RESEARCH PAPER

Drought increases heat tolerance of leaf respiration in *Eucalyptus globulus* saplings grown under both ambient and elevated atmospheric [CO₂] and temperature

Paul P. G. Gauthier¹,², Kristine Y. Crous¹,³, Gohar Ayub¹,⁴, Hongliang Duan³,⁵, Lasantha K. Weerasinghe³,⁶, David S. Ellsworth⁷, Mark G. Tjoelker³, John R. Evans¹, David T. Tissue³ and Owen K. Atkin¹,⁷,*

¹ Division of Plant Sciences, Research School of Biology, Building 46, The Australian National University, Canberra, ACT, 0200, Australia
² Department of Geosciences, Princeton University, Guyot Hall, Princeton, NJ 08544, USA
³ Hawkesbury Institute for the Environment, University of Western Sydney, Hawkesbury Campus, Locked Bag 1797, Penrith, NSW, 2751, Australia
⁴ Department of Horticulture, Agricultural University Peshawar, 25130, Khyber Pakhtunkhwa, Pakistan
⁵ Institute of Ecology & Environmental Science, Nanchang Institute of Technology, No. 289 Tianxiang Road, Nanchang 330099, China
⁶ Faculty of Agriculture, University of Peradeniya, Peradeniya, 20400, Sri Lanka
⁷ ARC Centre of Excellence in Plant Energy Biology, The Australian National University, Canberra, ACT, 0200, Australia

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Abstract

Climate change is resulting in increasing atmospheric [CO₂], rising growth temperature (T), and greater frequency/severity of drought, with each factor having the potential to alter the respiratory metabolism of leaves. Here, the effects of elevated atmospheric [CO₂], sustained warming, and drought on leaf dark respiration (R<sub>dark</sub>), and the short-term T response of R<sub>dark</sub> were examined in *Eucalyptus globulus*. Comparisons were made using seedlings grown under different [CO₂], T, and drought treatments. Using high resolution T-response curves of R<sub>dark</sub> measured over the 15–65 °C range, it was found that elevated [CO₂], elevated growth T, and drought had little effect on rates of R<sub>dark</sub> measured at T <35 °C and that there was no interactive effect of [CO₂], growth T, and drought on T response of R<sub>dark</sub>. However, drought increased R<sub>dark</sub> at high leaf T typical of heatwave events (35–45 °C), and increased the measuring T at which maximal rates of R<sub>dark</sub> occurred (T<sub>max</sub>) by 8 °C (from 52 °C in well-watered plants to 60 °C in drought-treated plants). Leaf starch and soluble sugars decreased under drought and elevated growth T, respectively, but no effect was found under elevated [CO₂]. Elevated [CO₂] increased the Q<sub>10</sub> of R<sub>dark</sub> (i.e. proportional rise in R<sub>dark</sub> per 10 °C) over the 15–35 °C range, while drought increased Q<sub>10</sub> values between 35 °C and 45 °C. Collectively, the study highlights the dynamic nature of the T dependence of R<sub>dark</sub> in plants experiencing future climate change scenarios, particularly with respect to drought and elevated [CO₂].

Key words: Dark respiration, drought, elevated CO₂, *Eucalyptus globulus*, Q<sub>10</sub>, temperature response.

Introduction

Around the world, the frequency and severity of droughts may increase as a result of global climate warming underpinned by rising atmospheric CO₂ concentrations ([CO₂]; IPCC, 2013). The average mean temperature of the Earth’s surface is increasing (Rahmstorf *et al.*, 2007), with heatwaves (such as the recent heatwaves in Australia (BOM-Australia, 2014]) predicted to become more common (Meehl and Tebaldi, 2004; Ciais *et al.*, 2005; IPCC, 2013). In C₃ plants,
drought leads to a rapid decrease in photosynthetic carbon gain due to stomatal closure (Hsiao, 1973; Lawlor and Cornic, 2002), with high temperature (T) further exacerbating reductions in net carbon gain (Sharkey, 2005; Rennenberg et al., 2006; Hüve et al., 2011); hence, plant productivity is typically lower during hot, dry periods. Collectively, such factors have important consequences for the growth and survival of plants, including economically important species in the forestry industry.

A major factor in determining the productivity and functioning of forest ecosystems is the response of leaf respiration in the dark (Rdark) to changes in the abiotic environment. Of the CO₂ fixed each day by net photosynthesis in well-watered plants, 20–80% is released back into the atmosphere by plant respiratory processes (Poorter et al., 1990; Atkin et al., 1996, 2007; Loveys et al., 2002; Gifford, 2003), with leaves accounting for ~50% of whole-plant Rdark (Atkin et al., 2007). Small changes in leaf Rdark (e.g. due to changes in atmospheric [CO₂], T, and/or water availability) could, therefore, have profound effects on functioning of forest ecosystems and the Earth system (Gifford, 2003; King et al., 2006; Atkin et al., 2008; Wythers et al., 2013). Indeed, because leaf Rdark is T sensitive (Atkin et al., 2005; Kruse et al., 2011), several studies have predicted large changes in terrestrial C storage and atmospheric [CO₂] in a future, warmer world (Cox et al., 2000; King et al., 2006; Huntingford et al., 2013).

In predicting the impacts of future climate change on plant respiration, Earth System Models (ESMs) often assume a constant Q₁₀ for leaf Rdark of 2.0 (i.e. Rdark doubles for every short-term 10 °C rise in T) (Huntingford and Cox, 2000) and that Rdark does not acclimate to sustained changes in growth T (Mahecha et al., 2010). While the assumption of a constant Q₁₀ of 2.0 may be appropriate for modelling rates of Rdark in some plant species, the assumption is unlikely to be valid for all scenarios, as the T response of Rdark can be highly variable. For example, sustained increases in growth T can result in declines in the Q₁₀ of Rdark (Atkin et al., 2000a, 2005; Zaragoza-Castells et al., 2008), underpinned by limitations in substrate supply and/or energy demand that restrict rates of Rdark at high measuring T more than at low measuring T (Atkin and Tjoelker, 2003). Moreover, the Q₁₀ often declines as measuring T increases (James, 1953; Forward, 1960; Tjoelker et al., 2001; Atkin and Tjoelker, 2003; Zaragoza-Castells et al., 2008).

The extent to which the Q₁₀ of leaf Rdark declines with increasing measuring T varies among species and environments, and is not well understood. However, a ‘generalized’ Q₁₀–T relationship proposed by Tjoelker et al. (2001) suggested that Q₁₀ declines with increasing T according to: Q₁₀ = 3.09 – 0.0437 T. Accounting for this Q₁₀–T relationship results in lower Rdark at T both lower and higher than a given reference temperature, leading to large decreases in predicted ecosystem Rdark compared with models that assume a constant Q₁₀ of 2.0 (Wythers et al., 2005, 2013). Moreover, variations in the Q₁₀–T relationship due to changes in the environment (e.g. in response to rising atmospheric [CO₂], growth T, and/or drought) that alter the balance between respiratory capacity, substrate supply, and/or energy demand could strongly affect the magnitude of plant Rdark estimated by ecosystem models and ESMs (Wythers et al., 2005, 2013; King et al., 2006). Given the link between substrates/energy demand and Q₁₀ values (Atkin and Tjoelker, 2003), it seems likely that higher substrate supply might result in an increased Q₁₀ in elevated atmospheric [CO₂], altering the T dependence of Rdark. Similarly, drought-mediated changes in photosynthesis, substrate supply, and energy demand (Ribas-Carbó et al., 2005) could, theoretically, affect the Q₁₀ of Rdark. In most studies, imposition of drought results in a decline in Rdark at a set measuring T (Flexas et al., 2005; Galmés et al., 2007; Atkin and Macherel, 2009); however, in some cases, drought results in no change (Gimeno et al., 2010) or an increase in Rdark at set measuring T (Zagdanska, 1995; Bartoli et al., 2005; Galmés et al., 2007; Slot et al., 2008; Metcalfe et al., 2010), with one report of drought-mediated increases in the Q₁₀ of Rdark (Slot et al., 2008).

