

# Seasonal acclimation of leaf respiration in *Eucalyptus saligna* trees: impacts of elevated atmospheric CO<sub>2</sub> and summer drought

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## Abstract

Understanding the impacts of atmospheric [CO<sub>2</sub>] and drought on leaf respiration (*R*) and its response to changes in temperature is critical to improve predictions of plant carbon-exchange with the atmosphere, especially at higher temperatures. We quantified the effects of [CO<sub>2</sub>]-enrichment (+ 240 ppm) on seasonal shifts in the diel temperature response of *R* during a moderate summer drought in *Eucalyptus saligna* growing in whole-tree chambers in SE Australia. Seasonal temperature acclimation of *R* was marked, as illustrated by: (1) a downward shift in daily temperature response curves of *R* in summer (relative to spring); (2)  $\approx$  60% lower *R* measured at 20°C (*R*<sub>20</sub>) in summer compared with spring; and (3) homeostasis over 12 months of *R* measured at prevailing nighttime temperatures. *R*<sub>20</sub>, measured during the day, was on average 30–40% higher under elevated [CO<sub>2</sub>] compared with ambient [CO<sub>2</sub>] across both watered and droughted trees. Drought reduced *R*<sub>20</sub> by  $\approx$  30% in both [CO<sub>2</sub>] treatments resulting in additive treatment effects. Although [CO<sub>2</sub>] had no effect on seasonal acclimation, summer drought exacerbated the seasonal downward shift in temperature response curves of *R*. Overall, these results highlight the importance of seasonal acclimation of leaf *R* in trees grown under ambient- and elevated [CO<sub>2</sub>] as well as under moderate drought. Hence, respiration rates may be overestimated if seasonal changes in temperature and drought are not considered when predicting future rates of forest net CO<sub>2</sub> exchange.

## Nomenclature:

*A*<sub>sat</sub> = net photosynthetic CO<sub>2</sub> assimilation under light saturation  
*R* = dark respiration  
*R*<sub>20</sub> = respiration measured at 20 °C  
LMA = leaf mass per unit leaf area  
*N*<sub>mass</sub> = nitrogen concentration per unit leaf dry mass  
*P*<sub>mass</sub> = phosphorus concentration per unit leaf dry mass  
VPD<sub>air</sub> = water vapour pressure deficit of air

**Keywords:** acclimation, drought, elevated CO<sub>2</sub>, leaf respiration, temperature

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## Introduction

Plant respiration (*R*) represents an important factor in the global carbon (C) cycle. Currently, several global land-surface climate models [e.g. those used by the UK

Met Office Hadley Centre (Cox, 2001)] assume that plant *R* increases exponentially with rising temperature with a constant *Q*<sub>10</sub> of 2.0 (i.e. *R* doubles for every 10 °C increase in temperature), with little account being given to possible effects of sustained changes in temperature, atmospheric CO<sub>2</sub> concentration and/or soil moisture availability. Accounting for the impact of such factors on plant *R* is important to refine process-based models

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of C cycling and account for how future climate change may affect net CO<sub>2</sub> balance over wide spatial and temporal scales (Cox *et al.*, 2000; Friedlingstein *et al.*, 2006).

Thermal acclimation of *R* is defined as the adjustment of respiration rates to sustained changes in temperature over time, and is an important element of seasonal respiratory CO<sub>2</sub> released to the atmosphere. While it is well-established that leaf *R* is sensitive to short-term changes in temperature, there is also long-standing evidence that leaf *R* thermally acclimates to changes in growth temperature (Rook, 1969; Billings *et al.*, 1971; Tranquillini *et al.*, 1986; Larigauderie & Körner, 1995; Atkin & Tjoelker, 2003). Thermal acclimation of *R* occurs within days of a change in growth temperature (Atkin *et al.*, 2000; Bolstad *et al.*, 2003; Lee *et al.*, 2005; Ow *et al.*, 2008a,b), resulting in rates of leaf *R* at a set measuring temperature (e.g. 20 °C) being lower in summer than in winter (Tjoelker *et al.*, 2009). This can lead to respiratory homeostasis, with winter- and summer-acclimated plants exhibiting similar rates of leaf *R* when rates are measured at the respective average daily growth temperature of each season (Zaragoza-Castells *et al.*, 2008).

Acclimation of leaf *R* to sustained changes in growth temperature might occur via altered temperature sensitivity (i.e. changes in the  $Q_{10}$ ) and/or via a shift in the overall intercept of the temperature response function (Atkin & Tjoelker, 2003). Changes in the  $Q_{10}$  are associated with temperature-mediated changes in energy demand and/or substrate supply (Covey-Crump *et al.*, 2002; Campbell *et al.*, 2007; Tjoelker *et al.*, 2008) whereas changes in the intercept are underpinned by temperature-mediated changes in respiratory capacity, reflecting changes in mitochondrial abundance, structure and/or protein composition (Miroslavov & Kravkina, 1991; Ribas-Carbó *et al.*, 2000; Armstrong *et al.*, 2006, 2008). Collectively, thermal acclimation of leaf *R* may help plants maintain a positive C balance by reducing C loss under hot conditions, while ensuring basal rates of leaf *R* are maintained in cold periods. Accounting for thermal acclimation is likely to be important when predicting rates of respiratory CO<sub>2</sub> release at global and regional levels, especially in response to changing environmental conditions (Wythers *et al.*, 2005; King *et al.*, 2006; Atkin *et al.*, 2008).

In spite of potentially large adjustments in plant function in elevated atmospheric [CO<sub>2</sub>], to date no study has investigated how elevated [CO<sub>2</sub>] affects the thermal acclimation of leaf *R* to seasonal changes in ambient temperature. By contrast, considerable attention has been given to the impacts of elevated [CO<sub>2</sub>] on specific rates of leaf *R* measured at a common temperature (Hamilton *et al.*, 2001; González-Méler *et al.*, 2004).

Leaf carbohydrate concentration and mitochondrial density are typically higher in plants grown under elevated [CO<sub>2</sub>], thereby increasing respiratory capacity and respiratory substrate availability (Griffin *et al.*, 2001). Conversely, leaf nitrogen and protein content is often lower under elevated [CO<sub>2</sub>] (Norby *et al.*, 1999; Ellsworth *et al.*, 2004), subsequently reducing the demand for respiratory ATP and thus increasing adenylate restriction of leaf *R* (Ryan, 1991). Collectively, the balance between these variable responses will determine the impact of elevated [CO<sub>2</sub>] on rates of leaf *R* measured at any given temperature but may potentially affect the response of leaf *R* to temperature as well. If thermal acclimation is underpinned by changes in substrate supply (Tjoelker *et al.*, 2008), then sustained high rates of photosynthesis under elevated atmospheric [CO<sub>2</sub>] may result in leaf *R* being substrate-saturated throughout much of the year. By contrast, leaf *R* in ambient [CO<sub>2</sub>] grown trees could likely become substrate-limited during warm periods, resulting in a decline of leaf *R* at a common temperature (Tjoelker *et al.*, 2008). Therefore, ambient [CO<sub>2</sub>] grown trees could exhibit greater downward adjustment of leaf *R* in summer compared with their elevated [CO<sub>2</sub>] grown counterparts.

As is the case with elevated [CO<sub>2</sub>], responses of leaf *R* to drought are also highly variable (Flexas *et al.*, 2005; Galmés *et al.*, 2007). In two thirds of studies reviewed in Atkin & Macherel (2009), drought reduced rates of leaf *R*, with the inhibitory effect often being greater in faster-growing, short-lived species than in slower-growing, evergreen species (Galmés *et al.*, 2007; Giméno *et al.*, 2010). What is less clear, however, is whether growth under elevated atmospheric [CO<sub>2</sub>] alters the response of leaf *R* to drought. Increased water savings of trees growing under elevated atmospheric [CO<sub>2</sub>] (Saxe *et al.*, 1998; Leuzinger & Körner, 2007) may reduce drought stress on leaf *R*. Moreover, while summer drought exacerbates the decline in leaf *R* in the deciduous *Fagus sylvatica* (Rodríguez-Calcerrada *et al.*, 2010), little is known about the impacts of drought on thermal acclimation of leaf *R* in longer-lived, evergreen tree species. Given the contrasting effects of drought on rates of leaf *R* in short- and long-lived leaves (Galmés *et al.*, 2007), one possibility is that there is little effect of drought on seasonal temperature acclimation of leaf *R* in long-lived, evergreen species.

The aim of our study was to investigate the impacts of elevated atmospheric [CO<sub>2</sub>] and summer drought on seasonal temperature acclimation responses of leaf *R* in an evergreen tree species (*Eucalyptus saligna* Sm.) growing in the field in whole-tree chambers (WTCs; Barton *et al.*, 2010) in SE Australia. Our study is the first to investigate the effects of elevated atmospheric [CO<sub>2</sub>] and a moderate summer drought on: (1) rates of leaf *R*

across a range of ambient temperatures; and, (2) seasonal shifts in the daily temperature response curve of leaf *R*. Given the potential for acclimation to be substrate-dependent and for atmospheric [CO<sub>2</sub>] to alter seasonal changes in substrate limitation, we tested the hypothesis that seasonal shifts in daily temperature response curves of leaf *R* will be greater in trees grown under ambient atmospheric [CO<sub>2</sub>] than their elevated [CO<sub>2</sub>] grown counterparts. Building on the findings of Galmés *et al.* (2007), we also tested the hypothesis that summer drought has little impact on rates of leaf *R* *per se*, or on the seasonal shift in daily temperature response curves of leaf *R*, in the evergreen *E. saligna*.

## 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). The site is characterized by a humid temperate climate (mean annual temperature, 17 °C; mean annual precipitation, 800 mm; Bureau of Meteorology, station 067105 in Richmond, NSW Australia; <http://www.bom.gov.au>). The annual precipitation in 2008 and 2009 was similar to the mean annual precipitation (801 mm). Soils are sandy loam at the surface, with clay deposits at a depth of about 1 m below the surface.

