliorate moderate drought stress in *E. globulus* by increasing photosynthesis and dry mass, but these impacts of elevated $[\text{CO}_2]$ were diminished as drought became severe (Chapter 2 and Table 5-1). However, elevated $[\text{CO}_2]$ did not modify drought stress in the other three species (i.e. *E. radiata*, *P. radiata* and *C. rhomboidea*). Leaf and whole-plant level water status in those three species were not improved by elevated $[\text{CO}_2]$, as indicated by similar drought responses of $A_{\text{sat}}$ and $g_s$ to decreased soil VWC, leaf water potentials and hydraulic functions between ambient and elevated $[\text{CO}_2]$ treatments (Chapters 3 to 4 and Table 5-1). Mortality rates in *E. radiata*, *P. radiata* and *C. rhomboidea* were not delayed under elevated $[\text{CO}_2]$. It is noted that leaf area and soil VWC in seedlings exposed to drought treatment were similar between $[\text{CO}_2]$ treatments (Chapters 2 to 4 and Table 5-1), indicating that leaf area did not confound the direct effects of elevated $[\text{CO}_2]$ on drought responses. On the other hand, contrary to current observations (e.g. Niinemets, 2010; Ayub *et al.*, 2011), carbohydrate reserves were generally not increased by elevated $[\text{CO}_2]$ in this study. Overall, this study highlights that elevated $[\text{CO}_2]$ *per se* may not ameliorate drought stress on tree species, particularly when drought becomes severe.

This study improves understanding of the interactive effects of elevated $[\text{CO}_2]$ and elevated temperature on tree responses. In the well-watered treatment, elevated $[\text{CO}_2]$ and elevated temperature in combination increased leaf area, dry mass and whole-plant TNC content (e.g. in *E. radiata* and *C. rhomboidea*) compared with elevated temperature treatment alone, suggesting that interaction of elevated $[\text{CO}_2]$ and temperature can benefit plant growth under favourable water conditions. More
importantly, this study contributes to current knowledge of how elevated [CO$_2$] would modify drought response to elevated temperature, which has been rarely studied and requires greater resolution (see Way, 2013). These findings indicate that elevated [CO$_2$] offset negative effects of elevated temperature on tree response to moderate drought stress in *E. globulus* by interactively increasing photosynthesis and whole-plant TNC content, but did not counteract effects of elevated temperature on drought responses of hydraulic and carbon traits in *E. radiata*, *P. radiata* and *C. rhomboidea*. Therefore, my study highlights that elevated temperature and consequent higher VPD may be the dominant contributors to tree response to drought in future climate change, independent of rising [CO$_2$].
Table 5-1 Summary of the main and interactive effects of elevated [CO₂] (640 µl l⁻¹) and elevated temperature (30 °C or 32 °C) on key traits of seedlings exposed to well-watered and drought conditions in four studied species, i.e. *E. globulus*, *E. radiata*, *P. radiata* and *C. rhomboidea*.