Finally, consideration needs to be given to acclimation to increased growth T on Rdark at a set measuring T and associated Q₁₀ values. Acclimation to sustained increases in growth T often results in a decline in basal rates of Rdark (Atkin et al., 2000a; Bolstad et al., 2003; Loveys et al., 2003; Tjoelker et al., 2008, 2009; Zaragoza-Castells et al., 2008), that are accentuated by drought (Rodríguez-Calcerrada et al., 2010; Crous et al., 2011). Although sustained changes in growth T are reported to have little impact on the Q₁₀ of Rdark in some species (Tjoelker et al., 2001; Zaragoza-Castells et al., 2008; Crous et al., 2011), several studies have reported lower average Q₁₀ values in warm- compared with cold-grown plants (Zha et al., 2002; Armstrong et al., 2008). What is unclear, however, is the extent to which sustained increases in growth T impact on Q₁₀–T relationships. The extent to which the T dependence of leaf Rdark is affected by potential interactive effects of atmospheric [CO₂]-growth T-drought is also not known.

Examining how abiotic factors impact on the T dependence of leaf Rdark, previous studies either (i) have quantified the impact of diel variations in T on leaf Rdark; or (ii) have measured rates of Rdark at defined T intervals (e.g. often every 5 °C) following equilibration of leaves to each T interval. While informative, both approaches have their limitations. For example, with approach (i) account needs to be taken of other diel changes, such as changes in irradiance and metabolic functioning of the leaf through the day (Peuke et al., 2013). The quality/resolution of data derived from approach (ii) is often low due to the coarse nature of the measurements, and the fact that such measurements are typically made over a restricted T range (e.g. <35 °C), making it difficult to detect significant differences in Q₁₀–T relationships among treatments. Given the limitations of these approaches, an alternative is to record rates of leaf Rdark as leaves are rapidly heated (e.g. 1 °C min⁻¹), following the example of numerous studies assessing thermal tolerance of photosynthesis (Havaux et al., 1991; Knight and Ackerly, 2002; Hüve et al., 2006) and Rdark (Hüve et al., 2011, 2012; O’Sullivan et al., 2013; Heskel et al., 2014; Weerasinghe et al., 2014). The resultant high-resolution
data sets enable the impact of the abiotic treatments on 
$R_{\text{dark}}$–$T$ curves (and associated $Q_{10}$–$T$ relationships) to be 
explained in detail.

At high $T$, leaf $R_{\text{dark}}$ reaches a maximum (at $T_{\text{max}}$) at which 
$Q_{10}$ = 1.0; this point indicates the maximum heat tolerance of 
$R_{\text{dark}}$, with further heating resulting in irreversible declines in 
$R_{\text{dark}}$ (i.e. $Q_{10}$ <1.0), ultimately leading to cell death (Atkin 
and Tjoelker, 2003; Hüve et al., 2011, 2012; O’Sullivan et al., 
2013). Recent studies have reported that the $T_{\text{max}}$ of leaf 
$R_{\text{dark}}$ is near 52 °C in Phaseolus vulgaris (Hüve et al., 2012), 
51–57 °C in Eucalyptus pauciflora (O’Sullivan et al., 2013), 
60 °C in several tropical rainforest species (Weerasinghe et al., 
2014), and 53–58 °C in an arctic shrub, Betula nana (Heskel 
et al., 2014); these values are markedly higher than the 48 °C 
value derived from the regression reported in Tjoelker et al. 
(2001). The extent to which leaves can tolerate such $T$ is 
important, as 23% of the Earth’s land surface exhibits 
maximum air $T >$ 40 °C (Larcher, 2004), and, in such habitats, 
sun-exposed leaves can be 10 °C hotter than the surrounding 
(singsaas et al., 1999; Wise et al., 2004), probably resulting 
in leaf $T$ exceeding 50 °C (Hamerlynck and Knapp, 1994; 
Valladares et al., 2007). Such extremes, while rare now, are 
likely to become more frequent in the future (meehl and 
tebaldi, 2004; Ciais et al., 2005; IPCC, 2013; Tingley and 
Huybers, 2013). Here, a crucial factor is the extent to which 
the $T_{\text{max}}$ of leaf $R_{\text{dark}}$ is affected by growth $T$, atmospheric 
$[\text{CO}_2]$; and/or drought.

Studies on photosynthetic metabolism have reported 
increased high $T$ tolerance in plants subjective to elevated 
growth $T$ (Downton et al., 1984; Seemann et al., 1984, 
1986; havaux, 1993), atmospheric $[\text{CO}_2]$ (Faria et al., 1996; 
Taub et al., 2000), and/or drought (Seemann et al., 1986; 
havaux, 1992), with the increased heat tolerance being associated 
with increases in leaf osmotic potential and soluble sugar 
consentations (Seemann et al., 1986; HÜve et al., 
2006). Moreover, recent work by Hüve et al. (2012) suggests 
that the $T_{\text{max}}$ of $R_{\text{dark}}$ is increased in leaves with enhanced osmotic potential or sugar concentrations (via protection 
of respiratory membranes). Given this, enhanced concentra-
tions of non-structural carbohydrates in plants grown 
under elevated atmospheric $[\text{CO}_2]$ (wullschleger et al., 1992; 
tjoelker et al., 1998; Vu et al., 2002; Tissue and Lewis, 2010; 
Smith et al., 2012; Xu et al., 2012) might be associated with 
an increase in the $T_{\text{max}}$ of leaf $R_{\text{dark}}$. In contrast, 
environments that lead to depletion of carbon reserves [e.g. elevated 
growth $T$ (Tjoelker et al., 2008) and, in some cases, drought 
(Adams et al., 2009; Duan et al., 2013; Mitchell et al., 2013)] 
could potentially lead to a decrease in high $T$ tolerance of 
leaf $R_{\text{dark}}$ depending on whether plants are grown under 
ambient or elevated atmospheric $[\text{CO}_2]$ (Hamerlynck et al., 
2000; Niinemets, 2010). Importantly, however, no study has 
yet investigated the impact of multiple climate change driv-
ers on the respiratory $T_{\text{max}}$.

The overall aim of the present study was to assess how 
increased atmospheric $[\text{CO}_2]$, growth $T$, and drought affect 
the shape of the short-term $T$ response of leaf $R_{\text{dark}}$ (ranging 
from 15 °C to 65 °C) of a widely distributed, commercially 
important tree species Eucalyptus globulus. The study tested 
the following hypotheses. First, given that substrates can limit 
$R_{\text{dark}}$ (Azcón-Bieto and Osmond, 1983), particularly at high 
measuring $T$ (Atkin and Tjoelker, 2003; Bunce, 2007), and 
because substrate availability may decrease under conditions 
of drought/high growth $T$, it was hypothesized that rates of 
leaf $R_{\text{dark}}$ at high measuring $T$ would be lower in drought-
treated plants, with the effects of drought being accentuated 
by growth of plants under elevated growth $T$ and ambient 
大气 $[\text{CO}_2]$ (which increase $C$ turnover and limit CO$_2$ 
uptake, respectively). Further, given the linkage between $Q_{10}$ 
values and substrate supply (Atkin and Tjoelker, 2003), it was 
expected that exposure to those treatments that reduced 
soluble sugar concentrations would also be associated with 
reduced $Q_{10}$ values. Finally, given the potential link between 
the concentration of soluble sugars and high $T$ tolerance of 
leaf $R_{\text{dark}}$ (Hüve et al., 2012), it was hypothesized that $T_{\text{max}}$ would 
be greatest in well-watered plants grown under elevated 
ambient atmospheric $[\text{CO}_2]$ and ambient $T$. 

Materials and methods

Site description, plant material, and experimental design

The study took place at the Hawkesbury Forest Experiment (HFE) in 
Richmond, NSW, Australia (33°36′40″S, 150°44′26.5″E, elevation 
30 m) in a warm humid temperate climate with a mean annual 
$T$ of 17 °C and mean annual precipitation of 800 mm. The HFE 
consisted of 12 CO$_2$-, humidity-, and T-controlled whole tree 
chambers (WTCs) surrounded by a continuous block of forest. Two treatments 
described in further detail in Crous et al. (2013) were applied 
to the WTC: (i) temperatures increased 3 °C above ambient $T$; and 
(ii) atmospheric $[\text{CO}_2]$ elevated 240 ppm above ambient concentra-
tions, with three replicates per atmospheric $[\text{CO}_2]$ and $T$ treatment 
combination.