The HFE consists of 12 CO<sub>2</sub>- and temperature-controlled WTCs. The technical design and the WTC chamber operation in this experiment has been described in Barton *et al.* (2010). The air temperature within all WTCs tracked the ambient temperature to within 0.5 °C for >90% of the time throughout the experiment. One uniform-sized *E. saligna* tree (Sydney Blue Gum) was planted in the centre of each WTC as a seedling (30 cm tall) in April 2007 with buffer trees surrounding the WTCs at a density of 1000 trees ha<sup>-1</sup> to form a block of continuous forest and avoid edge effects. At the beginning of our core measurement period in October 2008, trees were on average 5.1 m tall and grew an additional 3–4 m in height over the subsequent summer of 2008–2009 (i.e. October–March).

Water and atmospheric CO<sub>2</sub> concentration treatments were applied to the WTCs, with three replicates per atmospheric [CO<sub>2</sub>] and water treatment combination. Six chambers received elevated [CO<sub>2</sub>] (ambient + 240 μmol mol<sup>-1</sup>) since the trees were planted. For each [CO<sub>2</sub>] treatment, three WTCs received a well-watered treatment equivalent to an annual average rainfall of near 1200 mm (i.e. 10 mm precipitation added every third day). While above the average annual rainfall in Richmond NSW, 1200 mm is representative of the average rainfall *E. saligna* would experience in its natural range (Boland *et al.*, 1984). The drought treatment was achieved by withholding water from the assigned trees starting in October 2008 (i.e. spring) and extending through February 2009 (i.e. summer). To monitor the progression of drought, predawn leaf water potentials (Ψ<sub>leaf</sub>) were measured each month on three leaves in each chamber for all 12 WTCs

with a Scholander pressure chamber (PMS Corp., Corvallis, OR, USA). Ψ<sub>leaf</sub> measurements were taken immediately before or during gas exchange measurement campaigns and reflect plant-available soil moisture integrated over the tree rooting zone (Franco *et al.*, 1994; Williams & Ehleringer, 2000). Water withholding was stopped at the beginning of March 2009.

In seeking to understand how atmospheric [CO<sub>2</sub>] and water availability affected seasonal shifts in rates of leaf *R*, we conducted two complementary studies detailed below: (1) diel (24 h) measurements of leaf *R* and associated plant traits (including quantification of leaf photosynthesis) over a core 5-month period commencing in October 2008, during which the drought treatment developed – here, leaf *R* was measured at the prevailing night or day temperature to quantify temperature responses of leaf *R* as well as the effects of elevated CO<sub>2</sub> and drought on leaf *R*, using the protocol reported in Atkin *et al.* (2000); Zaragoza-Castells *et al.* (2008) and Rodríguez-Calcerrada *et al.* (2010) and (2) postsunset, nighttime measurements of leaf *R* at prevailing temperatures over a 12-month period starting March 2008 to gauge thermal acclimation responses of nighttime respiration throughout the year.

### *Leaf R measurements*

Gas exchange measurements for the 5-month core part of the study were conducted ca. monthly between October 2008 and March 2009 in five consecutive campaigns of 2–3 days. For each campaign, we selected the most recently fully expanded leaves (one leaf per tree) growing in the sun-facing lower third of the canopy to measure net CO<sub>2</sub> exchange. Different leaves were chosen in each month so that the measured leaves could be harvested for analysis of leaf structure and chemical composition at the end of each measurement campaign. In addition to the core 5-month study, nighttime leaf respiration was measured eight times over a 12-month period beginning in March 2008 at the prevailing growth [CO<sub>2</sub>], temperature and drought conditions in each WTC. These measurements were conducted 2–3 h after sunset on three mature leaves of each tree in the lower third of the canopy. The chamber temperature was set to the prevailing ambient nighttime temperature with the exception of winter measurements, where it was necessary to measure leaf *R* at a temperature a few °C above the ambient dew-point to avoid condensation.

Gas exchange measurements were conducted with portable infrared gas analyser systems (LiCor 6400, LiCor Inc., Lincoln, NE, USA) using 6 cm<sup>2</sup> leaf cuvettes. Measurements of leaf *R* were made using flow rates of 300 μmol s<sup>-1</sup> in the cuvettes, whereas a flow rate of 600 μmol s<sup>-1</sup> was used for photosynthesis measurements. To minimize diffusion gradients across the gaskets of the cuvette (Bruhn *et al.*, 2002), CO<sub>2</sub> levels inside the cuvettes were set to the prevailing conditions in each WTC before each measurement, based on readings from the constantly updated readings from the gas analysers (PP-systems SBA-1, Amesbury, MA, USA) of each WTC. Block temperatures of the gas analyser systems (Li-Cor 6400) were set to the prevailing temperature in each WTC.

To generate diel temperature response curves of individual leaves in 5 consecutive months of the study (spring–summer), measurements of leaf *R* were made 2–3 h after sunset and

proceeding regularly during the subsequent day from presunrise (i.e. in early morning darkness) until daily temperatures peaked late in the afternoon. For measurements made during the day period, we covered the leaves with a reflective sleeve 30 min before measuring CO<sub>2</sub> efflux. Past work on postillumination transients has shown that rates of leaf *R* often increased markedly over the first 2–5 min after leaves are placed in darkness, with steady-state rates achieved after 30 min of darkness (Azcón-Bieto & Osmond, 1983; Atkin *et al.*, 1998). After each set of leaf *R* measurements during the day, leaves were re-exposed to daylight to prevent potential limitations in substrate supply (Azcón-Bieto, 1992). In addition to diel leaf *R* measurements, light-saturated photosynthesis (*A*<sub>sat</sub>) was measured at the respective CO<sub>2</sub> growth concentration on the same leaves twice each day, around 09:00 and 15:00 hours, using a photon flux density of 1800 μmol m<sup>-2</sup> s<sup>-1</sup> using the Li-Cor 6400 light source (10 min before measurement) with the corresponding measurements of leaf *R* at the same temperature obtained after 30 min of darkness.

#### *Diel temperature responses of leaf R over the 5-month drought treatment period*

Temperature (*T*) responses of leaf *R* in individual leaves in each WTC (determined using the diel time course described above) were compared from the 5 monthly campaigns to assess whether seasonal acclimation of leaf *R* occurred. With the exception of October and March, large diurnal temperature ranges (typically 20–25 °C range between maximum and minimum temperatures) were achieved on each measurement day. Given no significant differences between first- and second-order linear regression fits to the log<sub>10</sub>-transformed leaf *R* vs. temperature plots in any month/treatment combination, 1st order linear regression equations were fitted according to:

$$\log_{10}R = a + bT, \quad (1)$$

where *a* represents the *y*-axis intercept (i.e. log<sub>10</sub> *R* at 0 °C) and *b* is the slope of the 1st order regression. The average temperature dependence of *R* (i.e. *Q*<sub>10</sub>) over the diurnal temperature range experienced by each leaf could then be calculated according to:

$$Q_{10} = 10^{10b}. \quad (2)$$

*Q*<sub>10</sub> values were determined for individual leaves (i.e. three replicates per treatment). Using these variables, rates of leaf *R* at any given temperature (*R*<sub>*T*</sub>) were predicted using:

$$R_T = R_0 Q_{10}^{[T/10]}, \quad (3)$$

where *R*<sub>0</sub> is the rate of *R* at 0 °C (i.e. 10<sup>3</sup>). To compare rates of leaf *R* among the treatments over the October 2008–April 2009 period, we used the output of Eqns (1)–(3) to calculate modelled leaf *R* rates at 20 °C because this temperature was encompassed in each diel measurement campaign of the 5 months of the core study.

The utility of the method above depends, in part, on whether rates of leaf *R* vary in darkness and illumination. By comparing rates of postsunset and predawn leaf *R* at common temperature, we assessed whether the measured rates of leaf *R*

decreased with extended darkness overnight. Typically, postsunset and predawn leaves experienced similar leaf temperatures (<1.5 °C of each other) with rates of leaf *R* at a common temperature not decreasing with duration of darkness. Moreover, no relationship was found between variations in rates of *R*<sub>20</sub> and the preceding 1 through 7 days average daily photon input (data not shown) indicating that variations in leaf *R*<sub>20</sub> were not associated with changes in daily irradiance before measurement.

#### *Leaf traits and chemical properties over the 5-month drought treatment period*

After completion of the gas exchange measurements, all measured leaves from the core study period were harvested to measure leaf area (using a LI-3100 Leaf Area Meter, LiCor Inc.), fresh mass and dry mass (oven dried at 70 °C) using the 6 cm<sup>2</sup> segment contained within the Licor 6400 cuvette. We then calculated the dry matter content (DMC, ratio of leaf dry mass to fresh mass), fresh mass per unit leaf area (FMA) and ratio of leaf dry mass per unit leaf area (LMA) of each leaf replicate. FMA is correlated with leaf thickness (Dijkstra *et al.*, 1989; Vile *et al.*, 2005). Concentrations of nitrogen and phosphorus in the individual 6 cm<sup>2</sup> leaf segments were then determined with a Technicon Auto-analyzer II (Bran + Luebbe Pty. Ltd., Norderstedt, Germany) using Kjeldahl acid digests. The remaining portion of each leaf (i.e. that not contained within the Licor 6400 cuvette) was used to analyse sugars, starch and total nonstructural carbohydrates (TNC) as described previously (Loveys *et al.*, 2003).