<table>
<thead>
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<th>Drought conditions</th>
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<td></td>
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<td></td>
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<td><em>E. radiata</em></td>
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<tr>
<td>Ψₚₛ</td>
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<tr>
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<tr>
<td>PLC</td>
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<tr>
<td>Aₛₜₜₜₚ</td>
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<tr>
<td>Rₛₜₜₜₚ</td>
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<td>-- --</td>
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<tr>
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<tr>
<td>Leaf WUE</td>
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<td>↑ -- --</td>
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<tr>
<td>Plant WUE</td>
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<tr>
<td>Time-to-mortality</td>
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</table>
Note There are three symbols in each cell. The first two symbols, i.e. blue and red symbols, represent the main effects of elevated [CO₂] and elevated temperature, respectively. “↑” means “increased” the measured trait and “↓” means “decreased” the measured trait. “--” means “no significant effects” on the measured trait. The third symbol, i.e. green symbol, represents the interactive effects of elevated [CO₂] and elevated temperature on measured trait. Specifically, “↑” means elevated [CO₂] and elevated temperature synergistically “increased” the measured trait and “↓” means elevated [CO₂] “offset” the effects of elevated temperature on the measured trait. “--” means “no significant interactive effects” on the measured trait. Blank cells mean that traits were not determined or captured. Water relation traits: $g_s$ – stomatal conductance (mol m⁻² s⁻¹); $Ψ_{pd}$ - pre-dawn leaf water potential (-MPa); $Ψ_{md}$ -Mid-day leaf water potential (-MPa); PLC- percentage loss of hydraulic conductivity (%); Plant water use (kg plant⁻¹). Carbon dynamics traits: $A_{sat}$ – photosynthesis at saturated light (µmol m⁻² s⁻¹); $R_n$– night time leaf respiration (µmol m⁻² s⁻¹); Biomass- whole-plant biomass (g); Leaf area (cm²); TNCₗ conc- leaf TNC concentration (mg g⁻¹); TNCₛ conc- stem TNC concentration (mg g⁻¹); TNCᵣ conc- root TNC concentration (mg g⁻¹); TNCₚₗₜₜ conc- whole-plant TNC concentration (mg g⁻¹); TNCₚₗₜₜ content- whole-plant TNC content (g). Other traits: Leaf WUE- leaf instantaneous water-use-efficiency ($A_{sat}/g_s$, µmol CO₂ mol⁻¹ H₂O); Plant WUE- whole-plant water-use-efficiency (biomass/ water use, g mass kg⁻¹ H₂O); Time-to-mortality (days). The effects of elevated [CO₂] and elevated temperature on $g_s$, $A_{sat}$ and $R_n$ are presented at a given soil water content.
5.2 Conclusions

This research addressed the main and interactive effects of elevated [CO₂] and temperature on tree response to drought and drought-induced tree mortality, by linking water relations and carbon dynamics in four tree species representing different taxa and functional groups. The study confirmed that hydraulic failure was the dominant mechanism generating tree mortality during severe droughts, irrespective of species of varying taxa and functional attributes. Increasing temperature (ambient + 4 °C) and consequent higher VPD exacerbated drought stress and led to more rapid mortality through hydraulic failure in most species in this study. Rising [CO₂] (ambient + 240 µl l⁻¹) ameliorated moderate drought stress in *E. globulus*, but the positive effects of rising [CO₂] were eliminated by increasing drought intensity. Further, elevated [CO₂] did not ameliorate drought stress in *E. radiata*, *P. radiata* and *C. rhomboidea* or delay the time-to-mortality. These results suggest that elevated [CO₂] may not ameliorate drought stress in these tree species, particularly when drought is prolonged and severe. Elevated [CO₂] partially offset the negative effects of elevated temperature during moderate drought stress in *E. globulus*, but did not ameliorate drought response to elevated temperature in the other three species in this study, suggesting that rising temperatures and associated higher VPD may be the predominant contributing factors to drought mortality in future climates. In summary, my research expands current knowledge regarding the interactive effects of elevated [CO₂] and temperature on tree response to drought. More importantly, this work will assist in developing more accurate predictions of how forests respond to future climate change with respect to more frequent and severe droughts, rising [CO₂] and increasing temperature.
Appendix A: Supplementary Figures and Tables

Chapter 2: Table A-1 Effects of atmospheric [CO₂] (400 µl l⁻¹ and 640 µl l⁻¹) and growth temperature (28 °C and 32 °C) on TNC concentration of organs (leaf, stem and root) of *Eucalyptus globulus* seedlings in different watering regimes (“well-watered”, “rewatered drought” and “sustained drought”) during the glasshouse experimental period (Day 63 to Day 103). Values are Means (n = 1). “----” shows missing data.
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<th>Water</th>
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<th>Day70</th>
<th>Day79</th>
<th>Day95</th>
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**Note** More details about watering regime refer to Figure 2-1.
Chapter 2: Table A-2 Effects of atmospheric [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and growth temperature (28 °C and 32 °C) on dry mass partitioning ratio of organs (leaf, stem and root) of *Eucalyptus globulus* seedlings in different watering regimes ("well-watered", “rewatered drought” and “sustained drought”) during glasshouse experimental period (Day 63 to Day 103). Values are Means ± SE (n= 2-6).

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| Note           | More details about watering regime refer to Figure 2-1.