A widely planted eucalypt, E. globulus Labill., was planted from 
forestry tube stock seedlings in 5 litre pots in early October 2010 and 
in the tree chambers for an 8 week experiment starting on 1 November 2010. Fertilization was applied every week until 
8 November 2010, and once more on 3 December 2010. At these 
times, each pot received ~140 mL of 23:4:18 NPK liquid fertilizer 
containing ~8 kg N ha$^{-1}$. Seedlings were ~30 cm tall at the start of 
the experiment and controls grew 3–4 cm per week thereafter. Each 
chamber had six potted seedlings, of which two pots received a 
well-watered regime (watered daily to field capacity) and four pots 
received a drought treatment. Drought periods were imposed during 
weeks 3 and 4 (first drought period; watering reduced from 
12 November) and weeks 6 and 7 (second drought period; watering 
reduced from 4 December) of the experiment by adding only 
water enough to maintain the stomatal conductance ($g_s$) between 
0 and 100 mmol m$^{-2}$ s$^{-1}$ relative to a well-watered conductance 
exceeding 500 mmol m$^{-2}$ s$^{-1}$; rewatering pots to full soil water 
capacity occurred in week 5 to separate the two drought periods. 
These drought periods are denoted by shaded areas in the relevant 
figures.

Leaf respiration and photosynthesis measurements

To monitor plant physiological performance under the different 
environmental treatments, gas exchange measurements were taken 
weekly over a 7 week period. For the first drought period, gas 
exchange measurements commenced on 15 November 2010, while 
those of the second drought period commenced on 7 December 
2010 (i.e. 3 d after onset of both drought periods). Leaves of similar 
physiological age were measured throughout the experiment repre-
senting the most recently fully expanded leaves (i.e. node 3 from the
terminal apex on the seedlings). *Eucalyptus globulus* seedlings with juvenile leaves are hypostomatous (i.e. leaves have stomata on their abaxial side only).

Gas exchange measurements were conducted using portable infra-red gas analyser (IRGA) systems (LiCor 6400; LiCor Inc., Lincoln, NE, USA) using 6 cm² leaf cuvettes. To minimize diffusion gradients across the gaskets of the cuvette (Bruhn et al., 2002; Flexas et al., 2007), CO₂ levels inside the cuvettes were set to the prevailing [CO₂] in each WTC. No correction was made for diffusion of water vapour across the gasket (Rodeghiero et al., 2007); however, any error in estimates of light-saturated photosynthesis (A_max), g_min, and internal CO₂ concentration (C_i) would have been minor and similar for both well-watered and drought-treated plants in each growth [CO₂]_growth × T treatment. Block T of the LiCor 6400 was set to the prevailing T in each WTC (~18–33 °C; see Supplementary Fig. S1 available at JXB online). Measurements were made in the late morning to early afternoon of each sampling day. Photosynthesis was measured at saturating light of 2000 μmol m⁻² s⁻¹ (A_max). Measurements of leaf R_ dark were made on dark-adapted leaves after being covered with foil for at least 30 min to achieve steady-state R_dark (Aezcón-Bieto and Osmond, 1983; Atkin et al., 1998). In week 7, a methodological error occurred when measuring gas exchange under light saturation; therefore, data presented for week 7 are limited to R_dark.

**Temperature–response curves of leaf R_ dark**

Short-term temperature–response curves of leaf R_ dark for individual leaves from each chamber were measured in well-watered and drought-treated plants between 15 °C and 65 °C in week 7. Temperature control in two custom-made 15.5 cm × 10.6 × 6.5 cm water-jacketed, aluminium leaf chambers (each connected to a LiCor 6400) was regulated by a temperature-controlled water bath (Julabo 32HL, Julabo Labortechnik, Seelbach, Germany) and programmed to increase by ~1 °C min⁻¹ (Julabo 32HL, Julabo Labortechnik, Seelbach, Germany) and depending on the chamber (at high leaf temperature). The exiting air-stream from the cuvette was rewarmed to replicate the start of each release. The sample and reference gas lines were matched prior to entering the water-jacketed chamber to ensure that there was no condensation in the sample gas line exiting the water-jacketed chamber (at high leaf T, leaves exhibited high rates of water release). The sample and reference gas lines were matched prior to the start of each T–response run and several times during the run, with rates of net CO₂ exchange taking into account dilution of CO₂ by water vapour.

Short-term T–response curves were measured at week 7 on both well-watered and drought-treated plants brought to the lab at least half an hour prior to measurement. In the week prior to the start of the R_ dark–T analysis, g_min of each leaf was measured to confirm their drought status (see week 6 values of g_min in Supplementary Fig. S2 at JXB online). To assess leaf area, an image of the leaf was taken before starting the T–response curve and leaves were oven-dried afterwards. Leaf area was determined using Image J Software Analysis (Davidson and PrometheusWikicollectors, 2011).

Previous experiments with another *Eucalyptus* species (*E. pauciflora*) have indicated that short-term T–response curves are fully reversible up to 45 °C, but not when irreversible metabolic damage occurred at leaf T exceeding 45 °C (O’Sullivan et al., 2013). Given this, modelling of the T–response curves (in order to calculate Q_10 values at each leaf T) was restricted to the 15–45 °C range. To model T responses of leaf R_ dark over the 15–45 °C range, a polynomial equation was used (Atkin et al., 2005; O’Sullivan et al., 2013) fitted to the natural log of R_ dark:

\[
\log_e (R) = a + bT + cT^2
\]

and where:

\[
R = e^{a+bT+cT^2}
\]

with T being leaf T (°C) and a, b, and c are coefficients that describe the T response of the natural log of R and where a represents the natural log of R_ dark at 0 °C. The differential of equation 1 can be used to model the Q_10 of leaf R_ dark at any measuring T:

\[
Q_{10} = \left(\frac{R_{max}}{R_{min}}\right)^{rac{1}{10}}
\]

In past studies using a similar heating protocol, a ‘burst’ in respiration occurred in the ramp up to T_ max (as shown by an inflection point in R_ dark–T curves) (Hüve et al., 2011, 2012; O’Sullivan et al., 2013). Such bursts can lead to the activation energies (E_a) of R_ dark being markedly higher above the inflection point (O’Sullivan et al., 2013). They can also result in observed rates of R_ dark exceeding those predicted from curves fitted over a lower range of non-enthalpic Ts [e.g. <45 °C (O’Sullivan et al., 2013)]. To assess the effect(s) of growth T, [CO₂], and/or water availability on the magnitude of potential respiratory bursts in *E. globulus*, E_a values were calculated over two different T intervals (each 5–10 °C in range, depending on the R_ dark–T characteristics of each replicate), which are below T_max values reported previously (O’Sullivan et al., 2013). For well-watered plants, where T_max values were ~52 °C, the two intervals were within the 30–40 °C (low range) and 40–50 °C (high range) ranges. For drought-treated plants, where T_max values were ~60 °C, the intervals were within the 40–50 °C (low range) and 50–60 °C (high range) ranges. In both well-watered and drought-treated plants, the aim was to compare E_a values over two T intervals in the immediate lead up to T_ max. In cases where the burst was minimal or non-existent, E_a values are likely to be lower at the high T range compared with the low T range (i.e. E_a–high T/E_a–low T ratios <1); in contrast, where a burst occurs, this ratio was expected to be either near unity or >1.0.

**Leaf carbohydrate analyses**

To assess the impact of each growth treatment on the concentration of soluble sugars and starch in week 7, the leaf adjacent to the leaf used in the short-term T–response curve measurements was sampled. The sampled leaves were oven-dried for a minimum of 2 d at 70 °C, then ground in a ball mill and analysed for soluble sugars and starch, as described in Loveys et al. (2003). To assess the likely amount of carbohydrates respired during each run of a T–response curve, the total amount of CO₂ respired during each T–response curve was calculated (mol C m⁻²); thereafter, these values were converted to the equivalent mass of carbohydrate respired during each T–response curve (g m⁻²), assuming 1 mol C equals 30 g of carbohydrate.

