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Relationships between climate of origin and photosynthetic responses to an episodic heatwave depend on growth CO₂ concentration for *Eucalyptus camaldulensis* var. *camaldulensis*

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Abstract. Stressful episodic weather is likely to affect the C balance of trees as the climate changes, potentially altering survival. However, the role of elevated CO₂ concentration ([CO₂]) in tolerating off-season episodic extremes is not clear. We tested for interactive effects of elevated CO₂ and springtime heat stress on photosynthesis for seven genotypes of *Eucalyptus camaldulensis* Dehnh. var. *camaldulensis*, representing its widespread distribution across south-eastern Australia. We grew clonal material under glasshouse conditions of ambient (aCO₂; 400 parts per million (ppm)) or elevated (eCO₂; 640 ppm) [CO₂], and air temperatures of 25 : 17°C (day : night), and measured the electron transport rate in PSII (ETR), stomatal conductance to water vapour (g_s) and net CO₂ assimilation (A). Measurements were made before, during and after a four-day temperature excursion of 35 : 27°C. ETR and A were ~17% higher for plants grown in eCO₂ than in aCO₂. Photosynthesis remained stable for plants in eCO₂ during the heatwave. Based on the effect size ratio (eCO₂ : aCO₂), g_s and ETR were temporarily affected more by the heatwave than A . A reduction in ETR in eCO₂ was the only lasting effect of the heatwave. There were no significant differences among genotypes. Correlations between photosynthesis and climate of origin differed for plants grown in aCO₂ compared with eCO₂, suggesting potential complex and multiple control points on photosynthesis.

Additional keywords: chlorophyll fluorescence, electron transport, seedling growth, stomatal conductance, temperature stress.

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Introduction

Atmospheric concentrations of CO₂ and tropospheric temperatures continue to rise, a trend that extends back to the 1850s (Intergovernmental Panel on Climate Change 2014). Among the many impacts of anthropogenic climate change, episodic heatwaves can have rapid, costly and long-lasting impacts on ecosystems and their ability to provide food and fibre for society (Meehl *et al.* 2000). Long-term observations and models suggest an increase in the frequency, intensity and duration of heatwaves in North America and Europe (Meehl and Tebaldi 2004). For Australia, climatic change scenarios for the future include increases in annual average temperatures of 0.4–2.0°C by 2030 (relative to 1990) and 1.0–6.0°C by 2070 (Hughes 2003). In addition to the chronic increase in average air temperature, there is a likelihood of increased frequency, intensity and duration of heatwave events for Australia (Cowan *et al.* 2014; Purich *et al.* 2014).

Although there is considerable uncertainty regarding the extent of long-term climate change (e.g. rainfall magnitude and timing, atmospheric warming and tropical cyclone activity),

these changes are likely to have significant impacts on most vegetation types, including native eucalypt forests in Australia. Plant growth may be directly responsive to elevated CO₂ (eCO₂) but often other factors (e.g. temperature, water or nutrient stress) modify the effects of eCO₂ (Gunderson *et al.* 2002; Shaw *et al.* 2002; Zeppel *et al.* 2012). Elevated CO₂ may mitigate some negative climate impacts by reducing water, temperature or nutrient stress, which could lead to increased forest growth (Hughes 2003). In addition to the long-term impacts of elevated CO₂ and an increase in mean growth temperature on plants, it is important to understand how elevated CO₂ may alter physiological responses to short-term, high-impact events such as episodic heatwaves (Gauthier *et al.* 2014). Heatwaves can lead to high-temperature stress within plant cells that impairs electron flow within PSII, caused in part by production of reactive oxygen species that affects the repair processes of PSII (Allakhverdiev and Murata 2004; Allakhverdiev *et al.* 2008). Photosynthetic CO₂ assimilation is sensitive to high-temperature events, largely through effects on Rubisco activation (Feller 2016). Whereas eCO₂ may result

in enhanced tolerance of thermal stress for some species (Shanmugam *et al.* 2013), improved heat stress tolerance in eCO₂ is not universal (Hamilton *et al.* 2008; Bauweraerts *et al.* 2014). With improved information about physiological tolerances to eCO₂ and short-term weather events, we can better model future patterns and processes associated with tree establishment, survival, growth and forest productivity (Asshoff *et al.* 2006; Polley *et al.* 2006).