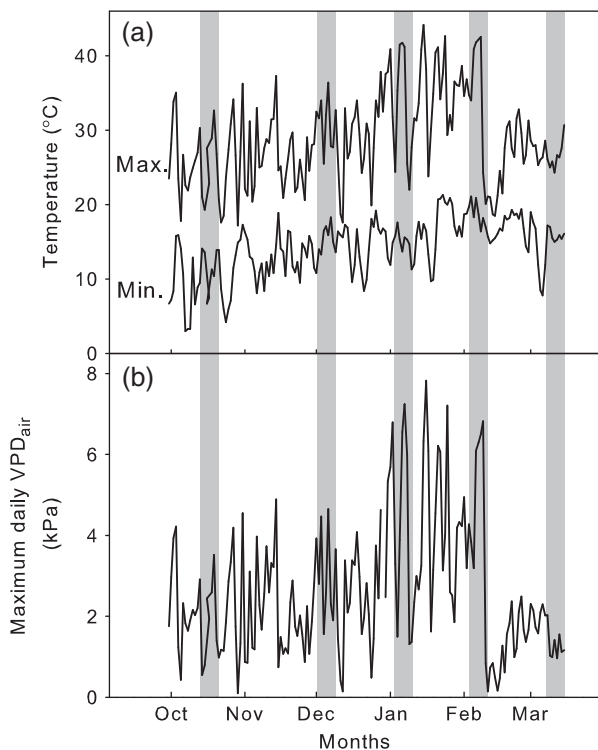
#### *Statistical analyses*

All statistical analyses were conducted in JMP v.5.0.1 (SAS Institute, Cary, NC, USA). To evaluate differences between atmospheric [CO<sub>2</sub>] treatments, watering treatments and sampling date, all variables were examined in a three-way full factorial ANOVA with month, CO<sub>2</sub> treatment and H<sub>2</sub>O treatment as main factors. Variables were transformed if necessary to meet the normality and equal variance assumptions (typically a log<sub>10</sub> transformation). Differences between means were considered significant at *P* < 0.05 using a Tukey *post hoc* test. Three-way interactions were never significant (*P* > 0.10). Within each month of water withholding, differences in the log-transformed respiration rates between well-watered and droughted trees as a function of diel variations in temperature were tested using linear regression analysis with dummy variables according to Neter *et al.* (1996) to address the question whether growth [CO<sub>2</sub>] and/or drought affected seasonal acclimation of leaf *R*.

## **Results**

### *Climate variables and predawn plant water status*

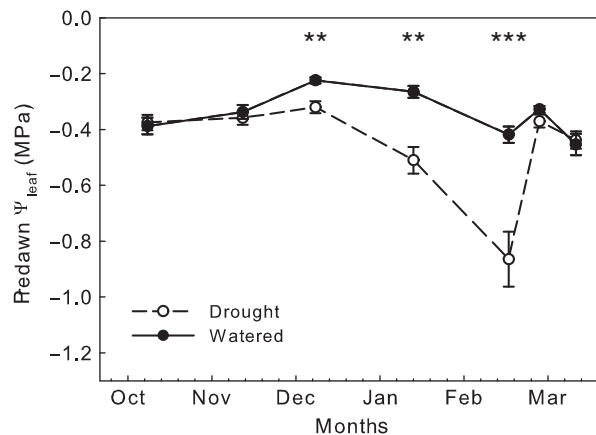
Meteorological data such as rainfall, temperature, vapour pressure deficit (*VPD*<sub>air</sub>) were collected at the site



**Fig. 1** Daily environmental conditions for the core 5-month study period from October 2008 to March 2009. The top panel (a) shows minimum and maximum daily temperatures, and the lower panel (b) shows the maximum daily vapour pressure deficit ( $VPD_{air}$ ). Vertical shaded regions indicate the timing of field campaigns when core measurements were taken.

since the beginning of the experiment (Barton *et al.*, 2010). Over the 5-month core experimental period of this study, average daily temperatures increased from  $12.3^{\circ}\text{C}$  at the beginning of the growing season in October 2008 to a maximum of *ca.*  $30^{\circ}\text{C}$  in late January/early February 2009. Daytime maximum temperatures exceeded  $40^{\circ}\text{C}$  in January and February 2009 (Fig. 1a) with daily photon input often exceeding  $60\text{ mol photons m}^{-2}\text{ day}^{-1}$  in summer months. The maximum daily  $VPD_{air}$  ranged between near  $0.5$  and  $7.8\text{ kPa}$ , with the highest maxima ( $>5\text{ kPa}$ ) in January and February 2009 (Fig. 1b).

Physiological effects of drought were apparent during the 5-month experimental period when water supply was withheld from half the trees, as shown by the significant decrease in predawn water potentials in this treatment ( $\psi_{leaf}$ ; open symbols in Fig. 2). As similar drought-mediated trends in  $\psi_{leaf}$  were observed within each  $\text{CO}_2$  treatment,  $\psi_{leaf}$  values were averaged across the two  $\text{CO}_2$  treatments (Fig. 2). Significant differences in predawn  $\psi_{leaf}$  between drought treated and well-watered chambers emerged in December 2008 ( $P = 0.004$ ) and became progressively stronger in January ( $P = 0.002$ )

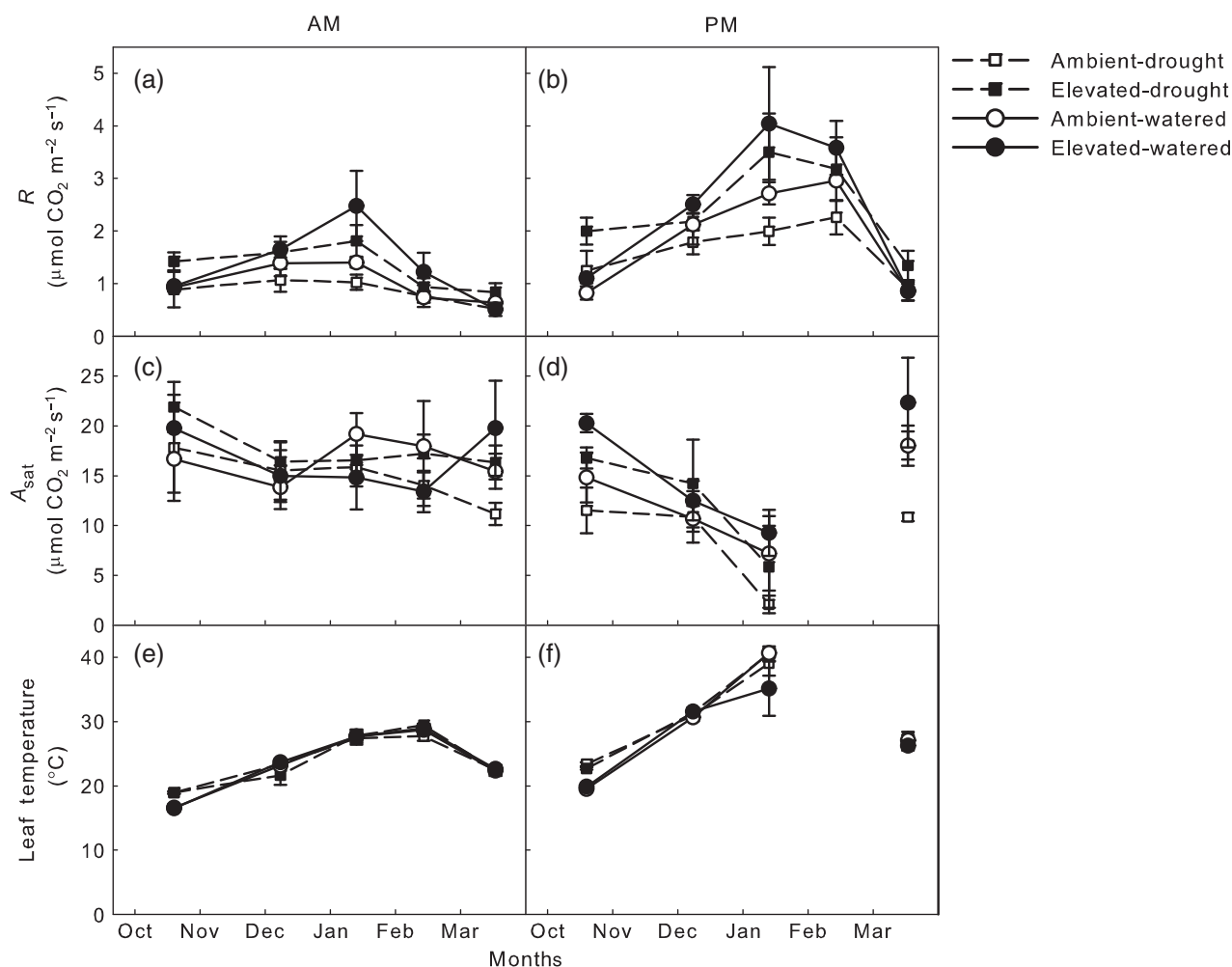


**Fig. 2** Mean predawn leaf water potentials ( $\psi_{leaf}$ ) ( $n = 6$  trees per drought treatment  $\pm$  SE) from October 2008 to March 2009, averaged across  $\text{CO}_2$  treatments. Open symbols and dashed lines are for trees subjected to the drought treatment and the well-watered trees are indicated by closed symbols and solid lines. Stars indicate significant differences between water treatments within sampling date, with  $**P < 0.005$  and  $***P < 0.001$  using Student's *t*-test. The vertical arrow indicates the timing of rewatering in March 2009.

and mid-February ( $P = 0.0005$ ) before rewatering in early March 2009 (indicated by the arrow in Fig. 2).

#### Effects of elevated $\text{CO}_2$ and drought on leaf gas exchange

Despite the significant difference in predawn  $\psi_{leaf}$  between watering treatments in December 2008 (Fig. 2), moderate drought treatment differences in leaf  $R$  for measurements made at the prevailing temperatures in the morning (AM) and afternoon (PM) in *E. saligna* were not detected until January 2009 (Fig. 3). Averaged across all 5 months, drought did not have a significant effect on leaf  $R$ . However, the effect of drought was substantially greater when considering only those months in which the drought treatment was fully apparent, with an average reduction of 24–39% in leaf  $R$  during January and February 2009 across  $\text{CO}_2$  treatments (Fig. 3a and b). Irrespective of whether leaf  $R$  was measured in the morning ( $R\text{-AM}$ ) or afternoon ( $R\text{-PM}$ ), strong monthly differences were found ( $P < 0.0001$ ; Table 1), with higher leaf  $R$  in the afternoon compared with the morning, especially during the summer months as temperatures increased (Fig. 3a and b). A significant effect of elevated  $[\text{CO}_2]$  on leaf  $R$  was observed in both morning and afternoon ( $P < 0.001$ , Table 1), with the overall average across well-watered and drought-treated  $R\text{-AM}$  and  $R\text{-PM}$  values being on average 34% higher in trees grown under elevated  $[\text{CO}_2]$  compared with their ambient  $[\text{CO}_2]$  counterparts measured at comparable temperatures. Although there was no clear drought effect on  $R\text{-AM}$ , rates of  $R\text{-PM}$  were signifi-



**Fig. 3** Mean leaf respiration in the dark (a, b), mean photosynthetic measurements at saturating light levels ( $A_{\text{sat}}$ ) (measured at  $1800 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) (c, d) and leaf temperatures in the cuvette (e, f) in morning (AM; left panels) and afternoon (PM; right panels) for designated atmospheric  $\text{CO}_2$  and drought treatments. Rates were measured at the prevailing ambient temperatures. Open symbols indicate ambient  $\text{CO}_2$  whereas closed symbols indicate elevated  $\text{CO}_2$ -grown trees ( $n = 3 \pm \text{SE}$ ). The designated drought treatment is shown separately for each time point, despite actual physiological effects occurring only in peak summer (December–February; see Fig. 2).

cantly lower in drought-treated trees in January and February compared with well-watered trees in those months ( $P = 0.024$ , Fig. 3a and b; Table 1). Importantly, the results highlight the inhibitory effect that summer drought has on leaf  $R$ , particularly under the hot conditions experienced in the afternoon when temperatures often exceeded  $40^{\circ}\text{C}$  (Figs 1b and 3h).