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Chapter 2: Table A-3 Effects of atmospheric \([\text{CO}_2\]) (400 \, \mu l \, l^{-1} and 640 \, \mu l \, l^{-1}) and growth temperature (28 °C and 32 °C) on TNC content partitioning ratio of organs (leaf, stem and root) of \textit{Eucalyptus globulus} seedlings in different watering regimes (“well-watered”, “rewatered drought” and “sustained drought”) during the glasshouse experimental period (Day 63 to Day 103). Values are Means ± SE (n = 2-6). “----” shows missing data.

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<th>Day70</th>
<th>Day79</th>
<th>Day95</th>
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**Note** More details about watering regime refer to Figure 2-1.
Chapter 3: Figure A-1 Stomatal conductance ($g_s$) of *Eucalyptus radiata* seedlings as a function of leaf to air VPD in Licor-6400 cuvettes in (a) well-watered (closed symbols) and (b) drought (open symbols) treatments exposed to two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments. All data points were raw data from the entire experimental period.
Chapter 3: Figure A-2 (a, b) Pre-dawn (\(\Psi_{pd}\)) and (c, d) mid-day (\(\Psi_{md}\)) leaf water potentials of *Eucalyptus radiata* seedlings in well-watered (closed symbols) and drought (open symbols) treatments exposed to two [CO\(_2\)] (400 µl l\(^{-1}\) and 640 µl l\(^{-1}\)) and two temperature (26 °C and 30 °C) treatments during the experimental period. Values are Means ± SE (n=3). In leaf water potential measurements, values were considered as -10 MPa when leaf water potential was more negative than -10 MPa. The grey areas represent the rewatered phase.
Chapter 3: Figure A-3 (a,b) Weekly added water and (c,d) cumulative added water of *Eucalyptus radiata* seedlings in well-watered (closed symbols) and drought (open symbols) treatments exposed to two [CO\(_2\)] (400 µl l\(^{-1}\) and 640 µl l\(^{-1}\)) and two temperature (26 °C and 30 °C) treatments during the experimental period. Water additions to drought seedlings were discontinued on Day 71 (i.e. week 11) when minimum water loss across [CO\(_2\)] and temperature treatments was less than 20 ml. Values are Means ± SE (n = 7-10). Data for well-watered seedlings were not available in week 11. The grey areas represent the rewatered phase.
Chapter 3: Figure A-4 Photosynthesis at saturating light ($A_{sat}$) as a function of stomatal conductance ($g_s$) of Eucalyptus radiata seedlings in (a) well-watered (closed symbols) and (b) drought (open symbols) treatments exposed to two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments. All data points were raw data from the entire experimental period. Data from well-watered treatments were fitted with linear relationships for each of the four [CO$_2$] and temperature treatments: $y = ax + b$ ($P < 0.001$, $R^2 = 0.07$-$0.425$, $n = 48$-$50$). Data from drought treatments were fitted with the two-parameter exponential rise to maximum relationships: $y = a \left(1 - e^{-bx}\right)$ ($P < 0.001$, $R^2 = 0.96$-$0.98$, $n = 45$-$47$).
Chapter 4: Figure A-5 Percentage loss of stem hydraulic conductivity (PLC) as a function of xylem pressure in (a) *Pinus radiata* and (b) *Callitris rhomboidea* seedlings in well-watered (closed symbols) and drought (open symbols) treatments exposed to two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments on Day 58 and Day 118. Values are Means ± SE (n = 3). The dash line shows 50 % loss of hydraulic conductivity.
**Chapter 4: Figure A-6** Total plant WUE as a function of mean leaf instantaneous WUE and of (a) *Pinus radiata* and (b) *Callitris rhomboidea* seedlings subjected to well-watered (closed symbols) and drought (open symbols) under two [CO$_2$] (400 µl l$^{-1}$ and 640 µl l$^{-1}$) and two temperature (26 °C and 30 °C) treatments.
References


years of continuous exposure to elevated [CO₂] and temperature. Global Change Biology, 15, 368-379.


Wertin TM, McGuire MA, Teskey RO (2012a) Effects of predicted future and current atmospheric temperature and [CO₂] and high and low soil moisture on gas exchange and growth of Pinus taeda seedlings at cool and warm sites in the species range. *Tree Physiology, 32*, 847-858.