Exposure to higher atmospheric CO₂ concentrations generally increases the instantaneous rate of CO₂ assimilation in many plant species by increasing CO₂ concentration ([CO₂]) at the site of carboxylation, which enhances carboxylation efficiency and reduces photorespiration for C₃ species (Sharkey 1985; Sage *et al.* 1989; Bowes 1991). In many cases, photosynthetic downregulation can occur, which coordinates photosynthesis with resource availability and growth patterns (Tissue and Oechel 1987; Arp 1991; Roden *et al.* 1999; Hamerlynck *et al.* 2000). Moreover, elevated CO₂ often leads to lower rates of stomatal conductance (g_s) (Medlyn *et al.* 2001), potentially affecting leaf energy budgets during heatwaves. Elevated CO₂ may also affect the relative amount of electron flow to photorespiration (Oberhuber and Edwards 1993), which generally reduces the ratio of electrons transported relative to the amount of CO₂ assimilated (Krall and Edwards 1992).

Given the importance of projected changes in CO₂ and heatwave characteristics for Australian forest health in the future (Hughes 2003; Canadell and Raupach 2008; Allen *et al.* 2010), climate-smart forest management requires a better understanding of the sources of variation in tree responses to increasing CO₂ concentrations and episodic air temperature extremes. Growth in eCO₂ can result in lower photosynthetic efficiency for some *Eucalyptus* species under conditions of high temperatures and irradiance, though the effects are dependent on plant water status (Roden and Ball 1996a, 1996b). Photosynthesis for some species of *Eucalyptus* grown in eCO₂ outside their home range may have a limited capacity for summertime temperature acclimation, which demonstrates the strong linkage with climate of origin (Crous *et al.* 2013). Responses to eCO₂ can also be complicated by interacting factors, such as when warmer temperatures increase the impact of drought (Duan *et al.* 2013). In other cases, positive or protective effects of eCO₂ on photosynthesis may be negated

by warming temperatures (Duan *et al.* 2014; Lewis *et al.* 2015). The deleterious effects of heatwaves on photosynthesis might be even more pronounced if they occur with greater frequency in cooler spring months when physiological mechanisms for coping with heat stress may be downregulated. Elevated CO₂ often does not lead to enhanced photosynthetic performance or survival of heatwave events (Bauweraerts *et al.* 2014; Duan *et al.* 2014). In some cases, photosynthetic and leaf area responses to growth temperature suggest intraspecific variation in the thermal response of some *Eucalyptus* species (Drake *et al.* 2015). These results indicate that there are diverse functional responses to eCO₂, weather, and climate among the eucalypts and neighbouring species of south-east Australia that could be important for their management.

This study was conducted to quantify genotypic variation in photosynthetic light energy processing and stomatal gas exchange for clonal cuttings of a widespread Australian eucalypt *Eucalyptus camaldulensis* Dehnh. var. *camaldulensis*. We grew seven genotypes of *E. camaldulensis* var. *camaldulensis* from locations spanning five degrees latitude between New South Wales (NSW), Victoria and South Australia (Table 1). Plants were grown under well-watered, controlled conditions in ambient or elevated levels of [CO₂] (aCO₂ or eCO₂, respectively) at day and night air temperatures resembling those of the spring months for these locations. Plants were grown for 3 months exposed to these conditions, followed by a 4-day, high-temperature ‘heatwave’ treatment (+10°C above the pretreatment day and night air temperatures) for half of the plants. Our goal was to determine whether growth in eCO₂ affects the sensitivity of the light harvesting processes of PSII and leaf-level photosynthetic gas exchange to a 4-day heatwave, and to determine variation across genotypes collected from locations with a range of climate conditions. We tested the hypotheses that: (1) plants in eCO₂ would have higher rates of photosynthesis (i.e. higher electron transport rates in PSII and greater rates of CO₂ uptake) relative to plants in aCO₂ conditions; (2) plants in eCO₂ would maintain higher rates of photosynthesis during the heatwave than plants in aCO₂; (3) plants grown in eCO₂ would have little or no follow-on effects of the heatwave, compared with plants in aCO₂, based on the assumption that lasting impacts on photosynthesis are alleviated by higher C availability (although we recognise the opposite could occur) and

