CO2 and temperature effects on morphological and physiological traits affecting risk of drought-induced mortality

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Research paper

CO$_2$ and temperature effects on morphological and physiological traits affecting risk of drought-induced mortality

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Despite a wealth of eco-physiological assessments of plant response to extreme drought, few studies have addressed the interactive effects of global change factors on traits driving mortality. To understand the interaction between hydraulic and carbon metabolic traits influencing tree mortality, which may be independently influenced by atmospheric [CO$_2$] and temperature, we grew *Eucalyptus sideroxylon* A. Cunn. ex Woolls from seed in a full-factorial [CO$_2$] (280, 400 and 640 µmol mol^{-1}, C_p, C_a and C_e, respectively) and temperature (ambient and ambient +4 °C, T_a and T_e, respectively) experiment. Prior to drought, growth across treatment combinations resulted in significant variation in physiological and morphological traits, including photosynthesis (A_{sat}), respiration (R_d), stomatal conductance, carbohydrate storage, biomass and leaf area (LA). C_e increased A_{sat}, LA and leaf carbohydrate concentration compared with C_a, while C_p generated the opposite response; T_e reduced R_d. However, upon imposition of drought, T_e hastened mortality (9 days sooner compared with T_a), while C_e significantly exacerbated drought stress when combined with T_e. Across treatments, earlier time-to-mortality was mainly associated with lower (more negative) leaf water potential (Ψ_l) during the initial drought phase, along with higher water loss across the first 3 weeks of water limitation. Among many variables, Ψ_l was more important than carbon status in predicting time-to-mortality across treatments, yet leaf starch was associated with residual variation within treatments. These results highlight the need to carefully consider the integration, interaction and hierarchy of traits contributing to mortality, along with their responses to environmental drivers. Both morphological traits, which influence soil resource extraction, and physiological traits, which affect water-for-carbon exchange to the atmosphere, must be considered to adequately predict plant response to drought. Researchers have struggled with assessing the relative importance of hydraulic and carbon metabolic traits in determining mortality, yet an integrated trait, time-dependent framework provides considerable insight into the risk of death from drought for trees.

Keywords: biosphere–atmosphere feedbacks, carbon starvation, drought mortality, global change ecology, global warming, hydraulic failure, pre-industrial [CO$_2$].

Introduction

Earth's atmospheric CO$_2$ concentration ([CO$_2$]) and temperature have been increasing since the start of the industrial revolution, and these trends are expected to continue into the next century, with important consequences for plant function (Christensen et al. 2007,

McDowell et al. 2010, McDowell 2011, Franks et al. 2013, IPCC 2014). In addition to their direct effects on plant function, rising [CO$_2$] and temperature may also greatly influence landscape water balance (Weltzin et al. 2003, Zeppel et al. 2011), thereby indirectly altering plant performance and subsequent survival (Davi and

Cailleret 2017). Reductions in precipitation over the last century have also been well documented (Huntington 2006, CCSP 2008), with projections for increasing aridity in many regions, including Australia (Seager et al. 2007, Hartmann 2011, The Bureau of Meteorology and CSIRO 2016). Indeed, regional-scale forest dieback, driven by water and heat stress interacting with biotic pressures, has been documented on all forested continents (Allen et al. 2010). Vegetation shifts at this scale have the potential to dramatically alter ecosystem structure and function (Huxman et al. 2005, Bonan 2008, Adams et al. 2010, Anderegg et al. 2013). However, we are only beginning to develop sufficient understanding of the physiological mechanisms responsible for drought-induced mortality to inform predictive models (McDowell et al. 2008, Sala et al. 2010, Mitchell et al. 2013, 2014), and to place that knowledge into projections of climate change and the consequences for biological features of landscapes (Allen et al. 2015, Mitchell et al. 2016).

Much research to date has focused on evaluating two non-mutually exclusive, inter-related physiological mechanisms of drought-induced mortality (McDowell 2011): (i) hydraulic failure, which occurs when reduced soil water supply and high evaporative demand generate sufficient cavitation in xylem conduits to impede water flow, desiccating plant tissue and leading to cellular death (Brodribb and Cochard 2009); and (ii) carbon starvation, which occurs when stomatal closure reduces photosynthetic carbon uptake such that metabolic consumption of carbohydrates (e.g., respiration) eventually depletes stored carbon reserves, leading to cellular death (Adams et al. 2013). Carbon starvation can be exacerbated by utilization of carbon for osmotic and defensive processes and failure of carbon mobilization (Sala et al. 2010, McDowell 2011). Additionally, these two mechanisms can be inter-related. Reduced carbohydrate availability may impede the creation of an osmotic gradient for xylem refilling, thereby increasing the risk of hydraulic failure (McDowell 2011). Mortality due to carbon starvation is primarily determined by stored carbohydrate content and rates of carbohydrate utilization, and hydraulic failure by access to soil water, stomatal control of water loss and xylem cavitation. Both processes are technologically challenging to document (Zeppel et al. 2015, Hartmann and Trumbore 2016). A growing body of work has focused on understanding how contemporary changes in mortality patterns can be attributed to these different processes (Hartmann et al. 2013b), and how they may be inter-related (McDowell et al. 2011, Anderegg et al. 2012, Duan et al. 2015). A recent meta-analysis of drought-induced mortality of 26 species from 19 studies suggests that hydraulic failure was the primary factor generating mortality of tree species, while reduction in non-structural carbohydrates (NSC), which differed among species or taxa groups, was more common in gymnosperms than angiosperms (Adams et al. 2017).

In order to develop predictive capabilities, research has focused on determining to what degree these mortality processes are sensitive to environmental features, such as atmospheric [CO_2] and temperature. In an initial study, Adams et al. (2009) observed that piñon pine saplings, grown at elevated temperature (+4 °C) and exposed to severe drought, died 28% faster than ambient temperature-grown saplings. Although this was a limited study (see Sala 2009), lack of evidence for hydraulic failure and increased leaf respiration in elevated temperature suggested carbon starvation was the physiological mechanism most sensitive to temperature, and best predictor of time-to-mortality between treatments. Subsequent evaluation of needle carbon pool dynamics showed starch–sugar inter-conversion (i.e., hydrolysis of starch to sugars for metabolic and osmotic requirements or starch synthesis from sugars) mediated changes in long-term carbon acquisition, use and total pool size upon the onset of mortality that were consistent with the notion of higher sensitivity of carbon dynamics under warming reducing time-to-mortality (Adams et al. 2013). Since these early studies, research on different tree systems have found divergent results (Hartmann et al. 2013a, 2013b), and the research community has begun to explore other factors of specific interest, such as how [CO_2] acts as a potential driver (Quirk et al. 2013, Duan et al. 2014).

Across plant biology and ecology, there is increasing interest in evaluating these mortality processes within the context of their integration into life cycle stages, and in the context of how traits interact within a whole plant. This focus includes assessments of how biomass allocation and morphology are altered by conditions prior to drought, how mortality processes and patterns are shaped by life history strategies, the role of plant–soil coupling and the extent of water stress experienced by a plant, and how population dynamics create a specific context for mortality (e.g., Huxman et al. 2013). In short, research in the last several years has evolved rapidly to consider complex interactions of drivers and demand a sophisticated treatment of trait inter-relationships affecting whole-plant function (Hartmann et al. 2015, Anderegg et al. 2016).

