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Drought increases heat tolerance of leaf respiration in *Eucalyptus globulus* saplings grown under both ambient and elevated atmospheric [CO$_2$] and temperature

Paul P. G. Gauthier[1,2]**, Kristine Y. Crous**[1,3]**, Gohar Ayub**[1,4]**, Honglang Duan**[3,5]**, Lasantha K. Weerasinghe**[1,6]**,
David S. Ellsworth**[3]**, Mark G. Tjoelker**[3]**, John R. Evans**[1]**, David T. Tissue**[3]** and Owen K. Atkin**[1,7,*]

[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

* To whom correspondence should be addressed E-mail: owen.atkin@anu.edu.au

Abstract

Climate change is resulting in increasing atmospheric [CO$_2$], 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 [CO$_2$], sustained warming, and drought on leaf dark respiration (*R*$_{dark}$), and the short-term *T* response of *R*$_{dark}$ were examined in *Eucalyptus globulus*. Comparisons were made using seedlings grown under different [CO$_2$], *T*, and drought treatments. Using high resolution *T*–response curves of *R*$_{dark}$ measured over the 15–65 °C range, it was found that elevated [CO$_2$], elevated growth *T*, and drought had little effect on rates of *R*$_{dark}$ measured at *T* <35 °C and that there was no interactive effect of [CO$_2$], growth *T*, and drought on *T* response of *R*$_{dark}$. However, drought increased *R*$_{dark}$ at high leaf *T* typical of heatwave events (35–45 °C), and increased the measuring *T* at which maximal rates of *R*$_{dark}$ occurred (*T*$_{max}$) 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 [CO$_2$]. Elevated [CO$_2$] increased the *Q*$_{10}$ of *R*$_{dark}$ (i.e. proportional rise in *R*$_{dark}$ per 10 °C) over the 15–35 °C range, while drought increased *Q*$_{10}$ values between 35 °C and 45 °C. Collectively, the study highlights the dynamic nature of the *T* dependence of *R*$_{dark}$ in plants experiencing future climate change scenarios, particularly with respect to drought and elevated [CO$_2$].

Key words: Dark respiration, drought, elevated CO$_2$, *Eucalyptus globulus*, *Q*$_{10}$, 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 CO$_2$ concentrations ([CO$_2$]; 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 C$_3$ 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 (R_{dark}) to changes in the abiotic environment. Of the CO_2 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 R_{dark} (Atkin *et al.*, 2007). Small changes in leaf R_{dark} (e.g. due to changes in atmospheric [CO_2], 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 R_{dark} is T sensitive (Atkin *et al.*, 2005; Kruse *et al.*, 2011), several studies have predicted large changes in terrestrial C storage and atmospheric [CO_2] 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 Q_{10} for leaf R_{dark} of 2.0 (i.e. R_{dark} doubles for every short-term 10 °C rise in T) (Huntingford and Cox, 2000) and that R_{dark} does not acclimate to sustained changes in growth T (Mahecha *et al.*, 2010). While the assumption of a constant Q_{10} of 2.0 may be appropriate for modelling rates of R_{dark} in some plant species, the assumption is unlikely to be valid for all scenarios, as the T response of R_{dark} can be highly variable. For example, sustained increases in growth T can result in declines in the Q_{10} of R_{dark} (Atkin *et al.*, 2000b, 2005; Zaragoza-Castells *et al.*, 2008), underpinned by limitations in substrate supply and/or energy demand that restrict rates of R_{dark} at high measuring T more than at low measuring T (Atkin and Tjoelker, 2003). Moreover, the Q_{10} 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 Q_{10} of leaf R_{dark} declines with increasing measuring T varies among species and environments, and is not well understood. However, a 'generalized' Q_{10}–T relationship proposed by Tjoelker *et al.* (2001) suggested that Q_{10} declines with increasing T according to: $Q_{10}=3.09-0.043T$. Accounting for this Q_{10}–T relationship results in lower R_{dark} at T both lower and higher than a given reference temperature, leading to large decreases in predicted ecosystem R_{dark} compared with models that assume a constant Q_{10} of 2.0 (Wythers *et al.*, 2005, 2013). Moreover, variations in the Q_{10}–T relationship due to changes in the environment (e.g. in response to rising atmospheric [CO_2], 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 R_{dark} estimated by ecosystem models and ESMs (Wythers *et al.*, 2005, 2013; King *et al.*, 2006).

Given the link between substrates/energy demand and Q_{10} values (Atkin and Tjoelker, 2003), it seems likely that higher substrate supply might result in an increased Q_{10} in elevated atmospheric [CO_2], altering the T dependence of R_{dark}. Similarly, drought-mediated changes in photosynthesis, substrate supply, and energy demand (Ribas-Carbó *et al.*, 2005) could, theoretically, affect the Q_{10} of R_{dark}. In most studies, imposition of drought results in a decline in R_{dark} 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 R_{dark} 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 Q_{10} of R_{dark} (Slot *et al.*, 2008).