Table 1. Seed-lot collection data (latitude, longitude and altitude (m)) for *Eucalyptus camaldulensis* var. *camaldulensis* used to establish an association population of ~500 unique genotypes, from which genotypes were selected for the heatwave experiment

For each seed-lot collection location, mean annual precipitation (MAP), mean annual relative humidity (MRH), mean annual air temperature (MAT), mean annual maximum air temperature (MXT), the mean temperature of the warmest month (January; TWM), the mean air temperature of spring months (October–December; TSM), the mean annual minimum air temperature (MMT) and the mean annual temperature range (ATR) were computed from meteorological station data for the years 1971–2010. Vic., Victoria; NSW, New South Wales; SA, South Australia

Seed-lot number	Location	State	Latitude (°S)	Longitude (°E)	Alt (m)	MAP (mm)	MRH (%)	MAT (°C)	MXT (°C)	TWM (°C)	TSM (°C)	MMT (°C)	ATR (°C)
19872	Nyngan	NSW	31.33	147.11	200	481	11.3	19.2	34.2	27.5	22.8	6.6	27.6
20429	Condobolin	NSW	33.06	147.09	180	459	11.3	17.6	33.9	26.1	20.9	5.0	28.9
19904	Yass River	NSW	34.53	149.02	0	675	18.7	14.0	28.4	21.8	16.5	2.6	25.8
19710	Coonawarrar	SA	37.2	140.42	200	646	13.9	14.5	30.0	19.3	15.6	6.0	24.0
20437	Barmah State Forest	Vic.	35.5	145.07	100	403	12.3	16.4	33.0	24.0	19.0	4.8	28.2
20436	Towong	Vic.	36.08	143	260	384	11.9	15.6	32.1	22.6	17.9	5.2	26.9
19912	Ovens Valley	Vic.	36.36	146.47	0	653	15.6	15.0	30.5	22.9	17.4	3.7	26.8

(4) in all these hypothesised relationships, plants grown from cuttings from the different provenances, representing different climates-of-origin (regions of varying temperature, rainfall, humidity and evapotranspiration), would exhibit genotypic variance in their photosynthetic physiology, based on the assumption they have adapted to microclimates at each location. In order to test these hypotheses, we quantified the effects of genotype, eCO_2 , and exposure to the simulated springtime heatwave (and all interactions) on plant photosynthesis in terms of the sensitivity of leaf-level gas exchange (g_s and leaf-level net CO_2 assimilation (A)), and electron transport rate (ETR) within chloroplasts of light-adapted leaves under glasshouse conditions.

Methods

Experimental design

We compared photosynthetic processes including leaf-level gas exchange (g_s and A), as well as light processing in PSII as the ETR of PSII for individuals from seven genotypes of *Eucalyptus camaldulensis* Dehnh. var. *camaldulensis* grown at aCO_2 or eCO_2 . The seven genotypes represent six locations, with one to three individuals per genotype per treatment combination. Measurements were collected after 3 months of growth in CO_2 -controlled glasshouses immediately before the onset of the heatwave. During the heatwave, air temperatures in the glasshouse were increased by $10^\circ C$ for 4 days and on the fourth day of the heatwave, measurements were repeated. Air temperatures were then returned to preheatwave levels, and 4 days later, photosynthetic processes were measured again.

Growth conditions

Plants were grown and experiments were conducted within four adjacent naturally lit temperature-controlled glasshouse bays ($3.0 \times 5.0 \times 3.5 m^3$) at the Hawkesbury Institute for the Environment, Western Sydney University, in Richmond, NSW. Seedlings from seven different genotypes of *E. camaldulensis* var. *camaldulensis* were prepared from clonal hedges by the Commonwealth Scientific and Industrial Research Organisation. Cuttings from the hedges represented half-sib plants originating from provenances representing different geographic and climatic origins. Plants were placed in the greenhouse in the last week of September; experiments occurred in the first full week of December 2013. After reaching an average height of 24.6 cm (± 0.97 s.e.) and a basal diameter of 1.86 mm (± 0.07), genotypes were transplanted into 6.9-L cylindrical pots and grown at the naturally lit glasshouse facilities. Incident PAR (400–700 nm) above the glasshouse averaged $1388 \pm 596 \mu mol m^{-2} s^{-1}$ at 13:00 hours ($n = 81$ days) and the glass attenuated $\sim 20\%$ of the incoming PAR. Plants were 9 weeks old at the time of the preheatwave measurements.