**Statistical analyses**

Statistical analyses were conducted in IBM SPSS® Statistics for Windows, Rel. 19.0.0.2010 (SPSS Inc., Chicago, IL, USA). First, seedlings of a given drought treatment (n=3 for drought and n=3 for well-watered) were averaged within each chamber. Then, a repeated-measures analysis of variance (ANOVA) was conducted to assess differences between [CO₂] and/or warming treatments (n=3) on weekly measurements of A_max and R_ dark. Kolmogorov–Smirnov and Levene’s test were used to test for normality and homogeneity of variance;
variables usually were log- or arcsine-transformed where necessary to meet the normality and homogeneity of variance assumptions at given time points. Over the 7 week experimental period, three-way ANOVAs were used to assess main effects of growth $T$ and atmospheric $[CO_2]$ at given time points. When three-way ANOVAs showed no significant effect of two of the three parameters studied (e.g. growth $T$ and $[CO_2]$), independent $t$-tests were used on the third parameter to determine whether there were significant differences between treatment and control plants at any given time points. Similarly, independent $t$-tests were used to assess whether water availability affected $Q_{10}$ values at any given measurement $T$ (within each growth $T$–atmospheric $[CO_2]$ treatment combination). Differences between means were considered significant at $P<0.05$.

**Results**

**Establishment of the drought phenotype**

Drought status of the drought-treated plants was assessed via measurements of $A_{sat}$, $g_s$, and $C_i$ each week, measured at the prevailing leaf temperature ($T$) in the mid-morning to early afternoon. In well-watered plants, $g_s$ and $A_{sat}$ exhibited week-to-week variations, reflecting concomitant variations in prevailing air/leaf $T$ and vapour pressure deficit (see Supplementary Fig. S1 at *JXB* online for week-to-week fluctuations of average leaf $T$). Developmental changes in photosynthetic capacity may have also contributed to declines in $A_{sat}$ (e.g. in the well-watered plants in weeks 5–6). Three-way ANOVAs showed no significant main effects of growth $T$ or $[CO_2]$ on $A_{sat}$ and $g_s$ during the first and second drought periods (Supplementary Table S1). Hence, average $A_{sat}$ and $g_s$ values were calculated across all four $[CO_2]–T$ treatments to illustrate the overall impact of drought (Fig. 1). Similar trends in $A_{sat}$ and $g_s$ in each $[CO_2]–T$ treatment combination are shown in Supplementary Figs S2 and S4; $C_i$ values also exhibited a similar trend, declining during each drought event (Supplementary Fig. S3). Over time, rates of $A_{sat}$ in well-watered plants declined, reaching minimum values of near 10 μmol CO$_2$ m$^{-2}$ s$^{-1}$ in week 5, with rates of $A_{sat}$ increasing slightly in week 6 (Fig. 1).

Imposition of drought during the first drought period resulted in marked reductions in $A_{sat}$ (compared with well-watered plants in week 4) in all $[CO_2]–T$ treatment combinations. Underpinning the drought-induced decreases in $A_{sat}$ in week 4 were declines in $g_s$ and $C_i$ in each $[CO_2]–T$ treatment combination (Supplementary Figs S2, S3 at *JXB* online). Thereafter, $A_{sat}$ of drought-treated plants recovered following rewatering in week 5 (Fig. 1; Supplementary Figures S2–S5).

**Fig. 1.** Effect of periodic drought on: (A) stomatal conductance under saturating irradiance ($g_s$); (B) light-saturated photosynthesis ($A_{sat}$); (C) leaf dark respiration ($R_{dark}$); and (D) $R_{dark}/A_{sat}$ ratios of *Eucalyptus globulus* fully expanded leaves (measured over several weeks commencing early November 2010). Within each plot, values of well-watered (filled circles) and drought-treated (open circles) plants are shown ($n=12$; sSE); values shown are averages of plants grown under two atmospheric CO$_2$ concentrations (400 μmol mol$^{-1}$ and 640 μmol mol$^{-1}$ for ambient and elevated CO$_2$, respectively) and two growth temperatures ($T$, ambient and ambient $+3^\circ C$) scenarios. The shaded regions designate two periods of controlled drought, with the intervening non-shaded region indicating when drought-treated plants were rewatered after the first drought period. Significant Student’s $t$-test $P$-values of comparisons between drought and well-watered values are indicated with * for $P<0.01$, ** for $P<0.05$, and *** for $P<0.001$. See Supplementary Figs S2–S5 at *JXB* online for plots of each parameter showing values within each $[CO_2]$ and growth $T$ combination. Note: in week 7, a methodological error occurred when measuring gas exchange under light saturation. As such, data presented for week 7 are limited to $R_{dark}$. 
Fig. S4). Rewatering also led to a recovery of $g_s$ and $C_i$ values to well-watered control plant values in all treatments, with the exception of the elevated [CO$_2$] and warming treatment, where $g_s$ values recovered to ~50% of well-watered controls (Supplementary Fig. S2). In week 6 (i.e. the first week of the second drought period), $A_{sat}$ and $g_s$ decreased significantly in drought-treated plants ($P<0.0001$; three-way ANOVA assessing main effect of H$_2$O supply; Supplementary Table S1), reaching values that were similar to those at the end of the first period of drought (week 4; Fig. 1; Supplementary Figs S2, S4). Associated with the drought-mediated declines in $g_s$ were concomitant declines in $C_i$ (Supplementary Fig. S3). Although there were no significant interactions between growth $T$, [CO$_2$], and/or H$_2$O supply (in the three-way ANOVA of week 6 data), cessation of water supply clearly resulted in a rapid response to water stress in the second period of drought. In contrast to the significant effect of drought in week 6, growth $T$ and [CO$_2$] treatments had no significant effect on $A_{sat}$ and $g_s$ measured in week 6.

Impact of drought, growth $T$, and elevated [CO$_2$] on leaf structure and carbohydrates

Sampled leaf dry mass per unit area (LMA) did not vary significantly among the growth treatments (Tables 1, 2); a cross all treatments, the average LMA was 80.0±4.0 g m$^{-2}$. Soluble sugar concentrations varied between 5.1 g m$^{-2}$ and 9.4 g m$^{-2}$, being significantly lower ($P=0.016$) in plants grown under the elevated growth $T$ treatment (compared with the ambient $T$-grown plants; Tables 1, 2). Neither atmospheric [CO$_2$] nor water availability affected the concentration of soluble sugars (Tables 1, 2). Starch concentrations exhibited values ranging from 0.9 g m$^{-2}$ to 12.5 g m$^{-2}$, with neither atmospheric [CO$_2$] nor growth $T$ having a significant effect; in contrast, less starch was found in leaf exposed to drought (Tables 1, 2). The absence of significant treatment interaction terms for sugars and starch (Table 2) suggests that the observed effect of growth $T$ and drought on sugars and starch, respectively, was consistent across the different treatments.

Impact of drought, growth $T$, and elevated [CO$_2$] on leaf respiration

Rates of $R_{dark}$ (measured at the prevailing mid-morning to early afternoon temperature) varied with time (Fig. 1), reflecting, in part, week-to-week variations of prevailing leaf $T$ (Supplementary Fig. S1 at JXB online). Importantly, the effect of the treatment combinations on $R_{dark}$ (Fig. 1; Supplementary Fig. S5) was considerably less than the drought-mediated changes in $A_{sat}$ (Fig. 1; Supplementary Fig. S4). Indeed, a three-way ANOVA showed no significant main effects of growth $T$, [CO$_2$], and/or drought $R_{dark}$ in week 6 (Fig. 1; Supplementary Fig. S5). Thus, in contrast to $A_{sat}$ and $g_s$ (Fig. 1; Supplementary Figs S2, S4), none of the growth treatments (including drought) had a significant effect on rates of leaf $R_{dark}$ measured at the prevailing $T$ occurring at the time of mid-morning/early afternoon measurements. Consequently, $R_{dark}:A_{sat}$ ratios were consistently higher in drought-treated plants (both drought periods) in all treatments, with rewathering after the first drought period (week 5) resulting in a sharp decline in $R_{dark}:A_{sat}$ in all growth $T$ and [CO$_2$] treatments (Fig. 1; Supplementary Fig. S6). In week 6, a three-way ANOVA (Supplementary Table S1) revealed a significant main effect of drought on the $R_{dark}:A_{sat}$ ratio ($P<0.001$). Thus, drought altered the instantaneous carbon balance of E. globulus leaves in the experiment, irrespective of the growth [CO$_2$] and/or growth $T$ treatment.