There were no significant differences in AM-measured rates of  $A_{\text{sat}}$  between trees growing in the ambient and elevated  $[\text{CO}_2]$  treatments, or between well-watered and drought treatments (Fig. 3c). However, the PM-measured rates of  $A_{\text{sat}}$  ( $A_{\text{sat-PM}}$ ) exhibited strong monthly differences (Fig. 3d;  $P < 0.0001$ , Table 1), declining in January 2009 in both well-watered and drought-treated trees in response to very high  $\text{VPD}_{\text{air}}$  in all treatments as air temperatures exceeded  $40^{\circ}\text{C}$  (Fig. 1).

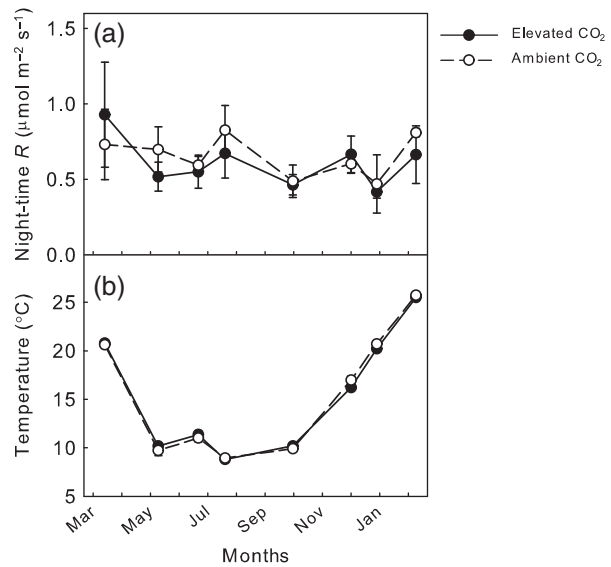
Although data for  $A_{\text{sat-PM}}$  are not available for February 2009 due to logistical problems on the day of measurement, it seems likely that the trend observed in January would have been maintained. Moreover, averaged across months where data were available, rates of  $A_{\text{sat-PM}}$  were  $38 \pm 5\%$  higher in trees grown under elevated  $[\text{CO}_2]$  than those grown under ambient  $[\text{CO}_2]$  conditions ( $P = 0.004$ ) and about 25% higher in well-watered trees compared with drought-treated trees ( $P = 0.027$ , Fig. 3d). Thus, while there were no treatment differences in rates of  $A_{\text{sat}}$  among treatments in the morning, significant differences in  $A_{\text{sat}}$  and leaf  $R$  emerged among the growth treatments when measurements were made in the afternoon (Fig. 3).

Results of our 12-month study indicate that nighttime leaf  $R$  at prevailing evening temperatures were not

**Table 1** *P*-values, whole-model error mean squares (MS) and goodness of fit from a three-way full factorial ANOVA using CO<sub>2</sub> treatment (CO<sub>2</sub>), water treatment (H<sub>2</sub>O) and month as the main factors, including degrees of freedom for the following variables: dark respiration in the morning (R-AM) and in the afternoon (R-PM), saturated photosynthesis measured in the afternoon (A<sub>sat</sub>-PM), modelled respiration rates at 20 °C (R<sub>20</sub>) according to Eqn. (3) and leaf traits of mass-based total soluble sugars (sugars), starch, total nonstructural carbohydrates (TNC) and leaf mass per area ratio (LMA), mass-based phosphorus concentrations (P<sub>mass</sub>) and fresh leaf mass per area (FMA)

Source	df	Daytime measurements			Modelled respiration			Mass-based leaf traits				
		A <sub>sat</sub> -PM	R-AM	R-PM	R <sub>20</sub>	Sugars	Starch	TNC	LMA	P <sub>mass</sub>	FMA	
Month	4	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.001	0.002	0.001	-	-	-
CO <sub>2</sub>	1	0.004	0.001	0.001	0.015	0.057	0.073	0.038	0.002	-	-	0.01
H <sub>2</sub> O	1	0.027	-	-	0.078	-	-	-	-	-	-	<0.001
Month × CO <sub>2</sub>	4	-	-	-	-	0.075	-	-	-	-	-	-
Month × H <sub>2</sub> O	4	-	-	0.024	-	-	-	-	-	-	-	-
CO <sub>2</sub> × H <sub>2</sub> O	1	-	-	-	-	-	0.014	0.084	-	-	-	-
Month × CO <sub>2</sub> × H <sub>2</sub> O	4	-	-	-	-	-	-	-	-	-	-	-
Sample size		47	60	59	60	60	60	60	60	60	60	47
Whole model R <sup>2</sup>		0.67	0.67	0.80	0.63	0.61	0.47	0.45	0.50	0.47	0.47	0.33

A<sub>sat</sub>-AM and N<sub>mass</sub> did not show any *P*-values <0.1 and are therefore omitted from this table. Dashes (-) represent nonsignificant values (*P* > 0.1), df indicate the degrees of freedom. See Tables 2 and 3 and Figure 3 for the mean values of data used for the analysis.

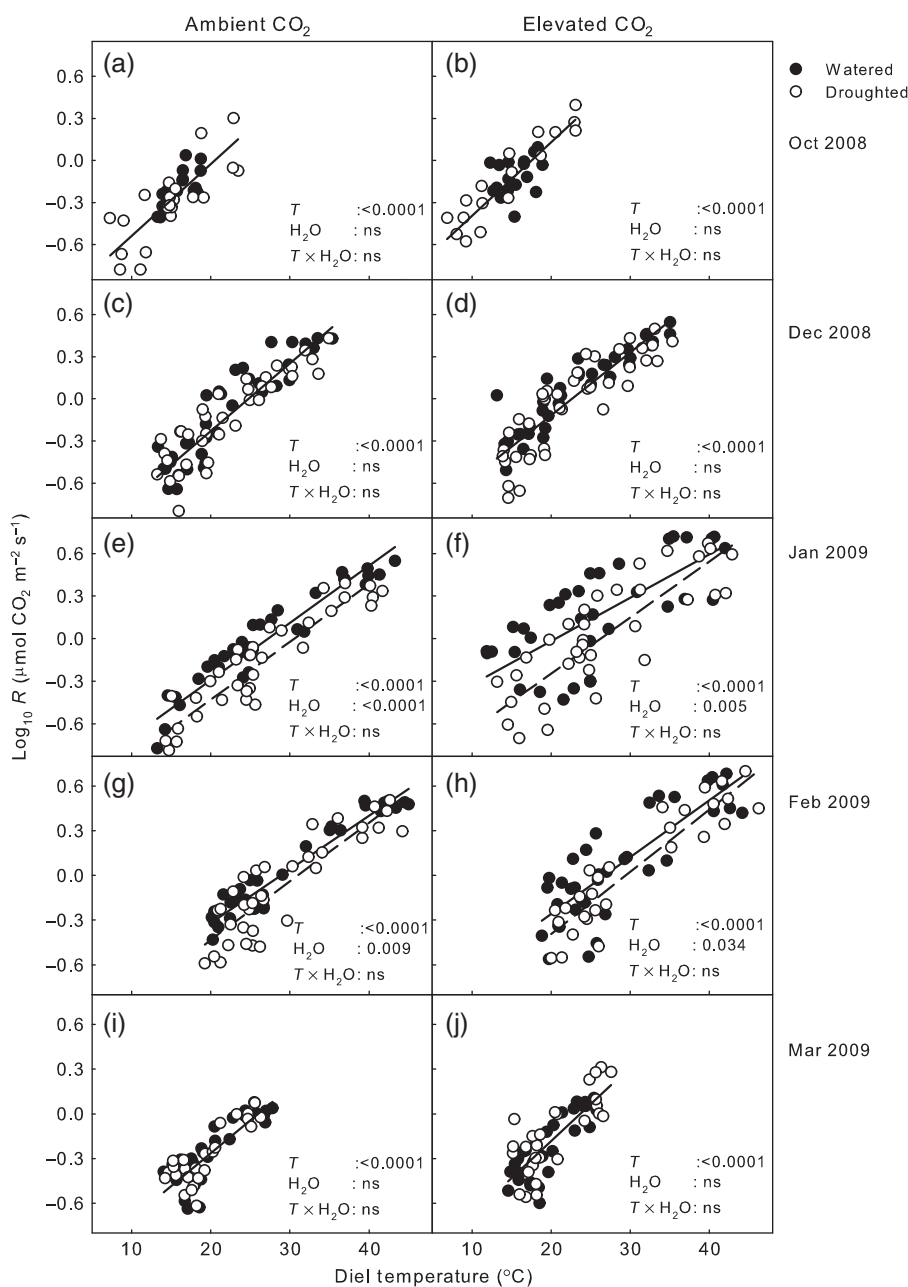


**Fig. 4** Mean nighttime dark respiration measurements from March 2008 to February 2009 for each treatment in well-watered plots (a). Ambient CO<sub>2</sub> treatment is represented by open symbols and dashed lines whereas the elevated CO<sub>2</sub> treatment is represented by closed symbols and solid lines (*n* = 3 ± SE). Near identical results were observed in droughted trees (data not shown). Average leaf temperatures during respiration measurements are shown in (b).

significantly different among the four [CO<sub>2</sub>] and drought treatment combinations, and nighttime leaf *R* varied little throughout the year (Fig. 4a). All early evening measurements were made at relatively low prevailing temperatures compared with the corresponding daily temperatures for any given season, with 25 °C the highest night temperature measured during the hottest summer month (February – when daytime temperatures exceeded 40 °C) (Figs 1a and 4b). Overall, the annual average evening rate of leaf *R* was  $0.62 \pm 0.02 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  (Fig. 4a).