Each pot contained 7.5 kg of coarse textured soil with a pH of 6.5. Soil was supplied by Australian Native Landscape and consisted of eight parts of 4-mm finely crushed sandstone, two parts Greenlife compost (Australia Native Landscapes), and $1.5 kg m^{-3}$ of sulfate of iron to maintain the pH below 6.5. Soil was added to pots (PVC pipes, 15 cm diameter \times 40 cm length), up to 2 cm below the rim of the pot. To ensure that no nutrient limitations occurred, the plants were fertilised every fortnight

with a commercial liquid fertilizer (500 mL Aquasol, at $1.6 g l^{-1}$; 23% N, 4% P, 18% K, 0.05% Zn, 0.06% Cu, 0.013% Mo, 0.15% Mn, 0.06% Fe and 0.011% B; Yates Australia). Plants were watered every other day to the point at which water flowed from drain holes in the bottom of the PVC containers.

Plants were grown in aCO_2 (400 parts per million (ppm)) or eCO_2 (640 ppm) and were randomly assigned to one of two glasshouse bays per CO_2 treatment, within a randomised block design. CO_2 concentrations are achieved by the controlled input of CO_2 gas from pressurised cylinders (food grade CO_2 , AirLiquide) through a solenoid connected to a CO_2 Infra Red Gas Analyzer (IRGA) and monitor and controller (Lambda T, ADC Bioscientific Ltd). Pots were rotated between (monthly) and within (weekly) glasshouse bays to further reduce potential glasshouse bay effects on plant performance. Glasshouse air temperatures (25:17°C, average day:night) and relative humidity (45:60%) were selected to simulate the daily temperature of the 30-year average of Richmond, NSW for the growing season (November–May). Air temperature was individually maintained in each growth bay with a PlantVisor PRO temperature control system (Carel Industries) and monitored using thermocouples. During the 4-day heatwave event, air temperatures in the experimental glasshouse bays were increased by $10^\circ C$ both day and night, after which air temperatures were returned to 25:17°C.

Further details on the glasshouse design and set-up are provided elsewhere (Ghannoum *et al.* 2010; Resco de Dios *et al.* 2016a). No differences in height and diameter occurred between genotypes at experiment initiation (with $P > 0.05$, ANOVA).

Photosynthetic measurements

We quantified the effects of genotype, concentration of CO_2 during growth, exposure to the heatwave, time and all interactions on photosynthetic gas exchange and chloroplast processes as indicators of heat tolerance for plants in spring. We chose to monitor these processes because decreased photosynthesis following a spring heatwave might shift the C balance towards respiration and result in reduced growth and survival. Photosynthetic responses to CO_2 growth concentration for each of the genotypes was characterised using instantaneous or spot measurements at the growth CO_2 concentrations. The photosynthetic gas exchange of leaves was measured with four cross-calibrated portable photosynthesis systems (LI-6400XT, Li-Cor Inc.). Light harvesting by PSII was assessed with integrated leaf chamber fluorometers (Model LI-6400-40, Li-Cor Inc.).

For all photosynthetic measurements, individual leaves (length, 3 cm; width ranging from 5 to 15 mm) were enclosed in the leaf cuvette using the $2 cm^2$ area of the leaf chamber fluorometers. The flow rate was set to $500 \mu mol s^{-1}$, the block temperature was maintained at $25^\circ C$, PAR (400–700 nm) was kept at a light-saturating $1800 \mu mol m^{-2} s^{-1}$ (generated by red and blue LEDs in the leaf chamber fluorometers) and the leaf-to-air vapour pressure deficit was maintained between 1.2 and 1.8 kPa by using desiccant adjustment. Leaf temperatures were recorded with a copper-constantan thermocouple (Type T, Model 6400-04, Li-Cor) pressed to the abaxial surface of the

leaf within the cuvette. Leaf temperatures were $25.4 \pm 1.9^\circ\text{C}$ in the control temperature glasshouse and $37.0 \pm 0.6^\circ\text{C}$ during the heatwave treatment. Photosynthetic measurements were recorded when all stability criteria were met when the coefficient of variation for A and g_s combined was $<0.5\%$, which generally required no longer than 4 min. Leaves were exposed to ambient glasshouse irradiation for at least 2 h before measurements of ETR. The steady-state conditions were confirmed in preliminary experiments. During the heatwave treatment, vapour pressure deficit was 2.0 kPa and other conditions were as described above. Measurements were made between 08:00 and 11:00 hours local time.