In this study, we grew *Eucalyptus sideroxylon* saplings from seed in a fully factorial atmospheric [CO_2] (280, 400 and 640 µmol mol^{-1}, herein referred to as C_p, C_a and C_e, respectively) and temperature (ambient and ambient +4 °C, herein referred to as T_a and T_e, respectively) experiment in a sun-lit glasshouse for 9 months in well-watered conditions, prior to being exposed to an extended, lethal drought (see Zeppel et al. (2012) for additional information). We used the range and combination of [CO_2] and temperature treatments to establish initial differences in key morphological and physiological traits, including light-saturated photosynthesis (A_{sat}), respiration (R_d), stomatal conductance (g_s), NSC, biomass and leaf area (LA), prior to imposition of the drought, thereby allowing a more expansive exploration of the relationships among traits associated with drought risk. Our objective was to elucidate the rate and sensitivity of drought-induced mortality mechanisms underlying

hydraulic failure and carbon starvation hypotheses to [CO$_2$] and temperature treatments. We assessed these hypotheses as a function of morphological and physiological attributes generated pre-drought, and the roles of environmental conditions and plant behavior during drought. Based on the significant body of research focused on elevated [CO$_2$] and temperature effects on plant function (see reviews by Ainsworth and Rogers 2007, Way and Oren 2010, Becklin et al. 2017), we expected that (i) despite reduction in g_s, saplings in C_e would use more water than those in C_a during drought given greater LA for whole-plant water loss from transpiration, thereby exhibiting higher susceptibility to hydraulic failure and shorter time-to-mortality. However, saplings in C_e would still experience lower susceptibility to carbon starvation given enhanced carbohydrate reserves in C_e saplings compared with those in C_a; (ii) saplings in C_p would have less whole-plant water loss than those in C_a given smaller LA, thereby exhibiting lower susceptibility to hydraulic failure and longer time-to-mortality, but nevertheless, saplings in C_p would experience higher susceptibility to carbon starvation given reduced carbohydrate reserves in C_p saplings compared with those in C_a; and (iii) compared with T_a, saplings in T_e would exhibit increased whole-plant water loss and enhanced carbohydrate consumption due to higher respiration during drought, thereby experiencing higher susceptibility to both hydraulic failure and carbon starvation and shorter time-to-mortality (Figure 1).

Materials and methods

Growing conditions

Red Ironbark (*E. sideroxylon* A. Cunn. ex Woolls) seeds obtained from CSIRO/ENSIS (Australian Tree Seed Centre, Canberra, ACT, Australia) were germinated in sun-lit glasshouse rooms with ambient growth conditions (i.e., 400 µmol mol^{-1} and ambient temperature; but ~10–15% of direct sunlight was reduced by the glasshouse) at Western Sydney University (Richmond, NSW, Australia) and grown for 8 weeks. *Eucalyptus sideroxylon* is a wide-spread medium-statured tree species, with fairly large lanceolate leaves, and ranging from south-eastern Queensland, through the western slopes and plains of New South Wales, extending into Victoria in Australia. After germination and establishment, saplings were placed into 75 l pots filled with local native forest soil and assigned to six climate-controlled bays of the same sun-lit glasshouse and grown in three [CO$_2$] (280 (C_p), 400 (C_a) and 640 (C_e) µmol mol^{-1}) and two temperature (ambient (T_a) and elevated (i.e., ambient +4 °C; T_e)) treatments in a factorial design. The glasshouse compartments were set to simulate ambient temperature, defined as the temperature of a 30-year average local (Richmond, NSW, Australia) day for the summer months of November to May, for T_a, with a + 4 °C for T_e. Temperatures were changed five times over 24 h to simulate natural diurnal variation (i.e., at 06:00, 10:00, 16:00, 20:00 and 24:00 h). Maximum and minimum temperatures for the T_a and T_e

treatments were 26/18 and 30/22 °C (day/night), respectively. The vapor pressure deficit (D) varied between 0.4 and 2.5 kPa in the T_a treatment and between 1.0 and 3.8 kPa in the T_e treatment. Saplings were rotated weekly within each of the six bays and monthly between bays to minimize environmental variation associated with specific positions within the glasshouse.

Saplings were watered once every 3 days to field capacity (~2 to 3 l), and fertilized on four occasions (50, 90, 120 and 170 days after planting (DAP)) with a liquid commercial fertilizer (Aquasol, Hortico Company, VIC, Australia). Following 7 months of additional growth (when saplings were 9 months old), irrigation was completely withheld for three saplings per treatment (i.e., drought treatment) until mortality was observed (detailed below), while the remaining three saplings (i.e., well watered control) continued receiving water every 3 days in a manner similar to the previous 7 months of growth. For additional facility design, growth conditions and coupled experiments, see Zeppel et al. (2012).

Physiological and growth measurements

Before initiation of the drought treatment and throughout the dry-down period, we measured photosynthesis under saturating light (A_{sat}) and stomatal conductance (g_s) during the daytime (09:00–13:00 h), along with dark respiration at night (R_d), using a Licor-6400XT Portable Photosynthesis System (Li-Cor Inc., Lincoln, NE, USA) with the red blue light source (6400-02B). For each measurement, the cuvette was held at the [CO$_2$] and temperature treatment associated with the specific sapling growth conditions, while the saturating photosynthetic photon flux density was set at 1800 µmol m^{-2} s^{-1}. Mid-day leaf water potential (Ψ_l) (11:00–13:00 h) was measured using a Scholander-type pressure chamber (PMS Instruments, Corvallis, OR, USA) on a weekly basis upon the onset of drought.

Leaf-level total non-structural carbohydrates (NSC) were measured weekly using the method described by Duan et al. (2014) and Quentin et al. (2015). In summary, ~20 mg oven-dried fine powder per sample was 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. Starch was determined on the pellets and assayed enzymatically using a total starch assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland). Soluble sugars were determined on the supernatants by the anthrone method (Ebell 1969). A standard glucose solution and standard tissue was used during each assay run to minimize potential variability. The area-based NSC presented in this paper was corrected by leaf mass per area (LMA). Leaf mass per area was calculated as leaf mass divided by leaf area. Non-structural
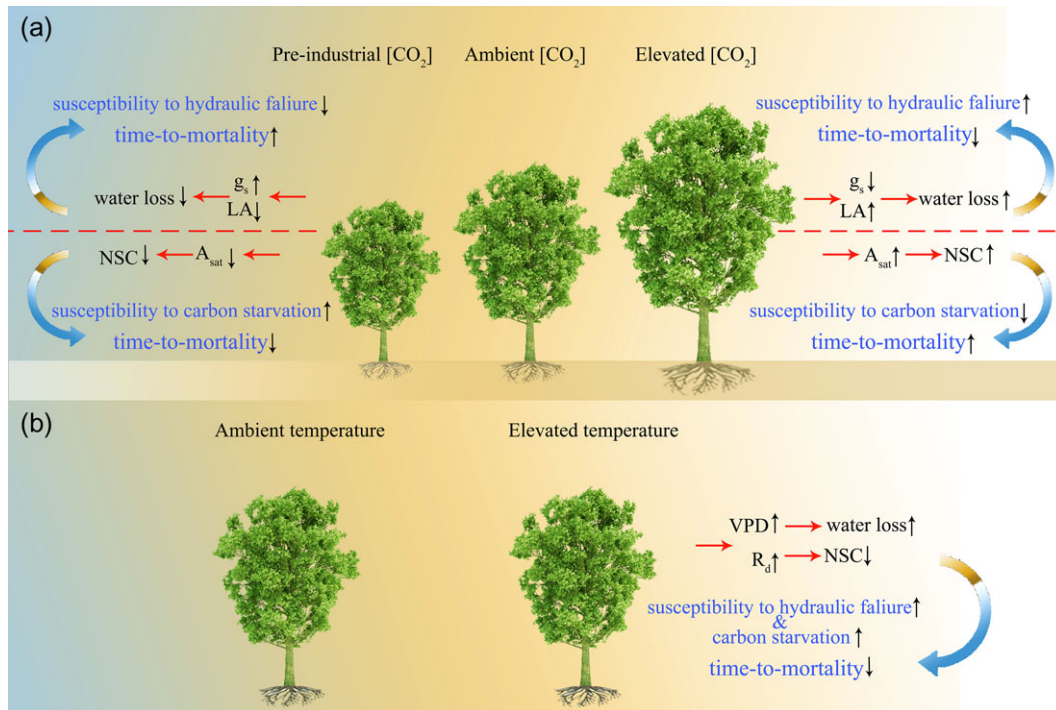
Figure 1. Hypotheses for effects of [CO₂] (a) and air temperature (b) on *Eucalyptus sideroxylon* saplings in response to lethal drought. Based on current literature, plants are predicted to die under lethal drought stress through hydraulic failure and/or carbon starvation. Changes in [CO₂] and temperature may have substantial effects on plant carbon and water relations, thereby potentially modifying the mechanisms underlying drought-induced mortality. In (a), we present the effects of elevated [CO₂] (right side) and pre-industrial [CO₂] (left side) on sapling carbon and water relations towards drought-induced mortality. A_{sat}, light-saturated photosynthesis; g_s, stomatal conductance; R_d, leaf dark respiration at night; LA, tree leaf area; NSC, non-structural carbohydrates; water loss, water loss at the whole-plant level. Up arrow means values of the related traits increased; down arrow means values of the related traits decreased.