#### *Diel temperature response curves over the 5-month period*

To gain further insights into the impacts of each treatment on diel temperature response curves of leaf *R*, we constructed plots of log<sub>10</sub> *R* vs. temperature for each treatment combination and month (Fig. 5). Acclimation was assessed in two ways: (1) comparing the elevation and slope of diel log<sub>10</sub> *R* vs. temperature plots of different seasons (Fig. 5) and (2) comparing month-to-month variations in rates of leaf *R*<sub>20</sub> (Table 2). Figure 5 shows that irrespective of [CO<sub>2</sub>] treatment or water availability, strong seasonal acclimation occurred, as evidenced by the substantive downward shift in elevation of the log *R*-*T* relationships over the spring-summer period.



**Fig. 5** Leaf  $R$  measured in darkness ( $\log_{10}$ -transformed) as a function of diel variations in temperature ( $^{\circ}\text{C}$ ) for trees grown under ambient  $[\text{CO}_2]$  (left panels) and elevated  $[\text{CO}_2]$  (right panels) for well-watered and drought-treated trees over the 5-month core period. First-order linear regression lines are shown for each treatment when treatment effects were significant at  $P < 0.05$  (solid line, well-watered; short-dashed line, drought treated). When treatments were not significantly different, a single regression relationship for all data combined is shown. Values are for October 2008:  $\text{Log}_{10} R = 0.05T - 1.05$ ,  $R^2 = 0.63$  in ambient  $\text{CO}_2$  and  $\text{Log}_{10} R = 0.05T - 0.92$ ,  $R^2 = 0.73$  in elevated  $\text{CO}_2$  (a, b), December 2008:  $\text{Log}_{10} R = 0.05T - 1.20$ ,  $R^2 = 0.79$  in ambient  $\text{CO}_2$  and  $\text{Log}_{10} R = 0.05T - 1.02$ ,  $R^2 = 0.76$  in elevated  $\text{CO}_2$  (c, d), January 2009: watered:  $\text{Log}_{10} R = 0.04T - 1.10$ ,  $R^2 = 0.88$ , droughted:  $\text{Log}_{10} R = 0.04T - 1.25$ ,  $R^2 = 0.35$  in ambient  $\text{CO}_2$  (e) and watered:  $\text{Log}_{10} R = 0.031T - 0.63$ ,  $R^2 = 0.50$  and droughted:  $\text{Log}_{10} R = 0.04T - 1.04$ ,  $R^2 = 0.70$  in elevated  $\text{CO}_2$  (f), February 2009: watered:  $\text{Log}_{10} R = 0.04T - 1.04$ ,  $R^2 = 0.94$ , droughted:  $\text{Log}_{10} R = 0.04T - 1.22$ ,  $R^2 = 0.78$  in ambient  $\text{CO}_2$  (g) and watered:  $\text{Log}_{10} R = 0.04T - 1.02$ ,  $R^2 = 0.69$  and droughted:  $\text{Log}_{10} R = 0.04T - 1.23$ ,  $R^2 = 0.85$  in elevated  $\text{CO}_2$  (h) and March 2009:  $\text{Log}_{10} R = 0.04T - 1.15$ ,  $R^2 = 0.71$  in ambient  $\text{CO}_2$  and  $\text{Log}_{10} R = 0.05T - 1.18$ ,  $R^2 = 0.65$  in elevated  $\text{CO}_2$  (i, j). Drought was most evident in January and February (Figs 2 and 3), with trees having been rewatered in March.



**Table 2** Short-term temperature sensitivity ( $Q_{10}$ , proportional change in  $R$  per 10 °C rise in temperature) and modelled rates of leaf respiration at a common measuring temperature 20 °C ( $R_{20}$ ) for each month and treatment ( $n = 3 \pm \text{SE}$ )

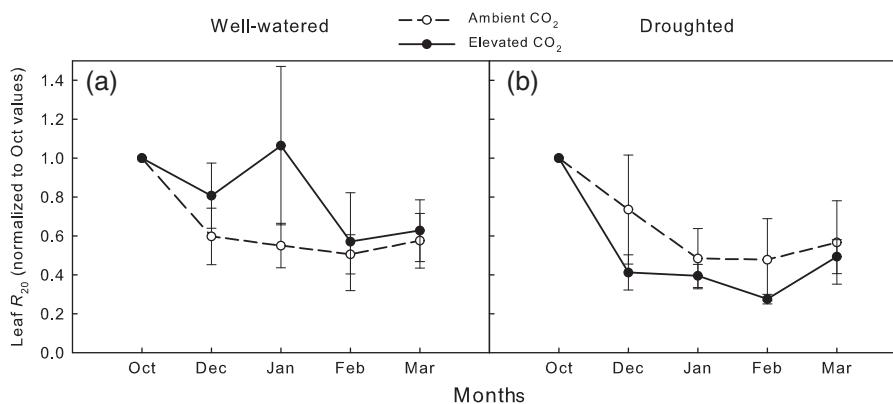
Month	Treatment	Leaf temperature range (°C)	$Q_{10}$	Modelled $R_{20}$	$E/A R_{20}$
October	Ambient	7.2–23.5	$3.23 \pm 0.25$	$0.96 \pm 0.12$	1.32
	Elevated	6.8–23.1	$3.00 \pm 0.41$	$1.27 \pm 0.12$	
December	Ambient	13.7–35.4	$3.06 \pm 0.31$	$0.57 \pm 0.08$	1.26
	Elevated	13.1–35.1	$2.66 \pm 0.30$	$0.73 \pm 0.11$	
January	Ambient-drought	13.2–43.3	$2.57 \pm 0.03$	$0.38 \pm 0.05$	Drought: 1.54
	Elevated-drought	11.8–41.9	$2.72 \pm 0.25$	$0.58 \pm 0.12$	
	Ambient-watered	14.3–41.7	$2.56 \pm 0.18$	$0.52 \pm 0.06$	Watered: 2.07
	Elevated-watered	13.2–42.9	$2.10 \pm 0.15$	$1.07 \pm 0.34$	
February	Ambient-drought	20.1–45.0	$2.51 \pm 0.12$	$0.38 \pm 0.09$	Drought: 1.08
	Elevated-drought	19.5–44.1	$2.65 \pm 0.05$	$0.41 \pm 0.07$	
	Ambient-watered	19.2–44.2	$2.32 \pm 0.03$	$0.47 \pm 0.04$	Watered: 1.16
	Elevated-watered	20.0–46.2	$2.62 \pm 0.37$	$0.55 \pm 0.17$	
March	Ambient-drought	14.6–26.3	$3.08 \pm 0.07$	$0.55 \pm 0.07$	Drought: 1.28
	Elevated-drought	15.3–27.6	$3.01 \pm 0.15$	$0.70 \pm 0.10$	
	Ambient-watered	15.6–27.8	$3.13 \pm 0.40$	$0.54 \pm 0.07$	Watered: 1.19
	Elevated-watered	14.8–25.8	$3.34 \pm 0.15$	$0.64 \pm 0.09$	

$Q_{10}$  and  $R_{20}$  values were calculated from plots of  $\log_{10} R$  versus temperature (Fig. 5) for individual leaves [see Eqns (1)–(3) in the Materials and methods]. Because of the absence of a drought treatment in October and December 2008, both water treatments (drought or watered) were pooled within a  $\text{CO}_2$  treatment (Ambient or Elevated). The leaf temperature range (°C) represents the range of temperatures during which the diel dark respiration ( $R$ ) data were collected and over which  $Q_{10}$  values were calculated.  $E/A$  represent the ratio of mean  $R_{20}$  values in elevated  $\text{CO}_2$  over mean ambient  $\text{CO}_2$  within each month for each water treatment.

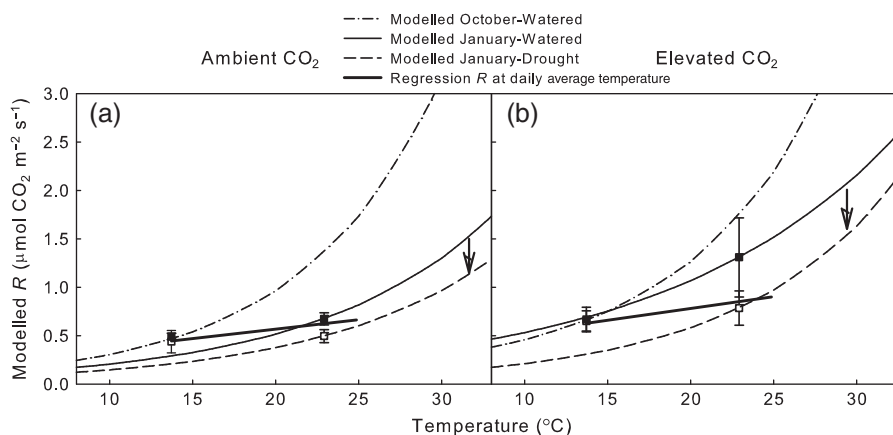
No significant differences in slope or intercept of the  $\log R$ – $T$  relationships were found between well-watered and drought-treated trees in the 2 months before the full onset of the drought treatment (October and December 2008), or in the month after rewatering (March 2009), irrespective of growth [ $\text{CO}_2$ ]. However, in January and February 2009, significant differences were found between well-watered and drought-treated trees ( $P < 0.05$  for each month); these drought effects were not due to a change in the slope of the  $\log_{10} R$ –temperature plots, as shown by the lack of a significant temperature–water treatment interaction ( $T \times \text{H}_2\text{O}$  in Fig. 5). Hence, there was no significant difference in the  $Q_{10}$  of leaf  $R$  between well-watered and droughted trees over the comparable diel temperature range (Table 2). Rather, the drought effect reflected a decrease in the  $y$ -axis intercept or elevation of the temperature response function (i.e. a decline in the basal rate of leaf  $R$ ,  $P < 0.0001$ ). Moreover, because drought resulted in reduced rates of  $R$  at temperatures experienced in the afternoon (Figs 3b and 5e–h), higher temperatures were needed before drought-treated trees exhibited the same rates of  $R$  as their well-watered counterparts. Thus, contrary to our working hypothesis that leaf  $R$  would be drought insensitive in the selected evergreen species, development of drought does result in lower rates of leaf  $R$  compared with well-watered trees.