Finally, consideration needs to be given to acclimation to increased growth T on R_{dark} at a set measuring T and associated Q_{10} values. Acclimation to sustained increases in growth T often results in a decline in basal rates of R_{dark} (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 Q_{10} of R_{dark} in some species (Tjoelker *et al.*, 2001; Zaragoza-Castells *et al.*, 2008; Crous *et al.*, 2011), several studies have reported lower average Q_{10} 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 Q_{10}–T relationships. The extent to which the T dependence of leaf R_{dark} is affected by potential interactive effects of atmospheric [CO_2]–growth T–drought is also not known.

Examining how abiotic factors impact on the T dependence of leaf R_{dark}, previous studies either (i) have quantified the impact of diel variations in T on leaf R_{dark}; or (ii) have measured rates of R_{dark} 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 Q_{10}–T relationships among treatments. Given the limitations of these approaches, an alternative is to record rates of leaf R_{dark} 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 R_{dark} (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_{dark}–T curves (and associated Q_{10}–T relationships) to be explored in detail.

At high T, leaf R_{dark} reaches a maximum (at T_{max}) at which $Q_{10}=1.0$; this point indicates the maximum heat tolerance of R_{dark}, with further heating resulting in irreversible declines in R_{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_{max} of leaf R_{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 air (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_{max} of leaf R_{dark} is affected by growth T, atmospheric [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 [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 concentrations (Seemann *et al.*, 1986; Hüve *et al.*, 2006). Moreover, recent work by Hüve *et al.* (2012) suggests that the T_{max} of R_{dark} is increased in leaves with enhanced osmotic potential or sugar concentrations (via protection of respiratory membranes). Given this, enhanced concentrations of non-structural carbohydrates in plants grown under elevated atmospheric [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_{max} of leaf R_{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_{dark}, depending on whether plants are grown under ambient or elevated atmospheric [CO_2] (Hamerlynck *et al.*, 2000; Niinemets, 2010). Importantly, however, no study has yet investigated the impact of multiple climate change drivers on the respiratory T_{max}.

The overall aim of the present study was to assess how elevated atmospheric [CO_2], growth T, and drought affect the shape of the short-term T response of leaf R_{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_{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_{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 atmospheric [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 hypothesized 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 R_{dark} (Hüve *et al.*, 2012), it was hypothesized that T_{max} would be greatest in well-watered plants grown under elevated atmospheric [CO_2] and ambient growth 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 [CO_2] elevated 240 ppm above ambient concentrations, with three replicates per atmospheric [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 put 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 enough water 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 representing 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 infrared 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), CO_2 levels inside the cuvettes were set to the prevailing $[CO_2]$ 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_{sat}), g_s, and internal CO_2 concentration (C_i) would have been minor and similar for both well-watered and drought-treated plants in each growth $[CO_2]$/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_{sat}). 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} (Azcón-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 × 11.0 × 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 (O'Sullivan *et al.*, 2013). Within each chamber, two fans mixed air (Micronel, Fellbrook, CA, USA), while leaf T was monitored using a small gauge wire copper–nickel–chromium thermocouple (type E) in contact with the abaxial surface of each leaf; the thermocouple was attached to a LI-6400 external thermocouple adaptor (LI640013, Li-Cor Inc.) to enable recording of leaf T. The exiting air-stream from each water-jacketed chamber was connected to the 'sample' gas line of the LI-6400 [fitted with an empty, sealed 3 × 2 cm cuvette (LI-6400-02B)]. Net CO_2 exchange (respiration) from the continuously warming, darkened cuvette was calculated via comparison of the 'sample' IRGA values with the 'reference' IRGA values. Flow rates through the water-jacketed chamber (700 µmol s⁻¹) and $[CO_2]$ of the incoming air were controlled via the LI-6400 console flow meter and LI-6400–01 CO_2 mixer. Incoming air was fully dried before 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 CO_2 exchange taking into account dilution of CO_2 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_s of each leaf was measured to confirm their drought status (see week 6 values of g_s 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 PrometheusWikicontributors, 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}:

$$\log_e(R) = a + bT + cT^2 \tag{1}$$

and where:

$$R = e^{a+bT+cT^2} \tag{2}$$

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:

$$Q_{10} = e^{10\times(b+2cT)} \tag{3}$$

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-lethal Ts [e.g. <45 °C (O'Sullivan *et al.*, 2013)].