Statistical analyses

Data for g_s , A and ETR were computed from the Model LI-6400XT software, and all data were analysed via mixed linear models with genotype, exposure to the heatwave, timing of the measurement (i.e. before, during or after the heatwave), glasshouse growth $[\text{CO}_2]$ and their interactions as fixed factors, and with glasshouse bay nested within CO_2 concentration as random variables. These analyses were performed after examining whether the data conformed to assumptions of homoscedasticity and normality. Analyses were conducted in the R software environment using the base packages and lme4 (Bates et al. 2015).

Results

Effects of elevated CO_2 on photosynthesis and g_s

Growth in $e\text{CO}_2$ affected certain aspects of photosynthesis compared with plants grown in $a\text{CO}_2$ (Fig. 1, Tables 2, 3). The internal partial pressure of CO_2 within leaves (C_i) was 55% higher for leaves of plants grown in $e\text{CO}_2$ than in $a\text{CO}_2$, and the $e\text{CO}_2$ to $a\text{CO}_2$ ratio ($e\text{CO}_2 : a\text{CO}_2$; Fig. 1) was consistently higher than 1.0 before, during and after the 4-day heatwave (Fig. 1, Table 2). Likewise, g_s was 35% lower and A was 16% higher for plants in $e\text{CO}_2$ than in $a\text{CO}_2$ before the heatwave. The ratio of ETR to A (ETR : A) was consistently higher for plants in $a\text{CO}_2$ than in $e\text{CO}_2$ at all times, but the s.e. is very large (Table 2).

Heatwave exposure

We simulated a springtime heatwave with four consecutive days of air temperatures that were 10°C above growth temperatures,

which led to variable changes to photosynthetic light energy processing and stomatal gas exchange (Table 3). Photosynthetic gas exchange and light harvesting differed significantly before,

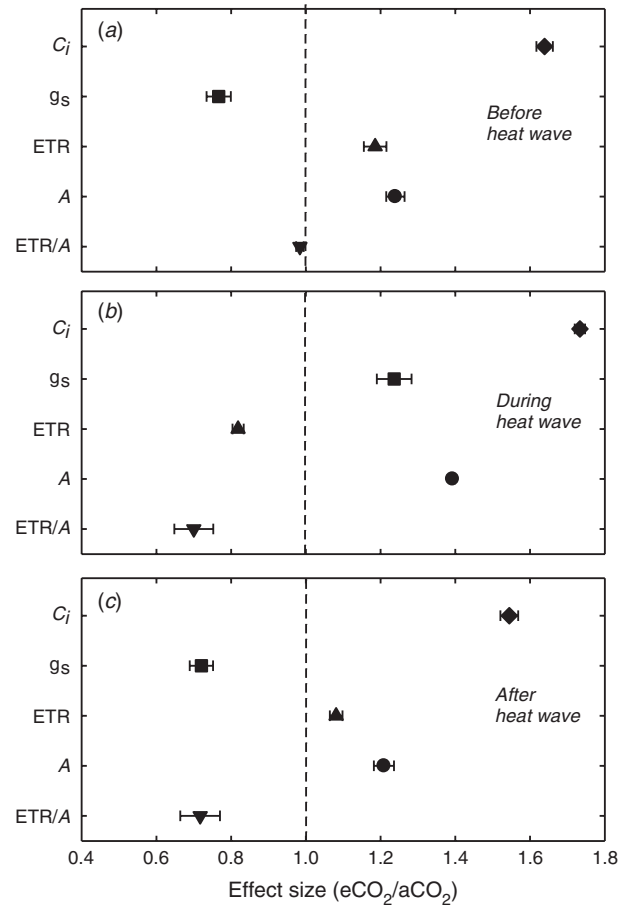


Fig. 1. Mean CO_2 response ratios of elevated CO_2 to ambient CO_2 ($e\text{CO}_2 : a\text{CO}_2$) for internal partial pressure of CO_2 (C_i ; circles), stomatal conductance (g_s ; squares) and electron transport rate (ETR; triangles), and net leaf-level CO_2 assimilation (A ; diamonds), for *Eucalyptus camaldulensis* var. *camaldulensis* grown at $a\text{CO}_2$ or $e\text{CO}_2$ (a) before, (b) during (4 days after treatment onset) and (c) 4 days after the cessation of the 4-day heatwave in the glasshouse (bottom). For each box, data are means ± 1 s.e. for all genotypes combined.