carbohydrates were calculated as the sum of leaf soluble sugars (L_{ss}) and starch (L_{st}).

Mortality definitions vary across studies; for the purposes of our study, we considered saplings dead when Ψ_l was below −10 MPa, all leaves were dry and no green stem remained. After death, plants were destructively harvested to determine total leaf area (LA) using a leaf area meter (Licor-3100A; Li-Cor, Lincoln, NE, USA). We additionally measured the mass of individual pots with intact saplings on a weekly basis to evaluate rates of whole-plant water loss (inclusive of soil evaporation, which was assumed to be near-zero following the first few days of drought treatment due to the diffusive barriers associated with rapidly drying soil surfaces). We used these rates of whole-plant water loss to understand the temporal dynamics of whole-plant function and how water-use strategies were associated with mortality risk.

Statistical analyses

All statistical analyses were conducted with the open-source statistical software platform R (version 3.4.1; 2017-06-30, R Foundation for Statistical Computing). To illustrate the collective behavior within each treatment, to make comparisons associated with the performance of these saplings and other studies in the literature, and to attempt to understand the mechanistic features

underlying the patterns evaluated with time-to-mortality, we used two-way analysis of variance (ANOVA) to test for CO_2 and temperature treatment effects on plant variables prior to the initiation of drought (A_{sat}, g_s, R_d, Ψ_l, biomass, LA). Pairwise contrasts (following significant effects identified by pre-drought ANOVA) were evaluated post-hoc with Tukey's HSD tests. Time series of parameters were analyzed using repeated-measures mixed-effects with the 'nlme' package. The fixed factors were CO_2, temperature, water and/or date. Seedling number was treated as a random factor in all analyses. Homoscedasticity and normality were checked prior to all statistical analyses. Values of A_{sat}, g_s and Ψ_l were log-transformed. Results were considered statistically significant at $P < 0.05$.

We also used stepwise regression (via backward elimination) to more fully assess leaf and whole-plant changes within and among treatments, consistent with our hydraulic and carbon hypotheses. This approach enabled us to consider the importance of traits in a hierarchical fashion, which is valuable in fully understanding proximate and ultimate drivers of the changes in time-to-mortality. We used the 'relaimpo' package in R to carry out this analysis. The purpose of the test is not limited to documenting or uncovering the best model and the variables associated with that model. The test is used to understand the partitioning of variance among variables in a stepwise approach,

where data are often more correlated than is appropriate for the basic multiple regression approach of using R^2 as indicative of the variance fraction. This approach is used to understand 'dispersion importance' (e.g., Gromping 2006), where we can assess the hierarchy of variable influence on time-to-mortality as it relates to our hypotheses and current theories for plant mortality. In this case, initial water potential represents an order of magnitude more variance than any other variable, which is three times greater than anything else. The 'relaimpo' package uses a bootstrapping approach that provides an averaging-over-ordering approach, which creates an importance value reflecting a more reliable estimate of variance partitioning. We purposely did not include the interactions, because our focus was on identifying plant physiological and morphological traits that allow us greater insight into the current carbon starvation and hydraulic failure hypotheses, and their traditional treatment with respect to plant function.

Results

Growth and physiology at pre-lethal drought stage

Saplings grown at C_e exhibited increased A_{sat}, LA and NSC when compared with C_a, but leaf R_d, g_s and total biomass did not exhibit clear differences (Figure 2). A_{sat} was reduced at C_p compared with C_a, and A_{sat}, LA, total biomass and NSC at C_p were lower than C_e (Figure 2). Overall, saplings at C_e had higher A_{sat} and NSC than C_a and C_p saplings prior to the imposition of drought. Although g_s did not clearly differ among [CO$_2$] treatments, initial whole-plant water loss generally increased with increasing [CO$_2$] (described in more detail below). Higher growth temperature did not clearly affect A_{sat}, g_s, LA, biomass or leaf NSC, but surprisingly decreased instantaneous rates of leaf R_d by ~10% when evaluated across all [CO$_2$] treatments (Figure 2).

Process dynamics during lethal drought

According to the earlier coupled study (Zeppel et al. 2012), soil water potential (Ψ_s) substantially declined in the drought treatment, with more negative Ψ_s associated with increasing [CO$_2$] and temperature. In particular, Ψ_s of drought-stressed saplings grown in $C_e T_e$ exhibited the greatest decline (to ca −0.9 MPa in the end), followed by $C_e T_a$ and $C_a T_e$ (ca −0.4 MPa), while those in $C_p T_a$, $C_p T_e$ and $C_a T_a$ showed the lowest decline (ca −0.2 MPa). Drought stress altered all major carbon and water physiological processes (Figure 3; $P < 0.001$ for all cases). The main effects of [CO$_2$] and temperature on Ψ_l were significant as drought was progressed and their interactive effects were marginally significant (Figure 3a–c, Table 1). $C_e T_e$ saplings were the first to exhibit substantial reductions in Ψ_l (Figure 3). By drought Day 30, Ψ_l in this treatment rapidly dropped to −10 MPa and these saplings experienced mortality by Day 43. Ψ_l declined more slowly in the remaining [CO$_2$] and temperature combinations, and these treatments were separated in time. $C_a T_e$ saplings reached −10 MPa by Day 53, while $C_p T_e$ saplings

exhibited similar values a few days later. Within T_a, saplings declined to low Ψ_l in the reverse order observed at T_e—beginning with $C_p T_a$, then $C_e T_a$ and finally $C_a T_a$. For both C_a and C_e at T_a and T_e, mean g_s values were indistinguishable from zero before Ψ_l reached −10 MPa (Figure 3). In contrast, mean Ψ_l in the $C_p T_e$ and $C_p T_a$ treatments dropped below −10 MPa before mean g_s was indistinguishable from zero. Overall, higher temperature led to more rapid reductions in Ψ_l at both C_a and C_e, but did not have a clear effect at C_p.