To assess the effect(s) of growth T, $[CO_2]$, 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 CO_2 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 $[CO_2]$ and/or warming treatments (n=3) on weekly measurements of A_{sat} 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; Supplementay
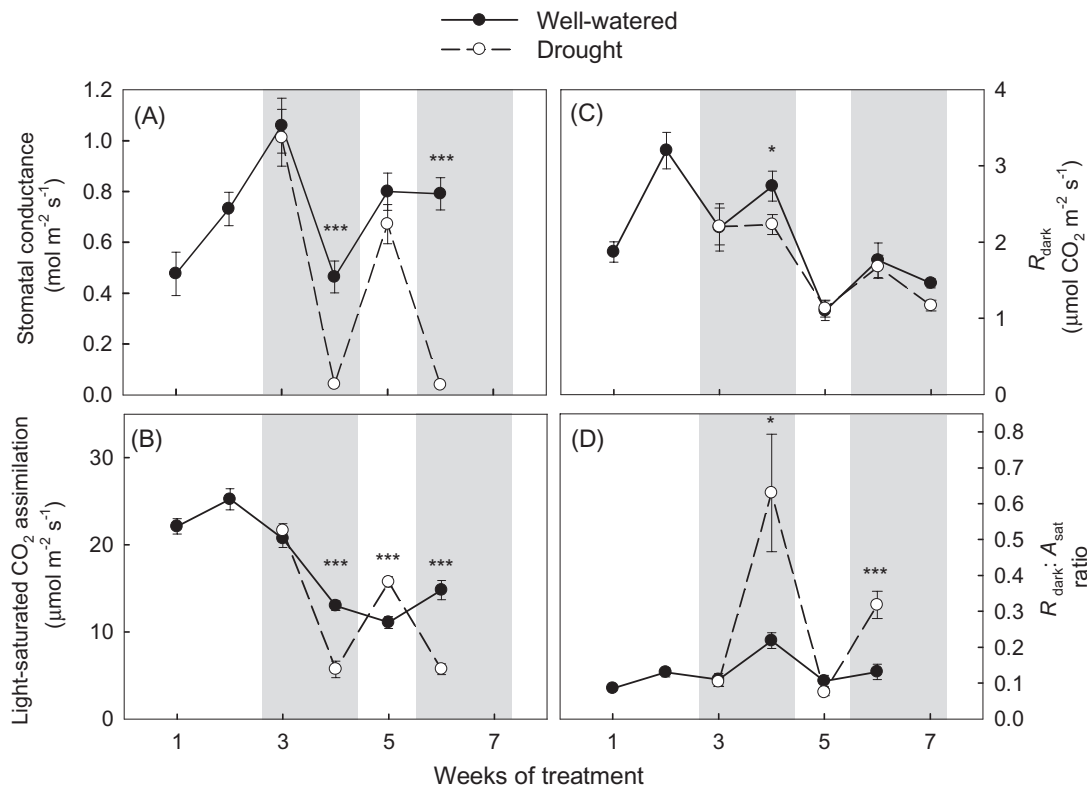

Fig. 1. Effect of periodic drought on: (A) stomatal conductance under saturating irradiance (g_s); (B) light-saturated photosynthesis (A_{sat}); (C) leaf dark respiration (R_{dark}); and (D) R_{dark}/A_{sat} ratios of *Eucalyptus globulus* fully expanded leaves (measured over several weeks commencing early November 2010). Within each plot, values of well-watered (filled circles) and drought-treated (open circles) plants are shown (n=12; ±SE); 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°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 $P<0.1$, ** for $P<0.05$, and *** for $P<0.01$. 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_2O 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_2O 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 [CO₂] on leaf structure and carbohydrates*

Sampled leaf dry mass per unit area (LMA) did not vary significantly among the growth treatments (Tables 1, 2); across 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 [CO₂] 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 rewatering 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 CO₂ 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)

The carbohydrate equivalent needed to supply all CO₂ respired during each run of a *T*–response curve (Fig. 2) is also shown. Also shown are: activation energy (*E*ₐ) values calculated over two measuring *T* intervals below *T*ₘₐₓ; for well-watered plants where *T*ₘₐₓ values were ~52 °C, the two intervals were in 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 in the 40–50 °C (low range) and 50–60 °C (high range) ranges. The ratio of these two *E*ₐ values is also shown.

CO₂ treatment	Growth *T* treatment	H₂O treatment	LMA (g m⁻²)	Sugars (g m⁻²)	Starch (g m⁻²)	Respired carbohydrate (g m⁻²)	*E*ₐ–low *T* (kJ mol⁻¹)	*E*ₐ–high *T* (kJ mol⁻¹)	*E*ₐ–high *T*/*E*ₐ–low *T* (ratio)
400 ppm	Amb	WW	75.8±14.0	7.2±1.5	9.8±1.4	0.14±0.03	41.45±1.39	30.28±3.35	0.73±0.05
		DR	95.7±22.6	9.0±3.1	3.1±1.8	0.25±0.06	35.70±8.51	43.86±12.48	1.19±0.12
	+3 °C	WW	71.5±5.4	5.4±0.4	10.0±0.7	0.14±0.01	30.21±6.32	21.20±4.98	0.78±0.22
		DR	66.0±4.7	5.1±0.6	0.9±0.3	0.20±0.02	28.26±7.25	47.39±7.03	1.76±0.17
640 ppm	Amb	WW	86.1±6.1	8.4±0.4	12.5±0.6	0.16±0.02	41.68±4.32	25.63±0.77	0.63±0.07
		DR	91.0±12.0	9.4±1.9	6.4±3.0	0.19±0.05	49.50±3.11	39.27±2.52	0.80±0.06
	+3 °C	WW	75.1±3.2	5.5±0.1	11.6±1.0	0.13±0.02	39.37±4.76	33.21±8.46	0.93±0.37
		DR	82.1±12.5	7.5±1.9	4.1±1.8	0.16±0.01	34.55±0.52	26.92±9.90	0.77±0.28

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_{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_{dark} reached its maximum (T_{max})*

Main factors used in the analysis were atmospheric growth CO_2 concentration (CO_2), growth temperature (T), and water availability (H_2O).