Table 2. Photosynthetic gas exchange and light harvesting for *E. camaldulensis* plants grown at ambient ($a\text{CO}_2$) or elevated CO_2 ($e\text{CO}_2$), measured before, during and after the heatwave

Data are means (s.e.) for all genotypes for internal partial pressure of CO_2 within leaves (C_i) the electron transport rate within PSII at $\text{PAR} = 1800 \mu\text{mol m}^{-2} \text{s}^{-1}$ (ETR), stomatal conductance to water vapour (g_s), photosynthetic net CO_2 assimilation (A) and the ratio of ETR to A (ETR : A). Within a row, values with the same superscript letter are not significantly different, based on Tukey's honestly significant difference. ppm, parts per million

Parameter	Units	Before		During		After	
		400 ppm $a\text{CO}_2$	640 ppm $e\text{CO}_2$	400 ppm $a\text{CO}_2$	640 ppm $e\text{CO}_2$	400 ppm $a\text{CO}_2$	640 ppm $e\text{CO}_2$
C_i	$\mu\text{mol mol}^{-1}$	280 (5) ^a	435 (18) ^b	271 (2) ^a	464 (6) ^b	277 (3) ^a	439 (8) ^b
g_s	$\text{mol m}^{-2} \text{s}^{-1}$	0.646 (0.037) ^a	0.416 (0.024) ^d	0.551 (0.017) ^{ab}	0.522 (0.029) ^{bc}	0.577 (0.017) ^{ab}	0.446 (0.022) ^{cd}
ETR	$\mu\text{mol m}^{-2} \text{s}^{-1}$	174 (2) ^c	204 (8) ^{ab}	222 (10) ^a	187 (9) ^{bc}	195 (8) ^{abc}	170 (4) ^c
A	$\mu\text{mol m}^{-2} \text{s}^{-1}$	31.2 (0.7) ^a	37.2 (1.2) ^b	31.7 (0.6) ^a	38.1 (1.4) ^b	31.0 (0.6) ^a	37.5 (0.844) ^b
ETR : A	Unitless	5.56 (5.79) ^a	5.47 (5.89) ^a	7.00 (7.44) ^a	4.90 (5.33) ^a	6.30 (6.68) ^a	4.52 (4.74) ^a

Table 3. Main effects of *E. camaldulensis* genotype (G), exposure to the heatwave (H), timing of the measurement (i.e. before, during or after the heatwave; T), glasshouse growth concentration ([CO₂] (C), and all interactions on stomatal conductance to water vapour, net CO₂ assimilation, electron transport rate through PSII and leaf internal [CO₂]. Data were analysed using linear mixed models that included G, H, T, C and their interactions as fixed variables, and with glasshouse bay nested within CO₂ concentration as random variables. Text in bold indicates statistically significant differences. df, degrees of freedom