In contrast, the temperature treatment significantly affected the responses of A_{sat} and g_s to drought stress (Figure 3d–i, Table 1). The imposition of drought sharply reduced A_{sat} across all [CO$_2$] levels, but especially for saplings grown at T_e when compared with T_a (Figure 3d–f). A_{sat} was indistinguishable from zero by drought Day 41 or 58 for T_e and T_a saplings (averaged across [CO$_2$]), respectively. The trend in g_s was similar to the trend observed in A_{sat} (Figure 3g–i). The response of g_s to Ψ_l across [CO$_2$] treatments and time was similar between temperature treatments (Figure 4a), indicating that the drought sensitivity of g_s was not altered by higher growth temperature. But, as determined gravimetrically, significant effects of [CO$_2$] (repeated measure ANOVA: $P = 0.002$) and temperature (repeated measure ANOVA: $P = 0.033$) on whole-plant water loss were observed (Figure 5). For example, C_e saplings lost more water in the first week of the experimental drought period compared with C_p (Figure 5; CO$_2$: $P = 0.003$). Subsequently, water loss in $C_e T_e$ saplings in the third week was smallest compared with those in other treatments (CO$_2 \times$ Temperature: $P = 0.030$), indicating that $C_e T_e$ saplings were first affected by drought stress at the whole-plant level. T_e saplings had larger water loss in the first few days, particularly during the first week. Interestingly, water-use patterns in the third week (when g_s reductions were significant despite continued substantial water loss in T_e) strongly predicted total water loss over the entire drought period for all treatments (Total water loss = (52.45 × water loss in the third week) + 7.16; $R^2 = 0.87$; $P < 0.05$).

Across all treatments and time periods, saplings grown at T_e had lower R_d than those grown at T_a, and T_e saplings reduced R_d to values indistinguishable from zero more quickly than saplings at T_a (Figure 3j–l). Nonetheless, due to differences in the time-course of changes in plant water status vs carbon metabolism, the response of R_d to changes in Ψ_l was smaller at T_e than T_a (Figure 4b). The [CO$_2$] and temperature treatments differentially affected A_{sat} and R_d, so that leaf carbohydrates varied significantly across the drought period (Figure 6a–f). L_{ss} did not vary much over the progression of drought. In contrast, L_{st} in drought saplings exhibited reductions relative to the well watered controls (Repeated measure ANOVA: $P < 0.001$), with greater difference occurring in T_a treatments (78% lower than well watered saplings) than T_e treatments (40% lower than well watered saplings). Starch values were typically lower at mortality than at the initiation of water stress, particularly in T_a treatments.
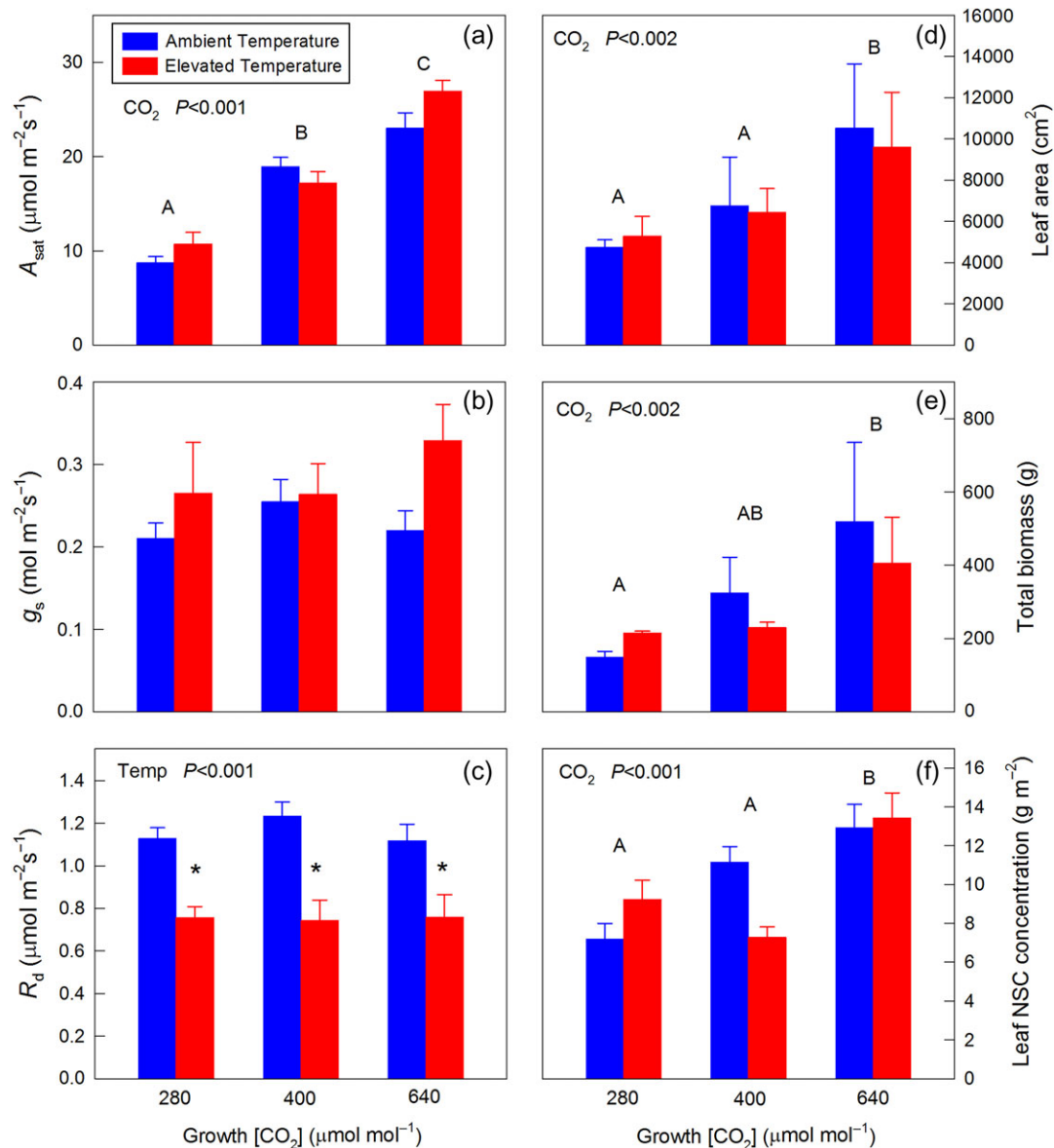
Figure 2. Patterns of light-saturated photosynthesis (A_{sat}) (a), stomatal conductance (g_s) (b), leaf dark respiration at night (R_d) (c), tree leaf area (LA) (d), tree biomass (e) and area-based leaf non-structural carbohydrates (NSC) (f) as a function of three [CO_2] (280, 400 and 640 µmol mol^{-1}) and two temperature (ambient and elevated) treatments during well watered conditions prior to the protracted drought experiment. Significant components of each ANOVA are indicated in each panel. Pairwise contrasts are illustrated for groups that are significantly different with upper case letters for [CO_2] contrasts, and with an asterisk for temperature treatments within [CO_2] levels.

Drought did not generally reduce leaf total NSC compared with well watered saplings throughout the experiment, except in C_p treatments at the final harvest (Figure 6g–i). Overall, across all [CO_2] and temperature treatments, drought reduced L_{st} relative to well watered controls, yet L_{ss} and total NSC was not as dramatically shifted (Figure 7).

Integrating carbon and water use into predictions of time-to-mortality

Following cessation of irrigation, saplings immediately began to experience water stress (Figure 3), and reached mortality between drought Days 43 and 63, depending upon treatments. T_e saplings reached mortality on average 9 days sooner than T_a saplings across all [CO_2] treatments (50.4 ± 2.0 vs 59.2 ± 2.8 days (mean ± SE), respectively). However, the difference in time-to-mortality between temperature treatments was increased with rising [CO_2]. For example, the time difference between C_pT_a (58 days) and C_pT_e (53 days) was smaller than that between C_eT_a (57 days) and C_eT_e (43 days). Saplings in C_eT_e died ~20 days sooner than those in C_aT_a (Figure 3).