Source (growth environment)	df	LMA	Sugars	Starch	T_{max}	E_a- low T	E_a- high T	Ratio
CO_2	1	0.319	0.213	0.106	0.962	0.063	0.399	**0.031**
T	1	0.159	**0.016**	0.370	0.568	**0.027**	0.620	0.126
H_2O	1	0.538	0.420	**<0.001**	**<0.001**	0.755	**0.032**	**0.019**
$CO_2 \times T$	1	0.802	0.939	0.804	0.684	0.924	0.970	0.544
$CO_2 \times H_2O$	1	0.961	0.656	0.275	0.824	0.479	0.132	**0.021**
$T \times H_2O$	1	0.509	0.959	0.407	0.324	0.558	0.725	0.734
$CO_2 \times T \times H_2O$	1	0.434	0.466	0.682	0.792	0.282	0.131	0.149
Error	16							

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_{max} values.

combination (Fig. 1; Supplementary Fig. S5 at *JXB* online). Typically, these T values were in the 18–35 °C range (with some leaf T values reaching 38 °C). However, it remained unclear whether the treatments affected the shape of the short-term T–response curves of leaf R_{dark} over a wider temperature range than 18–35 °C (including T exceeding T_{max}).

Different treatment combinations affected the shape of the R_{dark}–T curves (Fig. 2; shown at 5 °C intervals to simplify the presentation). Over the 15–45 °C range, there was little difference in rates of R_{dark} between well-watered and drought-treated plants (Fig. 2), consistent with treatment effects on R_{dark} measured at the prevailing temperatures. Moreover, a three-way ANOVA revealed that at leaf T of 15, 25, and 45 °C, there were no significant differences among any of the three treatments (growth T, growth [CO_2], and/or drought). In contrast, marked differences between well-watered and drought-treated plants were observed at leaf T values >45 °C, with drought-treated plants exhibiting markedly higher R_{dark} at T_{max} (i.e. R_{max}) than their well-watered counterparts (Fig. 2). Thus, while growth T, growth [CO_2], and water availability had little impact on R_{dark} at T <45 °C, drought increased R_{dark} at T >45 °C.

Drought increased T_{max} such that well-watered and drought-treated plants exhibited T_{max} values of 52.4±0.5 °C and 59.8±1.5 °C, respectively (when averaged across both growth [CO_2] and growth T treatments; Fig. 3). Importantly, only drought had a significant effect on T_{max} (*P*<0.0001), with no
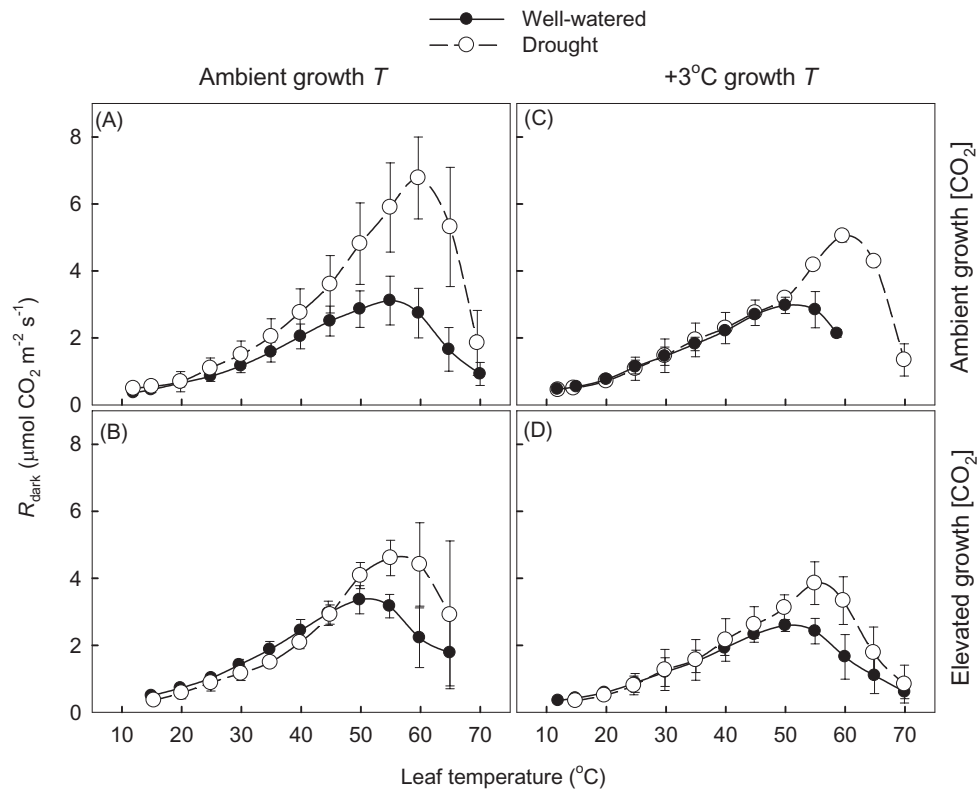