To further assess whether elevated [ $\text{CO}_2$ ] affected rates of leaf  $R$ , we used the parameters (i.e.  $Q_{10}$  and  $R_0$ ) from the diel temperature response curves to calculate rates of leaf  $R$  at 20 °C (i.e. leaf  $R_{20}$ ) for each individual leaf (Table 2). In all months, rates of leaf  $R_{20}$  were higher under elevated [ $\text{CO}_2$ ] compared with ambient [ $\text{CO}_2$ ] ( $P = 0.015$ , Table 1), irrespective of water supply. Averaged across all months, rates of  $R_{20}$  were 35% higher under elevated [ $\text{CO}_2$ ]. These findings support the results of the morning and afternoon gas exchange measurements, where rates of leaf  $R$  at ambient temperatures were consistently higher in trees grown under elevated [ $\text{CO}_2$ ] (Fig. 3a and b). Moreover,  $R_{20}$  declined as summer progressed with the lowest rates in February (Table 2). Compared with spring (October), average rates of  $R_{20}$  were 42% lower in December and 59% lower in February across  $\text{CO}_2$  treatments (Fig. 6). A three-way ANOVA of the normalized values shown in Fig. 6 (using arcsine square root transformed values), revealed significant differences compared with October values ( $P < 0.001$ ) and water treatments ( $P = 0.045$ ) but not between [ $\text{CO}_2$ ] treatments. Thus, all treatments exhibited substantive seasonal acclimation and elevated [ $\text{CO}_2$ ] did not alter the degree of seasonal acclimation.

To assess whether acclimation resulted in thermal homeostasis of leaf  $R$  (i.e. spring and summer accli-



**Fig. 6** Seasonal pattern in normalized leaf  $R$  at  $20\text{ }^{\circ}\text{C}$  (leaf  $R_{20}$ ) for well-watered (a) and droughted (b) trees. Values are normalized to the October estimate of leaf  $R_{20}$  (Table 2), which was arbitrarily set to 1.0 ( $n = 3 \pm \text{SE}$ ).



**Fig. 7** Curves of modelled leaf dark respiration in ambient (a) and elevated (b)  $\text{CO}_2$  as a function of diel temperature in the spring (October, dot-dash line) and summer (January, solid line for well-watered and dashed line for drought), with symbols indicating respiration calculated at the average daily temperatures of the 7-day period before each measurement campaign (drought, open symbols; watered, closed symbols). Curves were generated on the basis of measured diel temperature responses of leaf  $R$  in respective months, shifting downward as prevailing daily temperatures increase, modelled using Eqn. (3). Symbol values are computed using Eqn. (1) and individual  $Q_{10}$  and  $R_0$  values for each leaf ( $n = 3$  per treatment). Near homeostasis of leaf  $R$  within each  $\text{CO}_2$  treatment is shown by the regression line in each panel; each regression line was fitted through data points across  $\text{H}_2\text{O}$  treatments that represent the rate of leaf  $R$  at the 7-day daily average temperature for each of the 5-monthly campaigns between October and March (note: only data points for October and January are shown in the graph). Regression relationships are in ambient  $\text{CO}_2$ : leaf  $R = 0.020T + 0.17$ ,  $R^2 = 0.53$  and in elevated  $\text{CO}_2$ : leaf  $R = 0.024T + 0.30$ ,  $R^2 = 0.22$ .

mated plants exhibiting similar rates of leaf  $R$ , when rates are measured at the respective average daily growth temperature of each season), we used the regression outputs of  $\log R$  vs. temperature plots (Fig. 5) to calculate rates of leaf  $R$  at each monthly campaign's daily average temperature (symbols in Fig. 7). In the absence of acclimation, rates of leaf  $R$  at the daily average temperature are expected to rise in accordance with the modelled October values shown in Fig. 7. Despite large increases in daily temperature, little variation in calculated rates of leaf  $R$  were found among measurement campaigns (i.e. leaf  $R$  was relatively homeostatic over the 5-month period). This conclusion is supported by data from our 12-month study where

similar nighttime respiration rates were found throughout the year (Fig. 4a) despite large variation in prevailing temperatures (Fig. 4b).

Owing to changes in diurnal temperature regimes over the October–March experimental period, it was not possible to calculate average  $Q_{10}$  values over a common diel temperature range for all months and treatment combinations. Rather, we were limited to estimating  $Q_{10}$  values over the observed temperature range experienced by leaves on each sampling date (Table 2). In October,  $Q_{10}$  values over the 7–23  $^{\circ}\text{C}$  range were about 3.0, with no difference between the two  $[\text{CO}_2]$  treatments (Table 2). By contrast, average  $Q_{10}$  values declined to near 2.5 in January and February when daily

maximum temperatures exceeded 40 °C. Therefore, the monthly variation in  $Q_{10}$  (Table 2) appears to have been the result of changes in prevailing temperatures over which leaf  $R$  was measured. Importantly, there were no  $[\text{CO}_2]$  or water treatment effects on  $Q_{10}$  within any month, suggesting that the impact of intertreatment differences in  $R_{20}$  (Table 2; Fig. 6) were not due to differences in the short-term temperature dependence of  $R$ .

#### *Leaf traits and chemical properties over the 5-month period*

Over the 5-month experimental period during which water supply was withheld, differences were found in the leaf dry mass per unit leaf area (LMA) between leaves grown under ambient and elevated  $[\text{CO}_2]$ , particularly in over-wintered leaves sampled in October 2008 (Table 3). Averaged across all months and watering treatments, LMA was 11% higher in elevated  $[\text{CO}_2]$  grown leaves compared with those grown under ambient  $[\text{CO}_2]$  ( $P = 0.002$ , Table 1). This difference in LMA was mainly due to elevated  $[\text{CO}_2]$  grown leaves exhibiting a higher ratio of leaf fresh mass per unit leaf area (FMA; +14%,  $P = 0.007$ , Table 3) than their ambient  $[\text{CO}_2]$  grown counterparts, indicating that elevated  $[\text{CO}_2]$  leaves were thicker. Growth  $[\text{CO}_2]$  had no significant effect on leaf DMC (Table 3). Neither growth  $[\text{CO}_2]$  nor watering treatment had a significant effect on the concentration of leaf nitrogen expressed per unit dry mass ( $N_{\text{mass}}$ , Table 3). By contrast, leaf phosphorus concentration per unit dry mass ( $P_{\text{mass}}$ , Table 3) did not exhibit any  $[\text{CO}_2]$  treatment differences, but was 27% lower in drought-treated trees vs. well-watered trees ( $P = 0.0002$ , Table 1).

To evaluate potential variation in the supply of respiratory substrates as a driver of seasonal acclimation of leaf  $R$ , we analysed leaves for carbohydrate concentrations (Table 3). While significant month-to-month variations occurred in the concentration of soluble sugars (Table 1), no clear seasonal pattern was evident (Table 3). Moreover, analyses of soluble sugars indicated no differences between drought-treated and well-watered trees, and only marginally significant differences between ambient and elevated  $[\text{CO}_2]$  grown trees (Table 1). By contrast with sugars, distinct seasonal patterns were evident in the concentration of starch in leaf samples, with starch levels being lowest in the summer months (Table 3). No relationship was found between leaf  $R_{20}$  and soluble sugar concentrations (when both were expressed on a mass basis), irrespective of the growth  $[\text{CO}_2]$  or availability of water (Fig. 8b). By contrast, there was a significant overall relationship between variations in mass-based leaf  $R_{20}$  and

$N_{\text{mass}}$  ( $P < 0.0001$ ; Fig. 8a), though no significant differences were found between ambient or elevated  $[\text{CO}_2]$  grown trees, or trees that were well-watered or droughted. We conclude, therefore, that seasonal and treatment-dependent variations in leaf  $R$  were unlikely to be associated with concomitant variations in substrate supply or leaf N.

#### **Discussion**

Our study examined the impact of elevated atmospheric  $[\text{CO}_2]$  and summer drought on seasonal shifts in temperature acclimation of leaf  $R$  of an evergreen tree species (*E. saligna*). Although elevated  $[\text{CO}_2]$  increased leaf  $R$  when measured at temperatures experienced during the daytime (Table 2, Fig. 3), no significant effect of elevated  $[\text{CO}_2]$  on the degree of seasonal acclimation was detected (Figs. 5 and 6), rejecting our hypothesis that seasonal shifts in daily temperature response curves of leaf  $R$  would be greater in ambient atmospheric  $[\text{CO}_2]$  than in elevated  $[\text{CO}_2]$ . Moreover, summer drought reduced average rates of leaf  $R$  by 25–40% when measured at high temperatures during the daytime in the peak summer months of January and February (Figs. 3 and 5). As a result, summer drought exacerbated the seasonal shift (i.e. arising from thermal acclimation) in diel temperature response curves of leaf  $R$  (Figs. 5 and 7). Therefore, failure to account for a thermal acclimation response could lead to large overestimates of predicted leaf  $R$  (and hence underestimates of net primary productivity) in ecosystems experiencing summer drought (Ciais *et al.*, 2005; Atkin *et al.*, 2008).