Time-to-mortality did not clearly vary with R_d or A_{sat} across any combination of sampling dates in the experiment (Table 2). Using stepwise regression, employing all physiological and
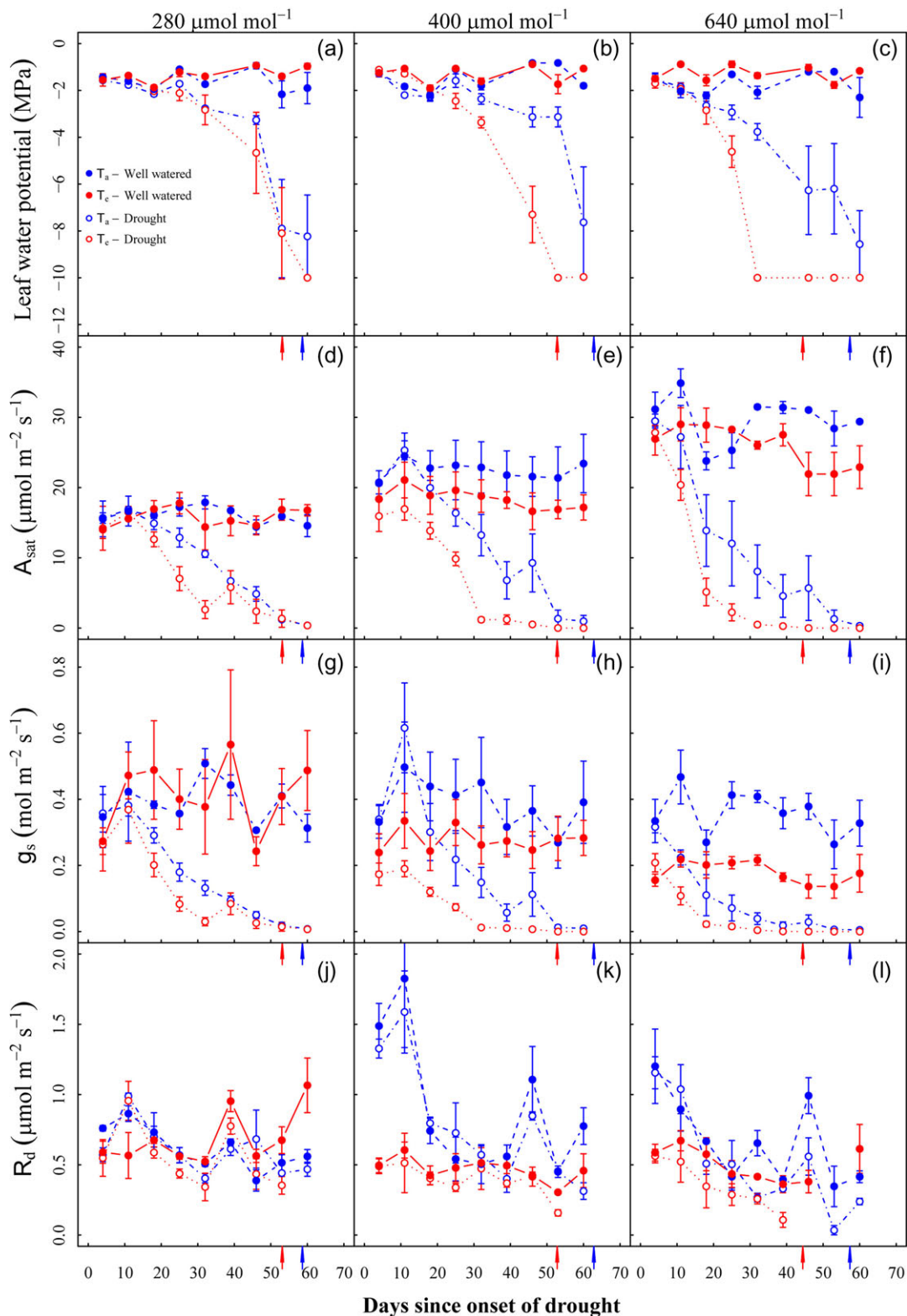
Figure 3. Changes in leaf water potential (Ψ_l) (a–c), light-saturated photosynthesis (A_{sat}) (d–f), stomatal conductance (g_s) (g–i) and dark respiration at night (R_d) (j–l) as a function of time for well watered and drought saplings exposed to three [CO₂] (280, 400 and 640 µmol mol⁻¹) and two temperature (ambient and elevated) treatments. Values are means ± SE ($n = 3$). Blue and red are used to distinguish T_a (ambient temperature) from T_e (elevated temperature) throughout, while closed and open symbols are used to represent well watered and drought, respectively. Minimum water potential values for plants were assigned a value of −10 MPa in order to accommodate missing data in the statistical design as trees died. Arrows on the x-axis reflect the date of mean drought mortality for T_a (blue) and T_e (red).

Table 1. Repeated-measure ANOVA results for effects of [CO_2] and temperature on leaf gas exchange and water relation variables of saplings grown in well watered and drought conditions throughout the experimental period.

			A_{sat}		g_s		R_d		Ψ_l	
	Factors	df	F	P	F	P	F	P	F	P
Well watered	CO_2	2/12	20.330	**<0.001**	1.567	0.249	7.460	**0.008**	0.345	0.715
	Temp	1/12	2.374	0.149	5.291	**0.040**	5.334	**0.040**	22.899	**<0.001**
	CO_2 × Temp	2/12	0.448	0.649	1.792	0.208	8.599	**0.005**	4.886	**0.028**
Drought	CO_2	2/12	1.306	0.307	0.667	0.531	25.109	**<0.001**	18.122	**<0.001**
	Temp	1/12	12.648	**0.004**	4.872	**0.047**	27.728	**<0.001**	8.623	**0.013**
	CO_2 × Temp	2/12	0.747	0.495	0.475	0.633	6.150	**0.020**	3.414	0.067

Significance at $P < 0.05$ are presented in bold. df, degrees of freedom (numerator/denominator); A_{sat}, light-saturated photosynthesis; g_s, stomatal conductance; R_d, leaf dark respiration at night; Ψ_l, leaf water potential.
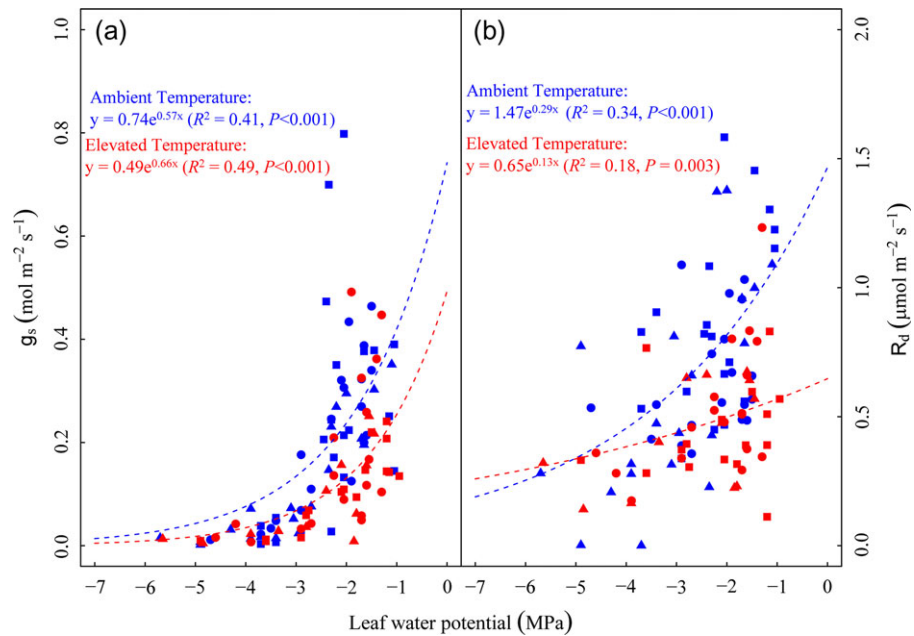

Figure 4. The relationship between leaf water potential (Ψ_l) and stomatal conductance (g_s) (a) and leaf dark respiration at night (R_d) (b) for saplings exposed to three [CO_2] (280, 400 and 640 µmol mol^{-1}; circles, squares and triangles, respectively) and two temperature (ambient and elevated; blue and red, respectively) treatments. Data are fitted with exponential regressions ($y = a \times e^{bx}$) and shown with blue or red dashed lines.

morphological variables that may influence time-to-mortality from the initial period of the experiment (Table 2), the minimal predictive model involved a combination of Ψ_l and L_{st} (mortality time = $114.7 \times \Psi_l + 4.08 \times L_{st} + 70.3$; $R^2 = 0.41$; $P = 0.018$; Table 2). Less negative values of Ψ_l and L_{st} on drought Day 4 combined to delay time-to-mortality. This initial drought Ψ_l pattern did not clearly differ among the temperature and CO_2 treatments (Figure 3). Moreover, these variables had different relative importance in the overall model (Table 2), with Ψ_l having a much larger effect on time-to-mortality compared with L_{st}.