Fig. 2. Effect of drought on short-term temperature (*T*)–response curves of area-based leaf respiration measured in darkness (R_{dark}) of *Eucalyptus globulus* fully expanded leaves for plants grown under two atmospheric CO_2 concentrations (400 μmol mol^{-1} and 640 μmol mol^{-1} for ambient and elevated [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 (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_{dark} and leaf *T* values to each 5 °C value. Values shown are the mean of three replicates (±SE).
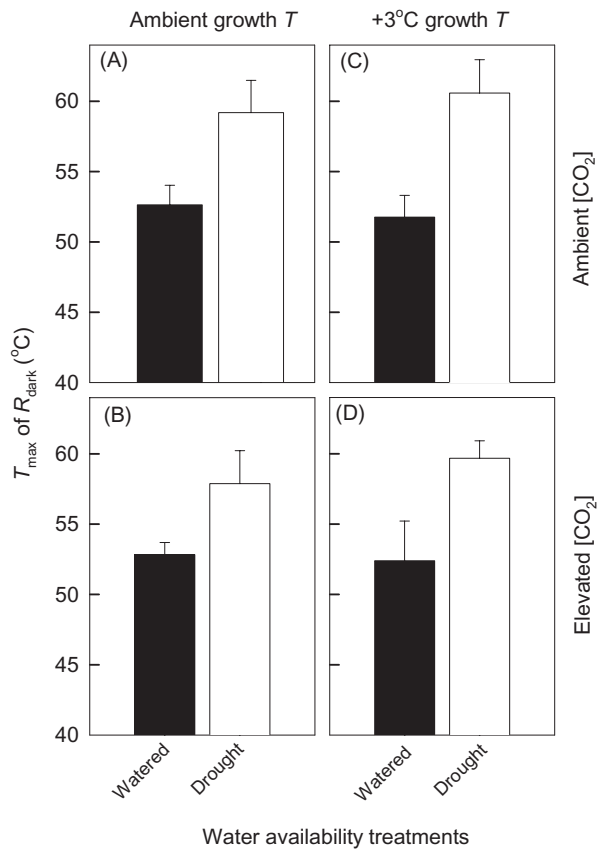
Ambient growth *T*   +3°C growth *T*

Fig. 3. Temperature where R_{dark} reached its maximum (T_{max}) of *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 (filled bars) and drought-treated (open bars) plants. Measurements took place in week 7 when drought-treated leaves were in the second week of the second period of drought. Data shown were derived from short-term *T*–response curves of mass-based R_{dark} (Fig. 5). Values shown are the mean of three replicates (±SE).

main or interactive effects of growth *T* and atmospheric [CO_2] on T_{max} (Table 2). Taken together, these results indicate that drought affected the shape of the temperature–response curve of R_{dark}, especially at high temperatures (>45 °C) in leaves of young *E. globulus* trees, whereas growth *T* and elevated CO_2 had no significant effect on R_{dark} at T_{max} or the value T_{max} *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 T_{max} (Fig. 3) and the concentration of soluble sugars (Table 1) was analysed using linear regression. No relationship was found (P=0.562, r^2=0.06) (Fig. 4B). However, a highly significant relationship was found between T_{max} and starch concentrations (P<0.001, r^2=0.84), with increased starch in well-watered plants associated with a decrease in T_{max} (T_{max}=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 CO_2 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, R_{dark} during the *T*–response curve runs 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 [CO_2] on the* Q$_{10}$ *and respiratory 'burst'*

To assess treatment effects on *T* sensitivity, individual plant log-transformed rates of R_{dark} were first plotted against *T*; thereafter, second-order polynomial equations were fitted to the log-R_{dark}–*T* plots over the 15–45 °C range, with the slope of those curve fits then used to calculate the Q_{10} of R_{dark} at any measuring *T* (see equations 1–3). Figure 5 shows the resulting Q_{10}–*T* plots for each treatment combination, comparing well-watered and drought-treated plants within each growth *T*–[CO_2] combination. In each panel, the Q_{10}–*T* relationship reported by Tjoelker *et al.* (2001) is shown for comparison (Q_{10}=3.05–0.045*T*). Regardless of the growth treatment combination, the observed Q_{10} 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 Q_{10} values (Table 3), with Q_{10} values being higher in drought compared with well-watered plants (Fig. 5). In contrast, growth *T* and [CO_2] had no significant effect on the *T* sensitivity of R_{dark} at high measurement *T* (Table 3). However, when comparisons of Q_{10} values were made at lower leaf *T* (<35 °C), significant differences were found between the two growth [CO_2] (Table 3), with Q_{10} values being higher in plants grown under elevated [CO_2] than in those grown under ambient [CO_2]. Taken together, these results demonstrate that: (i) the Tjoelker *et al.* (2001) relationship consistently underestimates the *T* sensitivity of leaf R_{dark} of *E. globulus* at high leaf *T* (>35 °C); (ii) the effectiveness of the Tjoelker *et al.* (2001) equation in predicting Q_{10} values at low measuring *T* differs between plants grown under ambient and elevated [CO_2] (being better under elevated [CO_2] conditions); and (iii) drought increases the *T* sensitivity of R_{dark} especially at high measurement *T*.