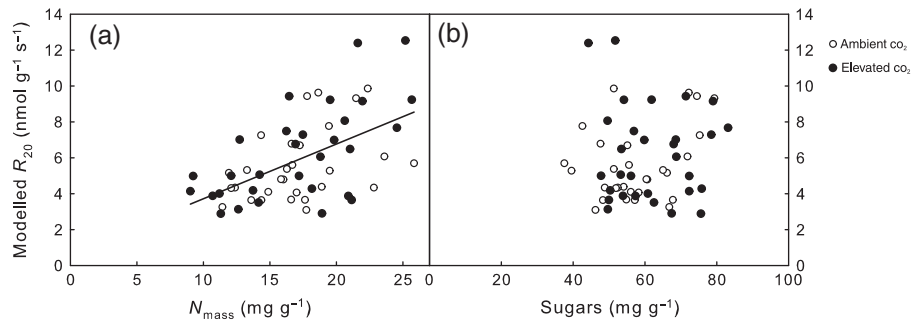
#### *Leaf R under elevated atmospheric CO<sub>2</sub>*

Nighttime measurements over an entire year under prevailing growth conditions indicated that elevated  $[\text{CO}_2]$  did not significantly affect leaf  $R$  at the cool temperatures experienced 2–3 h after sunset (typically below 20 °C; Fig. 3). Similarly, Tissue *et al.* (2002) found in *Liquidambar styraciflua* grown in FACE that nighttime leaf  $R$  was unaffected by elevated  $[\text{CO}_2]$ . However, when respiratory  $\text{CO}_2$  release was measured over a wide range of diel temperatures, we found that growth under elevated  $[\text{CO}_2]$  resulted in 30–40% higher leaf  $R$  in *E. saligna* compared with ambient  $[\text{CO}_2]$  grown trees. Consistent with these results, leaf  $R$  at 25 °C was stimulated by 37% in a fast-growing soybean grown in elevated  $[\text{CO}_2]$  in FACE (Leakey *et al.*, 2009). Stimulation of specific respiration rates at 25 °C in elevated  $[\text{CO}_2]$  (+11%) was also found by Davey *et al.* (2004) in *Phaseolus vulgaris*. Thus, while growth under elevated  $[\text{CO}_2]$  does not necessarily alter nighttime leaf  $R$  (when measured at the cooler nighttime temperatures), signif-

**Table 3** Means of leaf dry matter content (DMC, g dry mass g fresh mass<sup>-1</sup>), fresh leaf mass per area (FMA, g fresh mass m<sup>-2</sup>), leaf mass per area ratio (LMA, g dry mass m<sup>-2</sup>), mass-based N and P concentrations ( $N_{\text{mass}}$  and  $P_{\text{mass}}$ , mg g<sup>-1</sup>) and mass-based concentrations for soluble sugars, starch and nonstructural carbohydrates (TNC) for ambient and elevated CO<sub>2</sub> treatments in each month ( $n = 3 \pm \text{SE}$ )

CO <sub>2</sub> treatment	H <sub>2</sub> O treatment	Month	DMC	FMA	LMA (g m <sup>-2</sup> )	$N_{\text{mass}}$ (mg g <sup>-1</sup> )	$P_{\text{mass}}$ (mg g <sup>-1</sup> )	Soluble sugars (mg g <sup>-1</sup> )	Starch (mg g <sup>-1</sup> )	TNC (mg g <sup>-1</sup> )
Ambient CO <sub>2</sub>	Drought	October	–	–	124.5 ± 10.1	17.4 ± 0.1	1.3 ± 0.1	68.6 ± 8.7	39.3 ± 12.1	108.0 ± 18.1
		December	0.48 ± 0.01	224.1 ± 32.7	106.1 ± 14.0	17.6 ± 3.2	1.8 ± 0.2	57.4 ± 4.8	33.1 ± 10.9	91.1 ± 14.7
		January	0.39 ± 0.01	226.2 ± 10.9	89.7 ± 6.0	16.9 ± 0.5	1.3 ± 0.1	62.2 ± 2.8	7.2 ± 0.9	69.4 ± 2.1
		February	0.46 ± 0.02	209.4 ± 9.6	89.4 ± 5.5	17.0 ± 0.4	1.2 ± 0.2	52.2 ± 3.0	20.4 ± 2.0	62.1 ± 5.0
Ambient CO <sub>2</sub>	Well-watered	March	0.47 ± 0.01	243.3 ± 16.0	110.5 ± 10.8	12.5 ± 0.4	1.3 ± 0.2	63.7 ± 1.9	45.0 ± 6.1	108.7 ± 4.4
		October	–	–	120.4 ± 11.8	20.0 ± 0.3	2.0 ± 0.3	72.89 ± 0.8	45.6 ± 10.5	118.5 ± 11.3
		December	0.45 ± 0.004	251.3 ± 51.7	114.1 ± 23.7	17.3 ± 3.0	1.9 ± 0.2	49.6 ± 1.4	48.1 ± 18.0	97.7 ± 17.7
		January	0.40 ± 0.01	249.2 ± 16.9	99.0 ± 7.2	19.2 ± 3.4	2.1 ± 0.3	49.9 ± 6.2	27.1 ± 19.4	77.0 ± 22.7
Elevated CO <sub>2</sub>	Drought	February	0.45 ± 0.01	231.2 ± 7.9	104.4 ± 4.9	16.3 ± 1.7	1.8 ± 0.4	49.4 ± 6.0	29.7 ± 19.0	79.1 ± 24.8
		March	0.46 ± 0.01	223.6 ± 14.6	103.9 ± 8.7	15.5 ± 2.1	2.0 ± 0.5	50.2 ± 4.0	61.5 ± 14.3	111.7 ± 17.7
		October	–	–	168.4 ± 9.2	18.6 ± 0.2	1.4 ± 0.1	76.3 ± 2.4	66.6 ± 15.4	142.9 ± 14.0
		December	0.45 ± 0.03	287.8 ± 36.4	152.5 ± 28.3	15.3 ± 2.9	1.4 ± 0.3	49.1 ± 0.7	75.0 ± 24.0	124.1 ± 24.5
Elevated CO <sub>2</sub>	Well-watered	January	0.38 ± 0.01	269.4 ± 15.5	102.2 ± 2.4	18.3 ± 2.1	1.5 ± 0.3	58.6 ± 2.7	29.2 ± 12.3	87.8 ± 15.0
		February	0.45 ± 0.02	243.1 ± 20.6	108.9 ± 7.3	16.8 ± 2.8	1.1 ± 0.1	69.6 ± 6.1	40.3 ± 19.5	109.8 ± 25.2
		March	0.47 ± 0.03	282.1 ± 6.0	132.0 ± 6.4	15.0 ± 1.0	1.1 ± 0.1	57.2 ± 5.6	112.4 ± 27.6	169.6 ± 28.0
		October	–	–	165.3 ± 3.9	18.2 ± 0.3	1.6 ± 0.3	69.3 ± 7.8	62.4 ± 31.5	131.7 ± 31.5
Elevated CO <sub>2</sub>	Well-watered	December	0.43 ± 0.02	279.6 ± 33.0	122.3 ± 21.3	18.6 ± 4.3	1.9 ± 0.2	56.6 ± 2.6	31.0 ± 17.9	87.7 ± 16.8
		January	0.38 ± 0.01	275.0 ± 21.1	107.4 ± 5.7	19.3 ± 4.2	2.0 ± 0.03	52.3 ± 4.7	22.0 ± 14.5	74.2 ± 18.9
		February	0.44 ± 0.02	244.8 ± 24.4	109.6 ± 16.1	14.8 ± 2.9	1.3 ± 0.1	65.6 ± 4.5	33.0 ± 13.6	98.6 ± 18.1
		March	0.45 ± 0.01	239.2 ± 12.9	107.5 ± 6.4	16.1 ± 3.6	1.8 ± 0.4	63.5 ± 7.1	53.0 ± 33.4	116.5 ± 39.0

As fresh mass values were not measured in October 2008, it was not possible to calculate DMC or FMA values for that month.



**Fig. 8** Modelled rates of leaf respiration at a measuring temperature of 20 °C ( $R_{20}$ ,  $\text{nmol CO}_2\text{g}^{-1}\text{s}^{-1}$ ) across data collected during the core 5-month study plotted against leaf nitrogen concentration ( $N_{\text{mass}}$ ;  $\text{mg g}^{-1}$ ) (a), and against soluble sugar concentration ( $\text{mg g}^{-1}$ ) (b). Open symbols represent ambient  $[\text{CO}_2]$  leaves and closed symbols are leaves measured in elevated  $[\text{CO}_2]$ . The solid line in (a) shows the significant overall relationship between leaf  $R_{20}$  and  $N_{\text{mass}}$ , with no difference between ambient and elevated  $[\text{CO}_2]$  grown plants (overall regression: leaf  $R_{20} = 0.304 N_{\text{mass}} + 0.675$ ,  $R^2 = 0.29$ ,  $P < 0.0001$ ). (b) No significant relationship was found between  $R_{20}$  and sugar concentration, irrespective of the growth  $[\text{CO}_2]$ .

icant increases in leaf  $R$  are more likely resolved when measured at the higher temperatures experienced during the daytime.

Why were rates of area-based leaf  $R$  30–40% higher in trees grown under elevated  $[\text{CO}_2]$ ? The ratio of leaf mass to leaf area (LMA) was greater in trees grown under elevated  $[\text{CO}_2]$ , as is commonly observed (Poorter *et al.*, 2009) and often associated with higher area-based rates of  $A_{\text{sat}}$ , area-based leaf  $[\text{N}]$  and leaf  $R$  due to increased density of metabolic tissue (Ryan, 1995; Tissue *et al.*, 2002; Wright *et al.*, 2004; Reich *et al.*, 2006; Tjoelker *et al.*, 2008). Although leaf  $\text{N}$  concentration was not lower in elevated  $[\text{CO}_2]$  (Table 3) possibly due to the lower canopy position of the sampled leaves (Crous *et al.*, 2008), LMA and area-based leaf  $[\text{N}]$  were indeed higher in trees grown under elevated  $[\text{CO}_2]$ . However, because the effect of elevated  $[\text{CO}_2]$  on LMA (+11%) was considerably less than its effect on daytime measured rates of area-based leaf  $R$  (+34%; Fig. 3), rates of leaf  $R$  remained significantly higher under elevated  $[\text{CO}_2]$  even when expressed on a leaf mass basis. Thus, factors other than LMA such as increased substrate supply, energy demand and/or respiratory capacity were likely responsible for higher rates of leaf  $R$  per unit leaf area in elevated  $[\text{CO}_2]$ .