Discussion

In this experiment, we used saplings of *E. sideroxylon* grown in three [CO_2] levels and at two growth temperatures to understand the relative sensitivity of different components of the mortality process across a protracted, lethal drought. We were interested in how initial impacts of [CO_2] and temperature on morphology and physiology influenced the mortality process, as well as the physiological dynamics during the period of water stress. We anticipated that T_e would exacerbate the dynamics of drought-induced mortality through higher water loss and negative effects on plant carbon balance (as in Adams et al. 2009). We also anticipated that greater water loss due to larger LA and carbohydrate status enhancement from growth at increasing levels of [CO_2] would exacerbate or ameliorate negative processes leading to mortality. Partially consistent with our expectations, we found that saplings at C_eT_e died quickest, that water status shifts or carbohydrate dynamics were highly idiosyncratic by treatment, and that few of the predictive variables associated
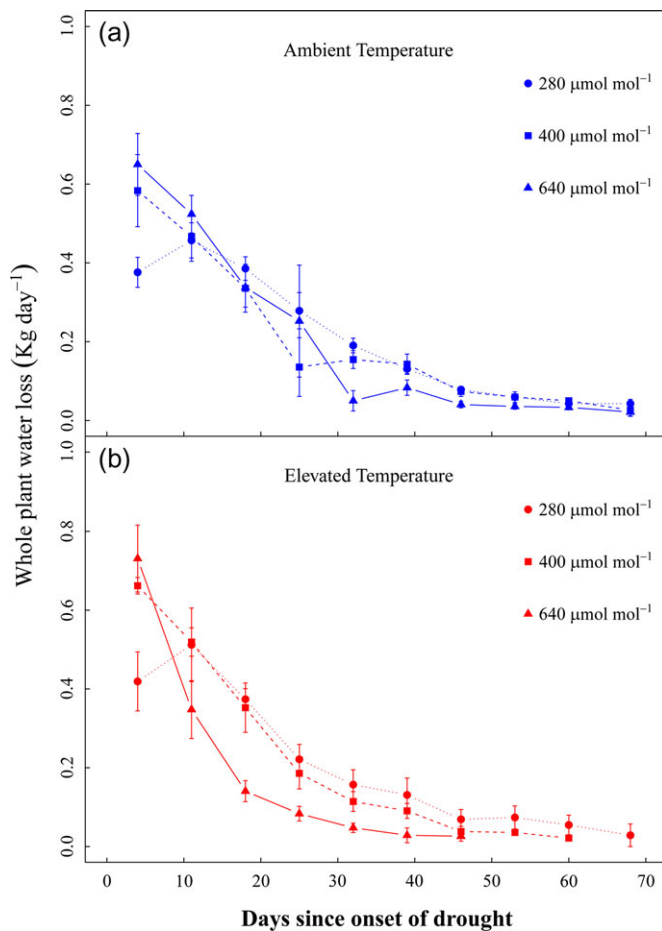
Figure 5. Water loss rates of whole plant grown in three levels of [CO$_2$] (280, 400 and 640 µmol mol^{-1}; circles, squares and triangles, respectively) and two temperature regimes ((a) ambient and (b) elevated; blue and red, respectively) during the drought. Values are means ± SE (n = 3). Water loss rates were determined gravimetrically for plants on a weekly basis.

temperature treatment in Adams et al. (2009), and, as such, the data do not support a straightforward interpretation of carbon metabolism driving variation in time-to-mortality across treatments. This reduction in R_d in T_e saplings was counter-intuitive because we expected an increase in carbon utilization and enhancement of metabolism due to higher activities of respiratory enzymes at T_e, which would have increased leaf R_d in T_e saplings or generated an acclimation response that would have resulted in similar R_d in T_a and T_e (Atkin et al. 2007, Heskel et al. 2016). However, several other factors such as substrate availability, adenylates capacity, nitrogen status and structural changes can also contribute to increased R_d in response to increasing temperatures (Atkin and Tjoelker 2003, Ayub et al. 2011, Way et al. 2015). Thus, temperature impacts on R_d may derive from a number of different feedbacks associated with alterations in plant size, demand (and lack of supply) for other limiting resources at T_e, and diurnal patterns of activity not captured by our measurements. Although we did not evaluate these potential mechanisms that might explain reduced R_d in T_e saplings, our more detailed carbohydrate dynamic data are available to evaluate drivers of mortality.

In contrast to R_d, Ψ_l exhibited a very clear separation among treatments consistent with expectations of time-to-mortality predicted from the hypothesized contribution of hydraulic features to the time series of mortality. Ψ_l closely matched the decline in Ψ_s measured with time domain reflectometry (Zeppel et al. 2012); however, Ψ_s remained nearly an order of magnitude less negative than Ψ_l throughout the study. When soil dries and shrinks, the root–soil interface becomes disconnected even as a plant continues to experience water loss (Passioura 1988). Thus, roots of drought saplings were likely hydraulically isolated from the soil profile, yet plants continued leaf gas exchange. This trait makes it difficult to simply identify hydraulic failure as the sole mechanism driving mortality, in part because of likely physiological mechanisms that maintain gas exchange despite disruption of the hydraulic pathway. Across all treatments, the significant variation in time-to-mortality, relative process dynamics between contrasting [CO$_2$] and temperature treatments, and pre-drought morphological and physiological attributes may help move us forward in partitioning the hydraulic and carbon metabolic forces influencing risk of mortality.

Differences in LA, biomass, A_{sat} and NSC generated by the [CO$_2$] and temperature treatments prior to initiation of the lethal drought led to large differences among treatments in the functional status of individual saplings, suggesting that growth at C_e increased tree susceptibility to hydraulic failure during prolonged drought, particularly when combined with T_e. This statement is counter to the argument based on C_e reductions in leaf g_s commonly described in the literature (e.g., Medlyn et al. 2001). In the present study, however, significant changes of g_s were not observed in either C_p or C_e conditions, which is consistent with some previous studies (e.g., see reviews Saxe et al. 1998,

with previously held hypotheses in the literature (e.g., such as R_d, as in Adams et al. 2009) explained time-to-mortality in the saplings in our study. However, initial values of plant water relations at the onset of drought explained most of the variation in time-to-mortality across treatments, followed by L_{st}, suggesting that various pre-drought environmental conditions and the associated plant morphological (i.e., growth) and physiological (i.e., carbon and water relations) changes contributed substantially to plant responses to following lethal drought stress. Sapling water status and leaf starch both greatly determined the degree of the response. These data and patterns provide insight into the current debate on mechanisms underlying mortality and help to explain the interactions among hydraulic and carbon metabolic traits influencing death from drought.