To assess whether a 'respiratory burst' occurred in each treatment, E_a values were calculated over two *T* intervals below the T_{max}. For well-watered plants, E_a 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, E_a values over the high-*T* range were lower compared with their low-*T*
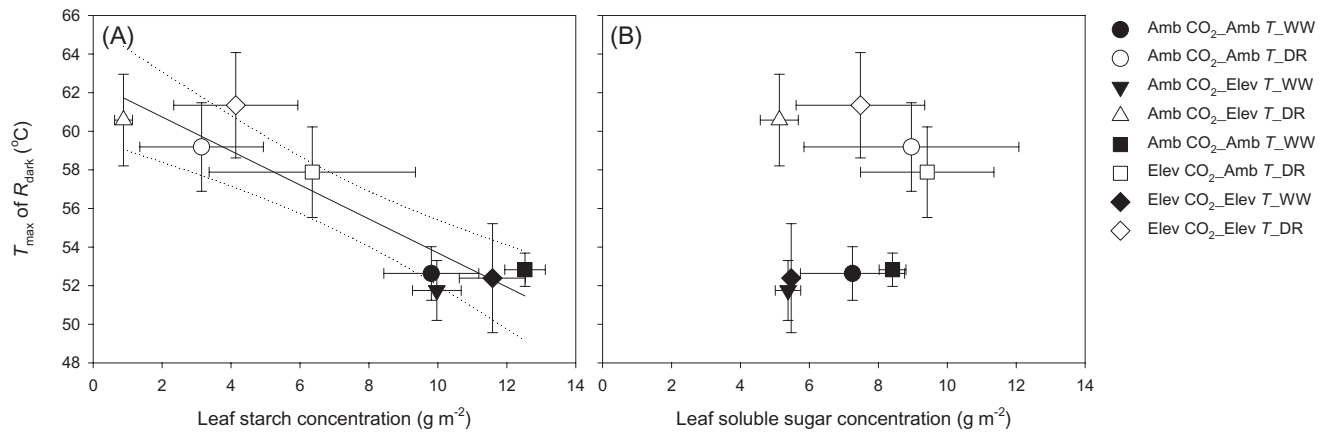
Fig. 4. Relationship between temperature where R_{dark} reached its maximum (T_{max}) and (A) leaf starch and (B) soluble sugar concentrations (g m^{-2}) of *Eucalyptus globulus* with values of T_{max} taken from Fig. 3 and starch/sugar concentrations from Table 1. Values shown are the mean of three replicates (±SE). Analyses of starch and sugar were carried out on a different set of (unheated) leaves from those used for the R_{dark}–T measurements. In (A), linear regression revealed a significant negative relationship ($P<0.001$; $r^2=0.82$) between T_{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_{max} and concentration of soluble sugars (B). See Table 1 for treatment means of sugar and starch concentrations.
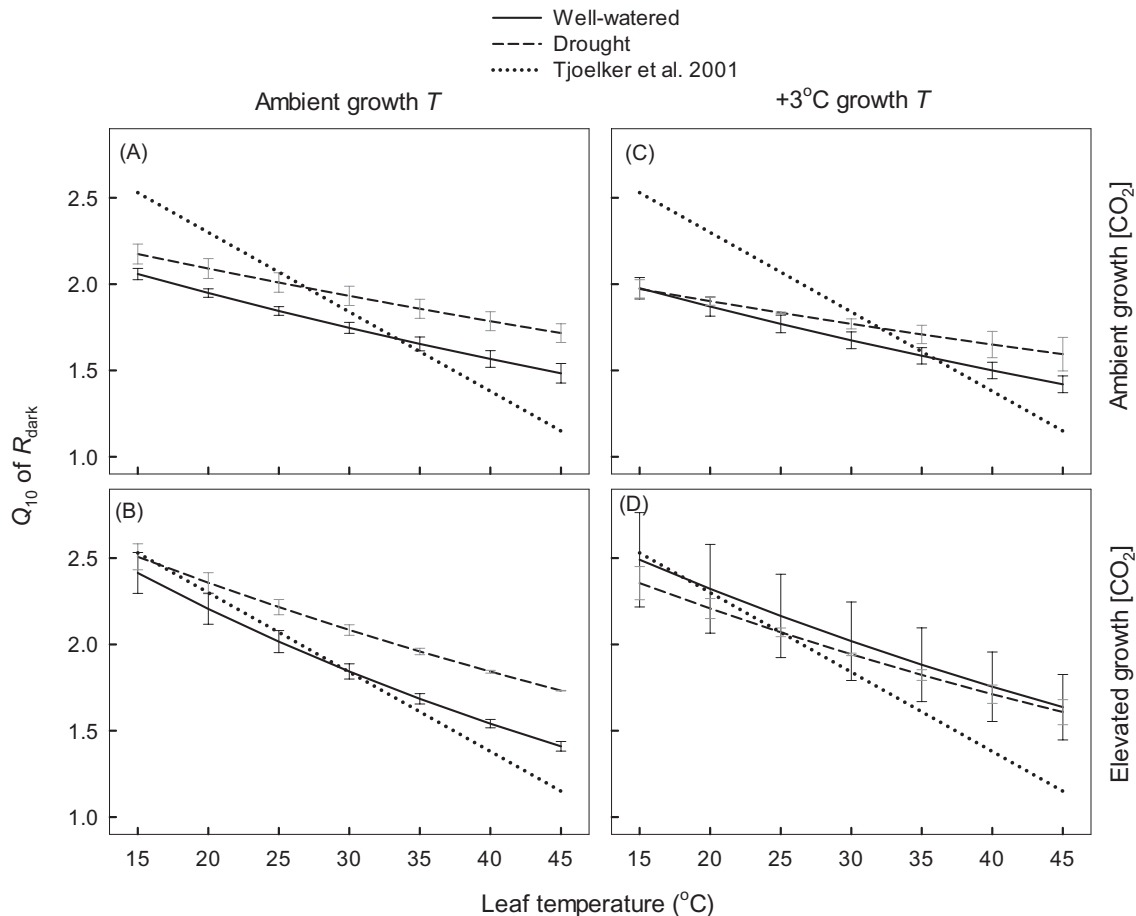