The lack of  $[\text{CO}_2]$ -mediated changes in sugar concentrations (Tables 1 and 3), and absence of any significant correlation between leaf  $R$  and soluble sugars (Fig. 8b) strongly suggest that increased substrate supply was not responsible for the higher rates of leaf  $R$  in elevated  $[\text{CO}_2]$  grown trees. Given the four- to eight-fold higher soluble sugar concentrations in our study compared with the leaf  $R$ -limiting concentration in *Eucalyptus* (<10  $\text{mg g}^{-1}$  in Atkin *et al.*, 2000) (Table 3, Fig. 8b), sugar concentrations were not limiting leaf  $R$  in either ambient or elevated  $[\text{CO}_2]$  grown trees. If correct, it

might explain the observed similar degrees of thermal acclimation in trees grown under ambient and elevated  $[\text{CO}_2]$  (Fig. 6). Alternatively, elevated  $[\text{CO}_2]$  trees may alter energy demand and/or respiratory capacity required for sucrose synthesis, phloem loading and protein turnover (Bouma *et al.*, 1994; Hoefnagel *et al.*, 1998). As protein turnover accounts for 20% of the energy produced by leaf  $R$  (Bouma *et al.*, 1994), increased protein content could explain the higher daytime rates of leaf  $R$  in trees grown under elevated  $[\text{CO}_2]$ . However, growth  $[\text{CO}_2]$  had no effect on  $N_{\text{mass}}$  of *E. saligna* leaves (Table 3) or relationships between leaf  $R$  and  $N_{\text{mass}}$  (Fig. 8a), suggesting that increased energy demand associated with higher rates of protein turnover were not responsible.

What seems more likely are increased energy demands and respiratory capacity associated with the  $\approx 40\%$  higher rates of  $A_{\text{sat}}$  exhibited by elevated  $[\text{CO}_2]$  trees in the afternoon (Fig. 3d) due to a tight coupling between  $R$  and  $A_{\text{sat}}$  (Gifford, 1995; Loveys *et al.*, 2003; Whitehead *et al.*, 2004; Noguchi & Yoshida, 2008). Higher rates of photosynthesis would increase flux through the sucrose synthesis pathway, thus increasing the demand for respiration energy (Krömer, 1995; Hoefnagel *et al.*, 1998), while also increasing energy requirements associated with phloem loading, which consumes up to 30% of respiratory ATP in starch-storing species (Bouma *et al.*, 1995). These higher ATP requirements could be met via increased respiratory capacity, mediated, in part, by the higher mitochondrial density found in plants grown under elevated  $[\text{CO}_2]$  (Griffin *et al.*, 2001). Although respiratory capacity was not measured, it seems likely that the higher rates of daytime measured leaf  $R$  in elevated  $[\text{CO}_2]$  were mostly associated with higher energy demand and respiratory capacity from increased photosynthesis rates. The lack

of substantive  $[\text{CO}_2]$ -mediated changes in the concentration of soluble sugars (Tables 1 and 3) might have reflected increased rates of sugar synthesis matched by increased rates of export, storage and/or consumption by mitochondrial respiration.

#### *Drought-mediated declines in leaf R*

Irrespective of whether trees were grown under ambient or elevated  $[\text{CO}_2]$ , the onset of summer drought resulted in reduced rates of leaf  $R$  during the daytime (measured at a common temperature; Figs. 3, 5 and 7). Past studies have suggested that decreased rates of leaf  $R$  under drought are likely due to a combination of decreased substrate availability (Lawlor & Fock, 1977) and/or increased adenylate restriction due to decreased ATP demand (Atkin & Macherel, 2009). In our study, concentrations of soluble sugars were not significantly affected by drought (Tables 1 and 3), suggesting that drought-induced declines in respiratory substrate supply were not responsible for lower daytime measured leaf  $R$ . Rather, decreases in the demand for respiratory products (e.g. ATP, NADH or TCA cycle intermediates) were most likely responsible (Atkin & Macherel, 2009). As drought was associated with reduced afternoon-measured  $A_{\text{sat}}$  (Fig. 3d), the demand for respiratory ATP for processes such as sucrose synthesis/phloem loading was probably reduced. Respiratory capacity may also have declined, as the intercept of log  $R$ -temperature plots was lower in droughted trees in peak summer (January and February; Fig. 5). Importantly, the proportional declines in  $R$  under drought were not as great as the proportional declines in  $A_{\text{sat}}$ , particularly when measured in the afternoon on hot, dry days in January 2009 (Fig. 3). However, given the constancy of soluble sugars concentrations among the treatments and the increased starch levels in droughted trees in elevated  $\text{CO}_2$  (Table 3), there appeared to be sufficient C to support the respiratory apparatus. Thus, the maintenance of  $R$  could play a crucial role in ensuring the subsequent recovery of net C gain following rewatering (Atkin & Macherel, 2009).

#### *Seasonal acclimation of R: the importance of both temperature and drought*

As is the case with the response of respiration to drought, acclimation to high temperatures is also associated with a decline in leaf  $R$ , resulting in downward shifts in the daily temperature response curve of leaf  $R$  as growth temperatures increase (Atkin & Tjoelker, 2003). Our results show that these seasonal shifts in the  $R$ -temperature curves occurred in all  $[\text{CO}_2]$  and drought treatments (Figs 5 and 7). Moreover, thermal

acclimation resulted in similar rates of leaf  $R$  in spring and summer using the 7-day average daily temperature (linear regression line in Fig. 7), which was consistent with field-grown *E. pauciflora* seedlings (Atkin *et al.*, 2000). Acclimation in preexisting leaves has been found to be near maximal 7 days after changing growth temperature (Atkin *et al.*, 2005). Such shifts could be common in most ecosystems, but they may especially be critical in low-productivity ecosystems where acclimation of  $R$  may help maintain a positive C balance under summer conditions when growth is limited (Zaragoza-Castells *et al.*, 2008). This could be particularly relevant to understand what factors underpin the positive rates of net  $\text{CO}_2$  uptake observed in a wide range of ecosystems experiencing abiotic stress (Valentini *et al.*, 2000; Reichstein *et al.*, 2002; Ciais *et al.*, 2005).

The  $\approx 60\%$  lower rate of  $R$  at  $20^\circ\text{C}$  ( $R_{20}$ ) in January compared with plants of the same treatment in October (Fig. 6) was indicative of the seasonal shift in the daily  $R$ -temperature response. Whereas our results showed little or no changes in the concentration of soluble sugars, significant changes in basal rates of  $R$  (i.e. predicted rates of leaf  $R$  at  $0^\circ\text{C}$ ), were observed via the significant change in  $y$ -axis intercepts in Fig. 5, suggesting that acclimation was associated with changes in respiratory capacity. Changes in respiratory capacity could result from changes in mitochondrial abundance, structure and/or protein composition (Griffin *et al.*, 2001, 2004; Armstrong *et al.*, 2006), which is broadly supported by the mass-based  $R_{20}$ - $N$  correlation in Fig. 8a.

Currently, it is unclear whether responses of leaf  $R$  to seasonal temperature changes under field conditions were a result of thermal acclimation *per se* or whether other abiotic factors (such as drought) contribute to the seasonal shift in  $R$ -temperature curves. Our results strongly suggest that thermal acclimation occurred in *E. saligna* trees, as illustrated both by the seasonal shift in  $R$ -temperature curves in well-watered trees (Figs. 5 and 7), and the seasonal decrease in modelled rates of  $R_{20}$  (Fig. 6), and that even a moderate summer drought further exacerbated the downward shift in daily  $R$ -temperature curves (relative to well-watered trees; Fig. 7). Because this effect was observed in both  $\text{CO}_2$  treatments, we conclude that the effects of  $[\text{CO}_2]$ ,  $\text{H}_2\text{O}$  and month are additive (i.e. no interactions). A similar finding for irradiance and  $\text{H}_2\text{O}$  supply was recently reported by Rodríguez-Calcerrada *et al.* (2010) for deciduous beech trees growing in Spain. Therefore, seasonal shifts in  $R$  could reflect both thermal acclimation and the effects of drought. Alternatively, drought-mediated stomatal closure increases leaf temperature which could drive a similar temperature acclimation response. Simple leaf energy balance models suggest

that the reduction in latent energy loss due to reduced stomatal conductance requires an increase in leaf temperatures in irradiated leaves. For example, following energy balance calculations in Nobel (2009), a 67% reduction in stomatal conductance of  $0.3 \text{ mol m}^{-2} \text{ s}^{-1}$  could increase leaf temperatures by  $1.5^\circ\text{C}$ . Such elevated leaf temperatures have consequences for thermal acclimation processes including freezing tolerance (Barker *et al.*, 2005; Loveys *et al.*, 2006). Given these energy balance arguments, it is possible that *in situ* daytime leaf temperatures would have been  $2\text{--}3^\circ\text{C}$  warmer in the drought-treated trees compared with their well-watered counterparts. If true for *E. saligna*, then the lower rates of *R* exhibited by drought-treated plants may have reflected, in part, a thermal acclimation response to elevated leaf temperatures under drought. Unfortunately, *in situ* leaf temperatures in the experiment were not monitored sufficiently to confirm this hypothesis; hence further work is needed.

## Conclusions

Our study provides insights into the effects of elevated  $[\text{CO}_2]$  and moderate summer drought on seasonal shifts in temperature response curves of leaf *R* in a fast-growing evergreen tree species. Seasonal temperature acclimation of *R* was evident in all treatments reducing leaf *R* when average growth temperatures increased in summer. Elevated atmospheric  $[\text{CO}_2]$  increased rates of leaf *R*, but did not appear to alter the degree of seasonal acclimation. Summer drought was found to exacerbate the seasonal downward shift in temperature response curves of leaf *R* in this evergreen tree species grown under ambient and elevated atmospheric  $[\text{CO}_2]$ . Persistent seasonal acclimation of leaf *R* in elevated  $[\text{CO}_2]$  and drought conditions should be incorporated into process-based models of C cycling (Cox, 2001; Pitman, 2003; Friedlingstein *et al.*, 2006). If general, our findings suggest that the combined effects of seasonal changes in temperature and water availability will need to be accounted for when predicting future rates of net  $\text{CO}_2$  exchange at local, regional and global scales.

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