The time series of R_d demonstrated opposite patterns to the trend in time-to-mortality; saplings grown at T_e had lower rates of R_d than those grown at T_a, but nonetheless died sooner. This pattern is opposite to those observed using a similar elevated
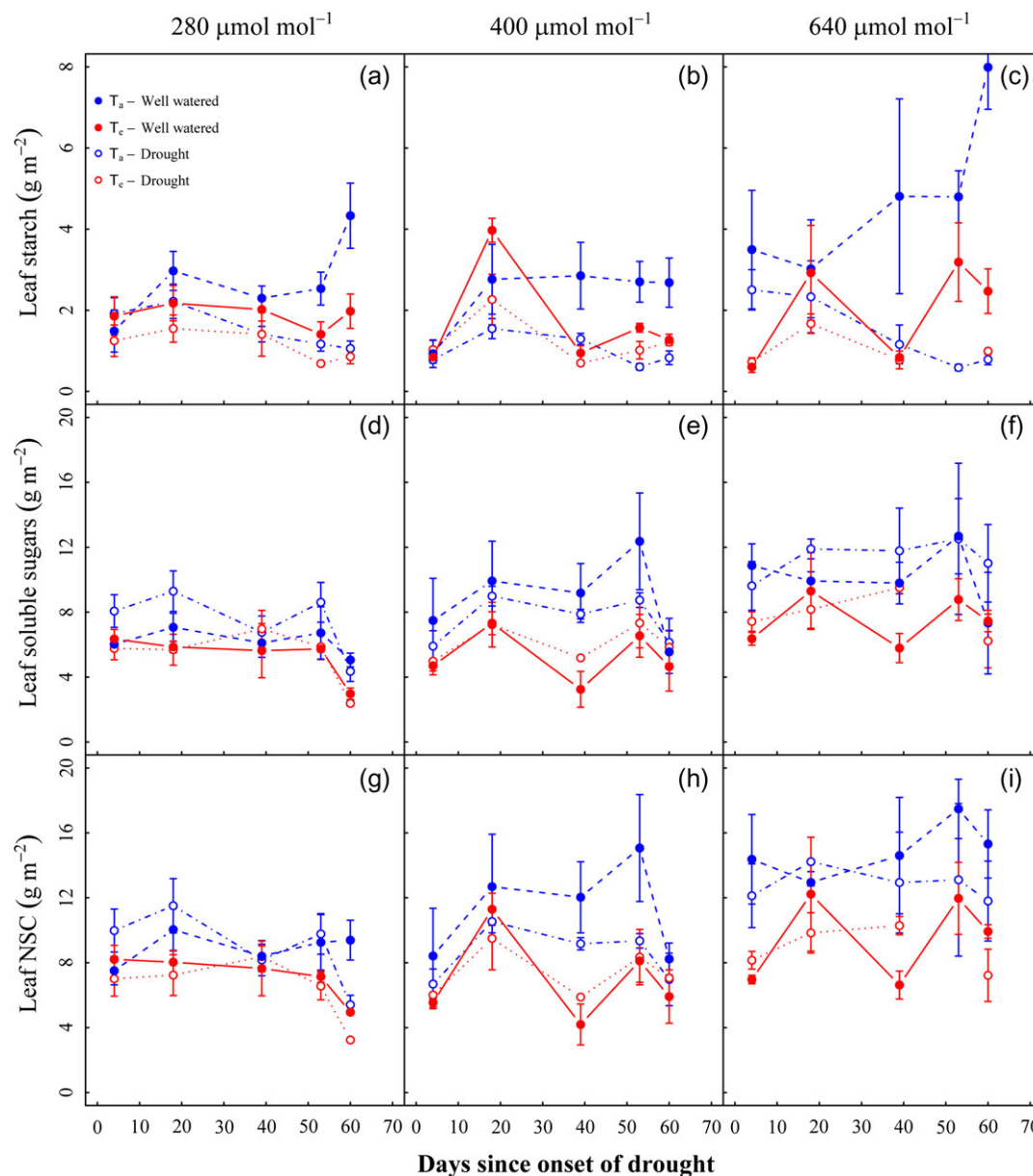
Figure 6. Changes in area-based concentration of leaf starch (L_{st}), soluble sugars (L_{ss}) and total non-structural carbohydrates (NSC) through time for well watered and drought saplings exposed to three [CO_2] (280, 400 and 640 μmol mol^{-1}) and two temperature (ambient and elevated) treatments. Values are means ± SE ($n = 3$). Blue and red are used to distinguish T_a (ambient temperature) from T_e (elevated temperature) throughout, while closed and open symbols are used to represent well watered and drought, respectively.

Franks et al. 2013; also see studies Ellsworth 1999, Ghannoum et al. 2010, Duan et al. 2014). Response of g_s to changes in [CO_2] may interact with other factors, such as stomatal sensitivity (Franks et al. 2013, Way et al. 2015), xylem water transport (Uddling et al. 2009) and plant functional groups (Ainsworth and Rogers 2007). For example, g_s was increased by C_e in the Aspen FACE experiment mainly due to the stimulated effects of C_e on hydraulic efficiency (Uddling et al. 2009). Given more LA for water loss from transpiration, without a specific reduction in g_s, trees in C_e should use more water than those in C_a during drought, thereby experiencing greater drought stress. Earlier

evidence showed that whole-plant characteristics, such as tree size and/or root distribution, can influence tree response to C_e × drought interactions (Duursma et al. 2011, Warren et al. 2011).

Across [CO_2] treatments, T_e saplings lost water at faster rates than T_a saplings in the initial phase of the drought, leading to more negative Ψ_l and rapid stomatal closure (Mencuccini 2003). Whole-plant water loss was more rapid in T_e treatments, particularly in C_eT_e, indicating that water stress at the whole-plant level occurred earlier in T_e treatments. Taken together, the correlated patterns of water status and time-to-mortality, temporal dynamics of g_s and whole-plant water-use rates (between
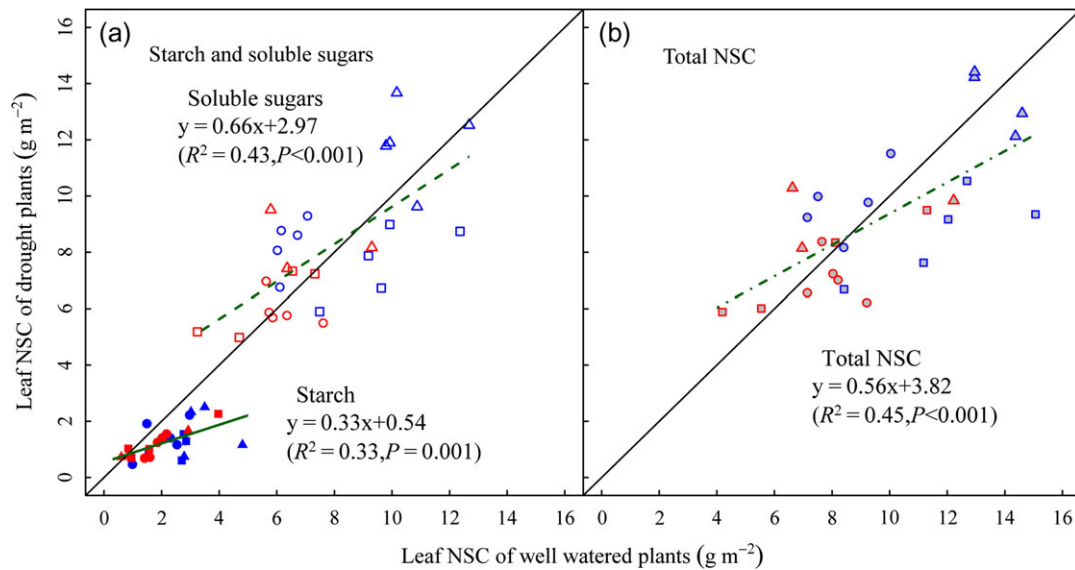
Figure 7. Treatment effects on the components of leaf non-structural carbohydrates (NSC) across the entire drought experiment, highlighting well watered control samples (x-axis) compared with drought samples (y-axis) for the combined [CO$_2$] (280, 400 and 640 µmol mol^{-1}; circles, squares and triangles, respectively) and temperature (ambient and elevated; blue and red, respectively) treatments (and the plotted 1:1 line). Values for starch (closed symbols) and soluble sugars (open symbols) (a), and total NSC (gray filled symbols) (b) are identified by a dashed (soluble sugars), solid (starch) or dot-dash (total NSC) line. Regression equations and R^2 values are documented in the figure.