### Table 1. Effect of atmospheric CO$_2$ treatment (400 ppm and 640 ppm), growth temperature (T) (ambient and +3 °C), and water treatments [well-watered (WW) and drought-treated (DR)] on leaf mass per unit leaf area (LMA), soluble sugars (sucrose, glucose, and fructose), and starch (±SE, n=3)

<table>
<thead>
<tr>
<th>CO$_2$ treatment</th>
<th>Growth $T$ treatment</th>
<th>H$_2$O treatment</th>
<th>LMA (g m$^{-2}$)</th>
<th>Sugars (g m$^{-2}$)</th>
<th>Starch (g m$^{-2}$)</th>
<th>Respired carbohydrate (g m$^{-2}$)</th>
<th>$E_a$-low T (kJ mol$^{-1}$)</th>
<th>$E_a$-high T (kJ mol$^{-1}$)</th>
<th>$E_a$-high T/$E_a$-low T (ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400 ppm</td>
<td>Amb</td>
<td>WW</td>
<td>75.8±14.0</td>
<td>7.2±1.5</td>
<td>9.8±1.4</td>
<td>0.14±0.03</td>
<td>41.45±1.39</td>
<td>30.2±3.35</td>
<td>0.73±0.05</td>
</tr>
<tr>
<td></td>
<td>+3 °C</td>
<td>WW</td>
<td>96.7±22.8</td>
<td>9.0±3.1</td>
<td>3.1±1.8</td>
<td>0.25±0.09</td>
<td>35.70±8.51</td>
<td>43.86±12.48</td>
<td>1.19±0.12</td>
</tr>
<tr>
<td>640 ppm</td>
<td>Amb</td>
<td>WW</td>
<td>71.5±5.4</td>
<td>5.4±0.4</td>
<td>10.0±0.7</td>
<td>0.14±0.01</td>
<td>30.2±1.32</td>
<td>21.2±4.98</td>
<td>0.78±0.22</td>
</tr>
<tr>
<td></td>
<td>+3 °C</td>
<td>WW</td>
<td>66.0±4.7</td>
<td>5.1±0.6</td>
<td>0.9±0.3</td>
<td>0.20±0.02</td>
<td>28.26±7.25</td>
<td>47.39±7.03</td>
<td>1.76±0.17</td>
</tr>
</tbody>
</table>

See Table 2 for results of three-way ANOVAs of selected traits.
Drought-mediated changes in the temperature response of dark respiration

Drought had little effect on rates of \( R_{\text{dark}} \) measured at the prevailing mid-morning/early afternoon \( T \) of each treatment.

Table 2. Three-way ANOVAs of leaf mass per unit leaf area (LMA), area-based concentrations of soluble sugars and starch, and the temperature where \( R_{\text{dark}} \) reached its maximum (\( T_{\text{max}} \))

Main factors used in the analysis were atmospheric growth \( \text{CO}_2 \) concentration (\( \text{CO}_2 \)), growth temperature (\( T \)), and water availability (\( \text{H}_2\text{O} \)).

<table>
<thead>
<tr>
<th>Source (growth environment)</th>
<th>df</th>
<th>LMA</th>
<th>Sugars</th>
<th>Starch</th>
<th>( T_{\text{max}} )</th>
<th>( E_{\text{R}_{\text{low}}} )</th>
<th>( E_{\text{R}_{\text{high}}} )</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{CO}_2 )</td>
<td>1</td>
<td>0.319</td>
<td>0.213</td>
<td>0.106</td>
<td>0.062</td>
<td>0.063</td>
<td>0.399</td>
<td>0.031</td>
</tr>
<tr>
<td>( T )</td>
<td>1</td>
<td>0.159</td>
<td>0.016</td>
<td>0.370</td>
<td>0.370</td>
<td>0.027</td>
<td>0.620</td>
<td>0.126</td>
</tr>
<tr>
<td>( \text{H}_2\text{O} )</td>
<td>1</td>
<td>0.538</td>
<td>0.420</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.755</td>
<td>0.032</td>
<td>0.019</td>
</tr>
<tr>
<td>( \text{CO}_2 \times T )</td>
<td>1</td>
<td>0.802</td>
<td>0.939</td>
<td>0.804</td>
<td>0.684</td>
<td>0.924</td>
<td>0.970</td>
<td>0.544</td>
</tr>
<tr>
<td>( \text{CO}_2 \times \text{H}_2\text{O} )</td>
<td>1</td>
<td>0.961</td>
<td>0.656</td>
<td>0.275</td>
<td>0.824</td>
<td>0.479</td>
<td>0.132</td>
<td>0.021</td>
</tr>
<tr>
<td>( T \times \text{H}_2\text{O} )</td>
<td>1</td>
<td>0.509</td>
<td>0.959</td>
<td>0.407</td>
<td>0.324</td>
<td>0.558</td>
<td>0.725</td>
<td>0.734</td>
</tr>
<tr>
<td>( T \times \text{H}_2\text{O} \times \text{H}_2\text{O} )</td>
<td>1</td>
<td>0.434</td>
<td>0.466</td>
<td>0.682</td>
<td>0.792</td>
<td>0.282</td>
<td>0.131</td>
<td>0.149</td>
</tr>
<tr>
<td>Error</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( P \)-values in bold indicate significant effects (\( P<0.05 \)). See Table 1 for trait values of LMA, sugars, and starch; see Fig. 3 for \( T_{\text{max}} \) values.

Fig. 2. Effect of drought on short-term temperature (\( T \))–response curves of area-based leaf respiration measured in darkness (\( R_{\text{dark}} \)) of Eucalyptus globulus fully expanded leaves for plants grown under two atmospheric \( \text{CO}_2 \) concentrations (400 \( \mu \text{mol mol}^{-1} \) and 640 \( \mu \text{mol mol}^{-1} \) for ambient and elevated \( \text{CO}_2 \), respectively) and two growth \( T \) (ambient and ambient +3°C) scenarios: (A) ambient [\( \text{CO}_2 \)] and ambient \( T \); (B) elevated [\( \text{CO}_2 \)] and ambient \( T \); (C) ambient [\( \text{CO}_2 \)] and elevated \( T \); and (D) elevated [\( \text{CO}_2 \)] and elevated \( T \). Within each [\( \text{CO}_2 \)]–growth \( T \) combination, values are shown for well-watered (filled symbols) and drought-treated (open symbols) plants. Measurements took place in week 7 when drought-treated leaves were in the second week of the second period of drought. Leaves were heated at a rate of 1°C min\(^{-1}\), starting at 10–15°C; data were recorded at minute intervals. To allow comparison of treatments at designated leaf \( T \), only values at 5°C intervals are shown, using the nearest \( R_{\text{dark}} \) and leaf \( T \) values to each 5°C value. Values shown are the mean of three replicates (±SE).
main or interactive effects of growth T and atmospheric [CO₂] on Tₘₐₓ (Table 2). Taken together, these results indicate that drought affected the shape of the temperature–response curve of Rₗₐₜₜ, especially at high temperatures (>45 °C) in leaves of young E. globulus trees, whereas growth T and elevated CO₂ had no significant effect on Rₗₐₜₜ at Tₘₐₓ or the value Tₘₐₓ per se (Fig. 3).

Previous studies have linked variations in high temperature tolerance of photosynthesis and respiration to variations in soluble sugar concentrations (Seemann et al., 1986; Hüve et al., 2006, 2012). Given this, the relationship between Tₘₐₓ (Fig. 3) and the concentration of soluble sugars (Table 1) was analysed using linear regression. No relationship was found (P=0.562, r²=0.06) (Fig. 4B). However, a highly significant relationship was found between Tₘₐₓ and starch concentrations (P<0.001, r²=0.84), with increased starch in well-watered plants associated with a decrease in Tₘₐₓ (Tₘₐₓ=62.51–0.88×[starch]; Fig. 4A) compared with drought-treated plants.