Fig. 5. Effect of drought on Q_{10} of R_{dark} [i.e. proportional increase in leaf R_{dark} per 10 °C increase in leaf temperature (T)] at 5 °C T intervals for *Eucalyptus globulus* fully expanded leaves for plants grown under two atmospheric CO_2 concentrations (400 µmol mol^{-1} and 640 µmol mol^{-1} 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. $Q_{10}=3.22–0.046T$). 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_{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.

Table 3. *Three-way ANOVAs of* Q_{10} *values of leaf* R_{dark} *at designated measuring temperatures in week 7 (i.e. second drought period)*

Q_{10} values (see Fig. 5) were calculated using second-order polynomial curves fitted to log R_{dark} versus T over the over the 15–45 °C range, with Q_{10} values being calculated from the slope at any set measuring T (using equations 1–3 in the main text). Main factors used in the analysis were atmospheric growth CO_2 concentration (CO_2), growth temperature (T), and water availability (H_2O).

Source (growth environment)	df	Instantaneous measurement T						
		7	20	25	30	35	40	45
CO_2	1	**0.000**	**0.001**	**0.002**	**0.007**	**0.038**	0.165	0.493
T	1	0.302	0.326	0.369	0.434	0.521	0.631	0.736
H_2O	1	0.836	0.488	0.230	0.093	**0.039**	**0.019**	**0.011**
$CO_2 \times T$	1	0.548	0.444	0.358	0.297	0.264	0.251	0.256
$CO_2 \times H_2O$	1	0.653	0.652	0.649	0.647	0.643	0.645	0.648
$T \times H_2O$	1	0.318	0.223	0.159	0.122	0.106	0.104	0.115
$CO_2 \times T \times H_2O$	1	0.753	0.607	0.479	0.375	0.307	0.269	0.249
Error	16							

P-values in bold indicate significant effects (*P*<0.05).

counterparts; the same was true for drought-treated plants grown under elevated growth [CO_2] (Table 1). In contrast, E_a values over the high-T range were greater than those over the corresponding low-T range in drought-treated plants grown under ambient [CO_2] (Table 1). Indeed, high-T/low-T ratios were significantly affected by growth [CO_2] and water availability; there was also a significant growth [CO_2]×water interaction (Table 2), suggesting that the extent to which drought affected the respiratory burst did indeed differ between the two growth [CO_2] treatments. There was no evidence of growth T affecting the presence/absence of a respiratory burst. Taken together, these observations suggest that while respiratory bursts were minor or absent in non-stressed *E. globulus* seedlings, more pronounced bursts occurred in ambient [CO_2]-grown plants subjected to drought.