Table 2. The univariate R^2, univariate slope of relationship and the relative importance of multiple regressions of different physiological and morphological variables in time-to-mortality. Relative importance is a measure of variance partitioning within the analysis. Variables illustrating a significant relationship are highlighted across the entire table in bold ($P < 0.05$). A_{sat}, light-saturated photosynthesis; g_s, stomatal conductance; g_{sn}, night-time stomatal conductance; R_d, dark respiration at night; Ψ_l, leaf water potential; LMA, leaf mass per area; L_{ss}, leaf soluble sugars; L_{st}, leaf starch; LA, leaf area.

Variable	R^2	Sign/slope of relationship	Relative importance
Ψ_l	**0.26**	**13.99**	**0.508**
L_{st}	**0.13**	**3.69**	**0.139**
LA	0.06	−0.0006	0.068
g_s	0.05	−18.10	0.067
A_{sat}	0.06	−0.31	0.058
L_{ss}	0.05	0.86	0.057
LMA	0.02	774.80	0.051
R_d	0.03	−4.95	0.030
g_{sn}	0.03	19.00	0.020

treatments) support a 'large-effect' role for hydraulic failure as the mechanism for environmentally induced shifts in mortality risk in *E. sideroxylon*.

While water status explained large-scale effects on time-to-mortality, fine-scale dynamics required further explanation by carbon metabolism. While R_d was not informative, L_{st} helped explain time-to-mortality. L_{st} was reduced by drought, while L_{ss} was not significantly affected. This pattern is conceptually consistent with the patterns of starch–sugar inter-conversion and the temperature effects on mortality during the extreme period of drought in piñon pine during mortality (Adams et al. 2013),

where, at high temperature, a lack of inter-conversion likely limited metabolic potential. In this case, declines in L_{st} in drought saplings, without alterations in L_{ss}, likely reflect conversion of storage carbohydrates to labile pools (Duan et al. 2014), which would be subsequently available for maintenance of cellular metabolism, osmotic adjustment and support for hydraulic function until time of mortality (Salleo et al. 2009, Sala et al. 2010). Changes in carbohydrates associated with lethal drought are difficult to interpret, in part because there are essentially two baselines required for evaluation: (i) the concentrations at the beginning of the water stress period and (ii) the trajectory associated with well-watered control plants (exhibiting continued photosynthesis) over the experimental period. However, we did not sample NSC from stems and roots throughout the experiment, which limits our ability to generate better understanding of carbon dynamics at the whole-plant level and NSC translocation among organs during drought stress. At the final harvest, we assessed NSC of all organs and found that, in particular, roots exhibited similar starch reduction in drought treatment compared with well watered treatment (see Table S1 available as Supplementary Data at *Tree Physiology* Online), confirming that reduced starch due to drought stress was consistent among organs. Future studies with greater temporal assessment of NSC among organs during drought progression in response to [CO$_2$] and temperature treatments are required.

Analyses of relationships among individual physiological and morphological traits provide evidence of a complex, hierarchical dynamic operating among water and carbon processes that drive mortality. C_e saplings, at both temperature treatments, reduced

g_s to values indistinguishable from zero prior to reaching a minimum Ψ_l, most likely to control the large potential fluxes of water associated with greater LA that was accumulated pre-drought. Yet, in other treatments, such patterns were not so easily interpretable. Taken as a whole, it seems that differences in the timing of g_s and Ψ_l responses to progressive lethal drought were partially driven by L_{st} and maintenance of photosynthesis to continue metabolic activity, despite continued unfavorable water status in some treatments. Thus, in this experiment, saplings experienced mortality due to hydraulic failure, but regulation of water loss was largely dictated by feedbacks from carbon metabolism and reserve status. Therefore, saplings with lower starch levels must maintain g_s to supply carbon for metabolism, despite increased risk of hydraulic failure; this strategy was most evident in the C_p treatment.

Taken together, it appears that rising [CO$_2$] and temperature, via long-term effects on plant surface area and leaf carbohydrate status, interactively generated the initial trait relationships and time-course of functional response that set the stage for differential mortality risk in response to severe water deficit. Understanding these initial plant conditions is essential for greater insight into the mortality process. The rate of soil water exhaustion and subsequent potential for loss of hydraulic functioning, likely determined the first-order time-constraints on time-to-mortality. However, changes in carbon dynamics fine-tuned the eventual outcome, changing the rank order of time-to-mortality within treatments with similar water loss potential. An important point that emerges from this analysis is that future studies should evaluate mechanisms within a hierarchical framework that stresses the interaction of physiological processes and morphological features of plants through time (e.g., Huxman et al. 2013). In this view, water and carbon dynamics reflect both past and current plant function, thus setting the risk for mortality as a function of the immediacy of water stress imposed by a drought.

Conclusions

Drawing upon the results herein, we provide a conceptual framework for assessing the risk of mortality from drought-induced stress. The primary effect of [CO$_2$] prior to the imposition of drought is observed in impacts on plant surfaces (e.g., LA) and physiological performance (e.g., A_{sat}, R_d, g_s). Increased photosynthesis leads to increased carbon storage, which ameliorates mortality via carbon resource limitation. Increased LA, on the other hand, may potentiate mortality via a predisposition to large water losses from transpiration leading to embolism and failure of water transport. Such counteracting responses of these variables to rising [CO$_2$] resulted in non-significant changes in time-to-mortality in *Eucalyptus* saplings. In addition, while elevated temperature did not greatly influence the surface area, it did lead to changes in g_s during the day and night (see Zeppel et al. 2012), and altered Ψ_l and leaf gas exchange. Importantly, it is the interaction of plant surfaces, physiological rates and whole-plant water status that

collectively generate the risk of drought-induced mortality. Overall, these data highlight the interactive nature of how we conceptualize mechanisms leading to mortality, the key role of plant functional traits prior to the onset of drought and the differential effects of environmental drivers on functional plant responses.

While data presented here are from saplings of a single species, they are part of a larger effort to understand this key demographic process that underlies vegetation change. Reports of forest mortality are increasing (Allen et al. 2010), with fundamentally negative impacts on humans, ecohydrology, biodiversity and ecosystem carbon fluxes (Adams et al. 2012), and unknown temporal changes in local-to-regional processes coupled to forest structure (Bonan 2008). Future climate scenarios suggest an increased likelihood of continued mortality events and likely pressures on society from changes in ecosystem services (IPCC 2014, IUFRO 2009). For example, local hydrology can be substantially impacted by forest death (Vertessy et al. 2001), and run-off patterns are not easy to predict and do not rapidly recover to rates prior to mortality events (Guardiola-Claramonte et al. 2011). Our results suggest that first-order predictions of the functional consequences of global change factors on plant processes that affect mortality do not provide substantial guidance in predicting outcomes. This is also true for the utility of conceptualizing the problem of predicting mortality from a dichotomous perspective of hydraulic failure or carbon starvation. Careful consideration of the inter-related nature of traits and processes associated with mortality, and coupling with key environmental features, is needed to make progress in predicting the manner in which drought results in plant death.

Supplementary Data

Acknowledgments

Conflict of interest

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