To gain insights into the percentage of leaf sugar and starch that may have been respired during each T–response curve, the total amount of CO₂ respired during each T–response curve (mol C m⁻²) was calculated; thereafter, these values were converted to an equivalent mass of carbohydrate respired during each T–response curve. The total carbohydrate respired ranged from 0.13 g m⁻² to 0.25 g m⁻² (Table 1), representing <4% of total soluble sugars present in non-heated leaves. Therefore, for all treatments, Rₗₐₜₜ during the T–response curve run is unlikely to have been limited by substrate availability. Moreover, starch degradation [which has been linked to abrupt increases in respiration rates at very high leaf T values (Hüve et al., 2012)] is likely to have continued during the entire T–response curve runs in all treatments, as the total carbohydrate respired represented only 1–23% of leaf starch (Table 1).

**Impact of drought, growth T, and elevated [CO₂] on the Q₁₀ and respiratory ‘burst’**

To assess treatment effects on T sensitivity, individual plant log-transformed rates of Rₗₐₜₜ were first plotted against T; thereafter, second-order polynomial equations were fitted to the log-Rₗₐₜₜ–T plots over the 15–45 °C range, with the slope of those curve fits then used to calculate the Q₁₀ of Rₗₐₜₜ at any measuring T (see equations 1–3). Figure 5 shows the resulting Q₁₀–T plots for each treatment combination, comparing well-watered and drought-treated plants within each growth T–[CO₂] combination. In each panel, the Q₁₀–T relationship reported by Tjoelker et al. (2001) is shown for comparison (Q₁₀=3.05–0.0457). Regardless of the growth treatment combination, the observed Q₁₀ values were consistently higher at leaf T >35 °C than those reported by Tjoelker et al. (2001). Moreover, three-way ANOVAs conducted at individual measuring T (at 35, 40, and 45 °C) revealed a significant effect of water supply on Q₁₀ values (Table 3), with Q₁₀ values being higher in drought compared with well-watered plants (Fig. 5). In contrast, growth T and [CO₂] had no significant effect on the T sensitivity of Rₗₐₜₜ at high measurement T (Table 3). However, when comparisons of Q₁₀ values were made at lower leaf T (<35 °C), significant differences were found between the two growth [CO₂] (Table 3), with Q₁₀ values being higher in plants grown under elevated [CO₂] than in those grown under ambient [CO₂]. Taken together, these results demonstrate that: (i) the Tjoelker et al. (2001) relationship consistently underestimates the T sensitivity of leaf Rₗₜₜ of E. globulus at high leaf T (>35 °C); (ii) the effectiveness of the Tjoelker et al. (2001) equation in predicting Q₁₀ values at low measuring T differs between plants grown under ambient and elevated [CO₂] (being better under elevated [CO₂] conditions); and (iii) drought increases the T sensitivity of Rₗₜₜ especially at high measurement T.

To assess whether a ‘respiratory burst’ occurred in each treatment, Eₛ values were calculated over two T intervals below the Tₘₐₓ. For well-watered plants, Eₛ values were calculated within two ranges of leaf T: within the 30–40 °C (low-T range) and within the 40–50 °C (high-T range) ranges; for drought-treated plants, the equivalent ranges used were 40–50 °C and 50–60 °C. For well-watered plants, Eₛ values over the high-T range were lower compared with their low-T
Effect of drought on the temperature response of leaf respiration

Fig. 4. Relationship between temperature where $R_{\text{dark}}$ reached its maximum ($T_{\text{max}}$) and (A) leaf starch and (B) soluble sugar concentrations (g m$^{-2}$) of Eucalyptus globulus with values of $T_{\text{max}}$ taken from Fig. 3 and starch/sugar concentrations from Table 1. Values shown are the mean of three replicates (±SE). Analyses of starch and sugar were carried out on a different set of (unheated) leaves from those used for the $R_{\text{dark}}$–$T$ measurements. In (A), linear regression revealed a significant negative relationship ($P<0.001$; $r^2=0.82$) between $T_{\text{max}}$ and starch concentration (regression shown with a solid line, with dotted lines showing 95% confidence intervals). In contrast, there was no significant relationship between $T_{\text{max}}$ and concentration of soluble sugars (B). See Table 1 for treatment means of sugar and starch concentrations.

Fig. 5. Effect of drought on $Q_{10}$ of $R_{\text{dark}}$ [i.e. proportional increase in leaf $R_{\text{dark}}$ per 10 °C increase in leaf temperature ($T$)] at 5 °C $T$ intervals for Eucalyptus globulus fully expanded leaves for plants grown under two atmospheric CO$_2$ concentrations (400 μmol mol$^{-1}$ and 640 μmol mol$^{-1}$ for ambient and elevated [CO$_2$], respectively) and two growth $T$ (ambient and ambient +3 °C) scenarios: (A) ambient [CO$_2$] and ambient $T$; (B) elevated [CO$_2$] and ambient $T$; (C) ambient [CO$_2$] and elevated $T$; and (D) elevated [CO$_2$] and elevated $T$. Within each [CO$_2$]–growth $T$ combination, values are shown for well-watered (solid line) and drought-treated (dashed line) plants. For comparison, the $Q_{10}$–$T$ relationship reported in Tjoelker et al. (2001) (i.e. $Q_{10}=3.22-0.0467T$). Measurements took place in week 7 when drought-treated leaves were in the second week of the second period of drought. At any measuring $T$, $Q_{10}$ values were calculated using second-order polynomial curves fitted to log $R_{\text{dark}}$ versus $T$ over the 15–45 °C range, with $Q_{10}$ values being calculated from the slope at any $T$ (using equations 1–3 in the main text). See Table 1 for a three-way ANOVA comparing treatment effects at any set measuring $T$. 
Discussion

The present study sought to assess the effects of three climate change drivers (elevated growth \([\text{CO}_2]\), elevated growth \(T\), and drought) on the short-term \(T\) sensitivity of leaf \(R_{\text{dark}}\) of \(E.\ globulus\). Although no significant effects of elevated \([\text{CO}_2]\) and growth temperature on \(R_{\text{dark}}\) were observed, the results highlight the importance of drought in: (i) increasing rates of \(R_{\text{dark}}\) at high leaf \(T\) typical of heatwave events; and (ii) increasing the \(T\) at which maximal rates of \(R_{\text{dark}}\) occur (\(T_{\text{max}}\)). It was also found that increased \([\text{CO}_2]\): (i) increased the slope of \(Q_{10}-T\) relationships of respiration, particularly at low to moderate measuring \(T\); and (ii) determined the extent to which the respiratory burst increases under drought. Collectively, the study highlights the dynamic nature of the \(T\) dependence of \(R_{\text{dark}}\) in plants experiencing future climate change scenarios, particularly with respect to drought and elevated \([\text{CO}_2]\). Importantly, the lack of interactive effects among the three treatments (elevated \([\text{CO}_2]\), growth \(T\), and drought) suggests that the effect of drought on rates of \(R_{\text{dark}}\) might not be altered in the near future by increases in atmospheric \([\text{CO}_2]\) or growth \(T\). The response to more extreme climate change scenarios is, however, not known.