Discussion

The present study sought to assess the effects of three climate change drivers (elevated growth [CO_2], elevated growth T, and drought) on the short-term T sensitivity of leaf R_{dark} of *E. globulus*. Although no significant effects of elevated [CO_2] and growth temperature on R_{dark} were observed, the results highlight the importance of drought in: (i) increasing rates of R_{dark} at high leaf T typical of heatwave events; and (ii) increasing the T at which maximal rates of R_{dark} occur (T_{max}). It was also found that increased [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_{dark} in plants experiencing future climate change scenarios, particularly with respect to drought and

elevated [CO_2]. Importantly, the lack of interactive effects among the three treatments (elevated [CO_2], growth T, and drought) suggests that the effect of drought on rates of R_{dark} might not be altered in the near future by increases in atmospheric [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_{dark} at moderate to high leaf T would be inhibited by drought (particularly under elevated growth T and ambient atmospheric [CO_2]) was based on past work showing that leaf R_{dark} can be substrate limited (Azcón-Bieto and Osmond, 1983), particularly at high measuring T (Atkin and Tjoelker, 2003; Bunce, 2007), and because, in many cases, drought reduces leaf R_{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_{dark} in the growth T and/or [CO_2] treatments (Supplementary Fig. S5 at *JXB* online). On first inspection, this result appears to contradict the assertion that drought inhibits leaf R_{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_{dark} at ≤25 °C (Galmés *et al.*, 2007; Gimeno *et al.*, 2010). Similarly, the resilience of R_{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_{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_{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 [CO_2] than in those grown under ambient [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_{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 [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_{dark} at high measuring leaf T >35 °C (Fig. 2), resulting in significant increases in R_{max} and T_{max} (Figs 2, 3) and increased Q_{10} of leaf R_{dark} at leaf T >35 °C (Fig. 4; Table 1). Past reports have reported drought-mediated increases in leaf R_{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_{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_{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_{dark} at very high T.

Coupling of respiratory and photosynthetic metabolism

Past studies have reported that variation in R_{dark} is often tightly coupled to variation in A_{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_{sat} in the juvenile leaves of *E. globulus* (Fig. 1), the absence of growth T and/or [CO_2] effect on R_{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_{dark} (Tissue *et al.*, 2002; Bunce, 2005). Less common is the absence of a significant growth T effect on rates of R_{dark}, as respiration more often than not acclimates to sustained changes in growth T (Atkin *et al.*, 2000a; Bolstad *et al.*, 2003; Loveys *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_{dark}. Hence, this changing weather pattern did not accommodate the potential acclimation of R_{dark} to sustained changes in growth temperature.

Although rates of R_{dark} (<35 °C) were relatively unaffected by any of the treatments, drought did have a marked inhibitory effect on A_{sat}. As such, $R_{dark}:A_{sat}$ ratios increased markedly under drought (Fig. 1; Supplementay Fig. S6 at *JXB* online). This finding is similar to that of a recent study assessing the effect of drought on *Eucalyptus saligna* saplings, $R_{dark}:A_{sat}$ increased 56% under drought (reflecting the greater inhibitory effect of drought on A_{sat} than on R_{dark}) (Ayub *et al.*, 2011). Similarly, $R_{dark}:A_{sat}$ ratios have been found to increase markedly under drought in evergreen and deciduous Mediterranean forests (Zaragoza-Castells *et al.*, 2008; Rodríguez-Calcerrada *et al.*, 2010). Variations in $R_{dark}:A_{sat}$ 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. $R_{dark}:A_{sat}$ 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 $R_{dark}:A_{sat}$ ratios to climate change factors, and the need for predictive dynamic vegetation–climate models to exercise caution when assuming a constant $R_{dark}:A_{sat}$ 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_{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_{dark}–T curves. At T <35 °C, little treatment (drought and CO_2) difference could be detected in specific rates of R_{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_{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_{dark} for carbon storage by terrestrial ecosystems (Huntingford *et al.*, 2013; Wythers *et al.*, 2013), such changes in the T dependence of R_{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_{dark}–T curves when T exceeded 45 °C. Drought-treated plants exhibited a 7 °C increase in T_{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 <35°C), it was predicted that a globally averaged T_{max} value was likely to be near 48 °C. Given that actual T_{max} values were not available in the data reported by Tjoelker *et al.* (2001), it seems unlikely that T_{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_{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_{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_{max} were near 48–50 °C for these species (Hamerlynck and Knapp, 1994; Hüve *et al.*, 2012), O'Sullivan *et al.* (2013) found that the T_{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_{max} values.

While the T_{max} of R_{dark} is not a measure of thermotolerance (i.e. the ability of metabolic processes to withstand high T), variations in T_{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 (Singsaas *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 (Meehl 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-suceptible 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_{max} of R_{dark} and concentrations of nonstructural carbohydrates? Although Hüve *et al.* (2012) found that additional leaf soluble sugars (supplemented via petiole uptake) increased the T_{max}, no relationship between initial sugar concentrations and T_{max} was found in the present study (Fig. 4). However, a strong negative relationship between T_{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 (Lichtenthaler, 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_{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_{dark} at very high leaf T (i.e. 'respiratory burst'). In their study, pulse–chase experiments strongly suggested that leaf R_{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_{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_{dark}, particularly when considering the effect of drought on R_{dark} at high leaf T indicative of heatwave events. Yet, changes in T dependence of R_{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_{dark} is constant (Huntingford and Cox, 2000). If more widespread, the present results suggest that dynamic changes in the shape of R_{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.

Acknowledgements

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