Drought impacts on respiration rates

The hypothesis that \(R_{\text{dark}}\) at moderate to high leaf \(T\) would be inhibited by drought (particularly under elevated growth \(T\) and ambient atmospheric \([\text{CO}_2]\)) was based on past work showing that leaf \(R_{\text{dark}}\) can be substrate limited (Azcón-Bíeto and Osmond, 1983), particularly at high measuring \(T\) (Atkin and Tjoelker, 2003; Bunce, 2007), and because, in many cases, drought reduces leaf \(R_{\text{dark}}\) at a set measuring \(T\) (Flexas et al., 2005; Atkin and Macherel, 2009; Crous et al., 2011; Rodríguez-Calcerrada et al., 2011; Duan et al., 2013). When measured at low to moderate \(T\) (15–35 °C), it was found that drought did not inhibit leaf \(R_{\text{dark}}\) in the growth \(T\) and/or \([\text{CO}_2]\) treatments (Supplementary Fig. S5 at JXB online). On first inspection, this result appears to contradict the assertion that drought inhibits leaf \(R_{\text{dark}}\) (Flexas et al., 2005; Atkin and Macherel, 2009). However, closer inspection of past studies reveals that in approximately one-third of cases, drought does not affect \(R_{\text{dark}}\) at ≤25 °C (Galmés et al., 2007; Gimeno et al., 2010). Similarly, the resilience of \(R_{\text{dark}}\) in drought-treated plants was observed over the low to moderate range of \(T\). Despite marked differences in total non-structural carbohydrates between well-watered and drought-treated plants, leaf \(R_{\text{dark}}\) remained unchanged over the 15–35 °C range, suggesting that respiratory metabolism was not substrate limited across this lower \(T\) range. Indeed, the analysis of the total amount of carbon respired during each run (0.13–0.25 g m⁻²) was <4% of the sugar present in leaves prior to measurements (Table 1), suggesting that for all treatments, \(R_{\text{dark}}\) was unlikely to have been substrate limited across all measurement \(T\) values.

When measured at leaf \(T\) below 35 °C, \(Q_{10}\) values were higher in plants grown under elevated \([\text{CO}_2]\) than in those grown under ambient \([\text{CO}_2]\). Why was this? Higher \(Q_{10}\) values have been linked to metabolic conditions where respiratory flux is more limited by enzymatic capacity than when \(R_{\text{dark}}\) is limited by substrate supply and/or turnover of ATP to ADP (Atkin and Tjoelker, 2003). Given this, one possibility is that growth under elevated \([\text{CO}_2]\) alters which factors limit respiratory flux over the low to moderate \(T\) range (i.e. away from substrate/ATP turnover to enzyme capacity). Further work that quantifies in vivo limitations in each of these factors is needed.

Interestingly, it was found that drought increased \(R_{\text{dark}}\) at high measuring leaf \(T\) >35 °C (Fig. 2), resulting in significant increases in \(R_{\text{max}}\) and \(T_{\text{max}}\) (Figs 2, 3) and increased \(Q_{10}\) of leaf \(R_{\text{dark}}\) at leaf \(T\) >35 °C (Fig. 4; Table 1). Past reports have reported drought-mediated increases in leaf \(R_{\text{dark}}\) at moderate measuring \(T\) (Zagdanska, 1995; Bartoli et al., 2005; Slot et al., 2008; Metcalfe et al., 2010) and drought-mediated increases in the \(Q_{10}\) (Slot et al., 2008) in a small number of species.
Thus, while the results differ somewhat from these studies, it is apparent that when leaves are exposed to high T (≥40 °C), drought may exacerbate/increase $R_{\text{dark}}$ rates (Slot et al., 2008; Metcalfe et al., 2010; Flanagan and Syed, 2011). While the underlying mechanisms responsible for these higher rates of leaf $R_{\text{dark}}$ and $Q_{10}$ values at high leaf T (in drought-treated plants) remain unclear, it seems unlikely that this response was linked to substrate supply differences (see above). Given this, it is suggested that drought-mediated increases in the demand for respiratory products (e.g. ATP and/or NADH) by cellular maintenance processes [e.g. high rates of protein turnover and maintenance of ion gradients (Amthor, 2000; Scheurwater et al., 2000) and membrane stability] may have played a role in the increased rates of leaf $R_{\text{dark}}$ at very high T.

**Coupling of respiratory and photosynthetic metabolism**

Past studies have reported that variation in $R_{\text{dark}}$ is often tightly coupled to variation in $A_{\text{sat}}$ (Gifford, 1995; Loveys et al., 2003; Whitehead et al., 2004; Noguchi and Yoshida, 2008). Given that neither growth T nor elevated [CO$_2$] resulted in significant changes in $A_{\text{sat}}$ in the juvenile leaves of *E. globulus* (Fig. 1), the absence of growth T and/or [CO$_2$] effect on $R_{\text{dark}}$ is perhaps not surprising. In the case of elevated [CO$_2$], other studies have also reported no effect of elevated [CO$_2$] on rates of leaf $R_{\text{dark}}$ (Tissue et al., 2002; Bunce, 2005). Less common is the absence of a significant growth T effect on rates of $R_{\text{dark}}$, as respiration more often than not accimates to sustained changes in growth T (Atkin et al., 2000a; Bolstad et al., 2003; Tjoelker et al., 2008, 2009; Zaragoza-Castells et al., 2008). However, given that the plants in the present study experienced weekly temperature changes of ~5–10 °C due to springtime weather at that time (Supplementary Fig. S1 at JXB online), it was not expected to see a growth T effect of +3 °C warming on rates of $R_{\text{dark}}$. Hence, this changing weather pattern did not accommodate the potential acclimation of $R_{\text{dark}}$ to sustained changes in growth temperature.

Although rates of $R_{\text{dark}}$ (<35 °C) were relatively unaffected by any of the treatments, drought did have a marked inhibitory effect on $A_{\text{sat}}$. As such, $R_{\text{dark}}$:$A_{\text{sat}}$ ratios increased markedly under drought (Fig. 1; Supplementary Fig. S6 at JXB online). This finding is similar to that of a recent study assessing the effect of drought on *Eucalyptus saliva* saplings, $R_{\text{dark}}$:$A_{\text{sat}}$ increased 56% under drought (reflecting the greater inhibitory effect of drought on $A_{\text{sat}}$ than on $R_{\text{dark}}$) (Ayub et al., 2011). Similarly, $R_{\text{dark}}$:$A_{\text{sat}}$ ratios have been found to increase markedly under drought in evergreen and deciduous Mediterranean forests (Zaragoza-Castells et al., 2008; Rodriguez-Calcerrada et al., 2010). Variations in $R_{\text{dark}}$:$A_{\text{sat}}$ ratios have also been reported in plants acclimated to contrasting growth T and [CO$_2$] in previous studies (Campbell et al., 2007; Tingey et al., 2007; Ow et al., 2008; Cai et al., 2010; Ayub et al., 2011). In contrast, no significant effect of elevated growth [CO$_2$] or elevated growth T on the ratio between respiration and photosynthesis was found (i.e. $R_{\text{dark}}$:$A_{\text{sat}}$ ratios were homeostatic), reflecting the lack of [CO$_2$] or growth T effects on C gain and C loss. Thus, these observations highlight the variable responses of $R_{\text{dark}}$:$A_{\text{sat}}$ ratios to climate change factors, and the need for predictive dynamic vegetation-climate models to exercise caution when assuming a constant $R_{\text{dark}}$:$A_{\text{sat}}$ ratios.

**Temperature response curves under future climate change scenarios**

Unlike past studies assessing the effect of climate change drivers on the T response of $R_{\text{dark}}$ that relied on low resolution data collected over a narrow T range, here high resolution curves were generated over a wide T range, including lethally high T where respiratory function was inhibited. The experiments revealed several marked effects of drought and elevated [CO$_2$] on the shape of the resultant $R_{\text{dark}}$–T curves. At T <35 °C, little treatment (drought and CO$_2$) difference could be detected in specific rates of $R_{\text{dark}}$ at any T (Fig. 2)—yet, analysis of the $Q_{10}$ values over the 15–35 °C range revealed significantly higher $Q_{10}$ values in elevated [CO$_2$] compared with plants grown under ambient [CO$_2$] (Fig. 5; Table 1). This finding was consistent across both growth T, suggesting that $R_{\text{dark}}$ may be more T sensitive (at T <35 °C) in a future, higher [CO$_2$] world, at least in juvenile leaves of fast-growing trees. Above 35 °C, other factors (e.g. drought) appear to play a more important role in determining the $Q_{10}$ response. Given the importance of the T dependence of $R_{\text{dark}}$ for carbon storage by terrestrial ecosystems (Huntingford et al., 2013; Wythers et al., 2013), such changes in the T dependence of $R_{\text{dark}}$ have the potential to alter the potential of managed forest ecosystems to sequester atmospheric CO$_2$ markedly. However, given that the present work was limited to seedlings, further work is needed to assess clearly whether the same responses occur in mature trees of managed and natural forests.

One of the most striking outcomes of this study was the effect drought had on the shape of $R_{\text{dark}}$–T curves when T exceeded 45 °C. Drought-treated plants exhibited a 7 °C increase in $T_{\text{max}}$ (59.8 ± 1.5 °C) compared with well-watered plants (52.4 ± 0.5 °C) when averaged across all growth [CO$_2$] and growth T treatments. In the survey of the literature by Tjoelker et al. (2001) (that relied on curve fits to low resolution $R$–T data for measuring $T_{\text{max}}$<35°C), it was predicted that a globally averaged $T_{\text{max}}$ value was likely to be near 48 °C. Given that actual $T_{\text{max}}$ values were not available in the data reported by Tjoelker et al. (2001), it seems unlikely that $T_{\text{max}}$ (i.e. the T where $Q_{10}$=1.0) can be accurately predicted from curve fits to $R$–T data over a sublethal range of T. In the present study, $T_{\text{max}}$ values predicted via extrapolation from curve fits over the 15–45 °C range were 59–69 °C for well-watered plants, and 76–94 °C for drought-treated plants (data not shown). Yet, actual measured $T_{\text{max}}$ values were markedly lower, being 52–53 °C for well-watered and 58–61 °C for drought-treated plants (Fig. 3). Moreover, while past studies on *Populus tremula* or *Quercus* sp. showed that actual values of $T_{\text{max}}$ were near 48–50 °C for these species (Hamelrynck and Knapp, 1994; Hüve et al., 2012), O’Sullivan et al. (2013) found that the $T_{\text{max}}$ of *E. pauciflora* trees growing in several thermally contrasting environments ranged from 51 °C to 57 °C (i.e. markedly greater than 48 °C). From these observations, it is suggested
that extrapolated curves fitted to $R-T$ plots over a range of sublethal $T$ values do not necessarily provide an accurate prediction of actual $T_{\text{max}}$ values.

While the $T_{\text{max}}$ of $R_{\text{dark}}$ is not a measure of thermotolerance (i.e. the ability of metabolic processes to withstand high $T$), variations in $T_{\text{max}}$ are positively correlated with variations in the temperature where disruption of electron transport in photosystem II occurs (typically in the 42–55 °C range; Havaux et al., 1991; Knight and Ackerly, 2002; Hüve et al., 2012; O’Sullivan et al., 2013). Thus, adding to past reports on drought-induced increases in photosynthetic high $T$ tolerance (Seemann et al., 1986; Havaux, 1992), the present study shows for the first time that drought can also increase respiratory heat tolerance. This finding has relevance given that: (i) 23% of the Earth’s land surface habitats exhibit air temperatures of >40 °C (Singaas et al., 1999; Wise et al., 2004), which in turn can result in leaf $T$ exceeding 50 °C (Hamerlynck and Knapp, 1994); and (ii) high leaf $T$ are likely to become more common in the future based on radiative warming (Mechel and Tebaldi, 2004; IPCC, 2007; Duffy and Tebaldi, 2012). Further work is needed to assess whether the present findings are representative of a wider range of species growing in drought-susceptible biomes around the world.

Past studies have reported that increases in leaf osmotic potential and soluble sugar concentrations are associated with increases in enhanced heat tolerance of both photosynthesis and respiration, possibly via sugars increasing protection of chloroplast and mitochondrial membranes (Seemann et al., 1986; Hüve et al., 2006, 2012). More broadly, increased tolerance of heat stress can occur via changes in membrane fluidity that result from modifications in membrane lipid and protein composition (Björkman et al., 1980; Sung et al., 2003). Increased synthesis of isoprene at high $T$ may also help stabilize membranes (Sharkey, 2005; Velikova et al., 2011), with isoprene synthesis linked to increased use of starch and soluble sugars in some drought-stressed plants (Funk et al., 2004; Monson et al., 2012; Rodriguez-Calcerrada et al., 2013). Given this, what might we expect from a relationship between the $T_{\text{max}}$ of $R_{\text{dark}}$ and concentrations of non-structural carbohydrates? Although Hüve et al. (2012) found that additional leaf soluble sugars (supplemented via petiole uptake) increased the $T_{\text{max}}$, no relationship between initial sugar concentrations and $T_{\text{max}}$ was found in the present study (Fig. 4). However, a strong negative relationship between $T_{\text{max}}$ and starch concentrations was found (Fig. 4). One possible explanation was that the maintenance of soluble sugar concentrations was facilitated by starch degradation (leading to lower concentrations in drought-stressed plants; Table 1), with products of sugar metabolism providing the glycolytic molecules [e.g. phosphoenolpyruvate (PEP)] needed for isoprene synthesis (Lichtenhaller, 1999). Alternatively, starch degradation and homeostasis of sugar pools may have provided the carbon molecules necessary for synthesis of compatible solutes [e.g. methylated cyclic amino acids (Lippert and Galinski, 1992), glycine betaine (Wani et al., 2013), or trehalose (Penna, 2003)], several of which are known to increase heat tolerance in plant cells. Irrespective of the mechanism via which $T_{\text{max}}$ was increased in drought-treated plants, most probably the products of starch degradation played a role.

Recently, Hüve et al. (2012) proposed that accelerated starch degradation might be responsible for the abrupt increase in $R_{\text{dark}}$ at very high leaf $T$ (i.e. ‘respiratory burst’). In their study, pulse–chase experiments strongly suggested that leaf $R_{\text{dark}}$ of $P. tremula$ was substrate limited at moderate and high leaf $T$ values, with accelerated starch degradation at high leaf $T$ probably alleviating substrate limitations of respiratory metabolism, resulting in a ‘burst’ of $R_{\text{dark}}$. In the present study, little evidence of a strong respiratory ‘burst’ was found in a majority of the treatments (Table 1), despite most treatments exhibiting similar area-based starch concentrations (in leaves that were not subjected to the $T$–response curve protocol) to those in Hüve et al. (2012). Thus, it may be premature to attribute presence/absence of a respiratory burst in all species to accelerated rates of starch degradation at high leaf $T$.

Conclusions

The present study has shown that predicted climate change scenarios may markedly alter the shape of $T$–response curves of $R_{\text{dark}}$, particularly when considering the effect of drought on $R_{\text{dark}}$ at high leaf $T$ indicative of heatwave events. Yet, changes in $T$ dependence of $R_{\text{dark}}$ were also apparent when comparing $Q_{10}$ values at low to moderate $T$ of plants grown under ambient and elevated [CO$_2$] (being highest under elevated [CO$_2$] over the 15–35 °C range), but not in ambient and elevated temperature treatments. Interestingly, no evidence of interactive effects between atmospheric [CO$_2$], growth $T$, or water availability was found, suggesting that the stimulatory effects of elevated [CO$_2$] (low to moderate $T$) and drought (at high $T$) on the $Q_{10}$ are generalized phenomena, at least for *E. globulus* seedlings grown under semi-controlled environment conditions. Collectively, these results challenge the prevailing assumption in most climate models that the $T$ dependence of $R_{\text{dark}}$ is constant (Huntingford and Cox, 2000). If more widespread, the present results suggest that dynamic changes in the shape of $R_{\text{dark}}$–$T$ curves may occur in the future, in response to rising levels of atmospheric [CO$_2$] and increasing frequency and severity of drought. Such changes, if realized, have important implications for terrestrial C storage and atmospheric [CO$_2$] in a future, warmer world.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Temporal variations in leaf temperature.

Figure S2. Temporal variation in stomatal conductance.

Figure S3. Temporal variation in internal CO$_2$ concentration.

Figure S4. Temporal variation in light-saturated photosynthesis ($A_{\text{sat}}$).

Figure S5. Temporal variation in leaf respiration ($R_{\text{dark}}$).

Figure S6. Temporal variation in $R_{\text{dark}}$: $A_{\text{sat}}$ ratios.

Table S1. Statistical analyses of leaf gas exchange.
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