Physiological response	Source	χ^2	df	Pr > χ^2	
Leaf internal [CO ₂] (µmol mol ⁻¹)	G	0.9457	1	0.3308	
	H	1.9338	2	0.1643	
	Time	1.6047	2	0.4483	
	C	73.437	1	2 × 10⁻¹⁶	
	G × H	0.9215	1	0.3371	
	G × T	0.8514	2	0.6533	
	H × T	0.711	2	0.7008	
	G × C	0.095	1	0.7579	
	H × C	0.6868	1	0.4072	
	T × C	4.0684	2	0.1308	
	G × H × T	0.6906	2	0.708	
	G × H × C	0.9877	1	0.3203	
	G × T × C	1.0164	2	0.6016	
	H × T × C	1.4268	2	0.49	
	G × H × T × C	0.6272	2	0.7308	
	Stomatal conductance (mol m ⁻² s ⁻¹)	G	1.9911	1	0.1583
		H	0.3603	1	0.5483
T		1.0204	2	0.6004	
C		1.6241	1	0.2025	
G × H		1.2697	1	0.2598	
G × T		0.8401	2	0.657	
H × T		4.0207	2	0.1339	
G × C		0.3615	1	0.5477	
H × C		2.7272	1	0.0986	
T × C		18.771	2	8.39 × 10⁻⁵	
G × H × T		1.1744	2	0.5559	
G × H × C		0.0118	1	0.9134	
G × T × C		2.4145	2	0.299	
H × T × C		12.0129	2	0.0025	
G × H × T × C		1.8762	2	0.3914	
Electron transport rate (µmol m ⁻² s ⁻¹)		G	0.6351	1	0.4255
		H	21.164	1	4.22 × 10⁻⁶
	T	33.1839	2	6.23 × 10⁻⁸	
	C	1.7939	1	0.1805	
	G × H	0.177	1	0.674	
	G × T	4.4976	2	0.1055	
	H × T	155.51	2	2 × 10⁻¹⁶	
	G × C	1.5119	1	0.2188	
	H × C	5.1191	1	0.0236	
	T × C	44.604	2	2.06 × 10⁻¹⁰	
	G × H × T	1.6509	2	0.4381	
	G × H × C	0.0097	1	0.9216	
	G × T × C	1.0809	2	0.5825	
	H × T × C	31.583	2	1.39 × 10⁻⁷	
	G × H × T × C	1.4443	2	0.4857	
	CO ₂ assimilation (µmol m ⁻² s ⁻¹)	G	0.1283	1	0.7202
		H	0.5593	1	0.4545
T		1.8789	2	0.3908	
C		59.6954	1	1.11 × 10⁻¹⁴	
G × H		1.3738	1	0.2412	
G × T		0.0697	2	0.9657	
H × T		7.0984	2	0.0287	

(continued next column)

Table 3. (continued)

Physiological response	Source	χ^2	df	Pr > χ^2
	G × C	1.8725	1	0.1712
	H × C	10.6252	1	0.0011
	T × C	0.2989	2	0.8612
	G × H × T	3.5029	2	0.1735
	G × H × C	0.2389	1	0.625
	G × T × C	0.3255	2	0.8498
	H × T × C	7.9341	2	0.0189
	G × H × T × C	3.1203	2	0.2101

during, and after the 4-day heatwave. Internal [CO₂] was 16% higher during the heatwave than before it. Notably, g_s was higher during the heatwave than before it for eCO₂ plants. For plants grown in aCO₂, the average ETR increased 27% during the heatwave than before it and then returned to preheatwave levels 4 days after the experimental heatwave ended (Table 2). However, this was not the case for plants grown in eCO₂, for which ETR decreased by 17% after the heatwave. Net CO₂ assimilation by leaves was consistently higher before, during and after the heatwave for plants in eCO₂ than those in aCO₂.

Four days after the heatwave ended, C_i and A were at the same levels as before the heatwave for both aCO₂ and eCO₂ plants. Stomatal conductance values were slightly (but not significantly) different from values before the heatwave. However, the ETR for plants in eCO₂ after the heatwave was significantly lower (by 17%) than those before the heatwave (Table 2).

The time effect is likely to stem from daily variation in ambient irradiance. Overall, there were significant interactions of heatwave exposure and time on ETR and A because the patterns over the course of the heatwave cycle were different between plants grown in aCO₂ and those grown in eCO₂.

Interactive effects of CO₂ and heatwave

There were numerous interactive effects among growth in eCO₂, exposure to the 4-day heatwave treatment and time (i.e. before, during and after the heatwave) on light processing in PSII and photosynthetic gas exchange (Table 3); however, the patterns were not consistent among the different aspects of photosynthesis. There were significant interactions between heatwave exposure and time, as well as between heatwave exposure and growth in eCO₂ for ETR and A . There were significant interactions between growth in eCO₂ and time of the heatwave cycle for g_s and ETR. Most of the parameters exhibited significant interaction effects between growth in eCO₂ with exposure to the heatwave and time of the heatwave cycle (i.e. a significant growth condition × heatwave × time interaction effect for ETR, g_s and A).

Climate of origin meteorology

Mean annual air temperature, mean air temperature of the warmer months (TWM) and mean air temperature of the spring months were negatively correlated with latitude (Table S1, available as Supplementary Material to this paper). Mean annual minimum air temperature and mean relative humidity were both correlated

with elevation. No meteorological variables were correlated with longitude.

Before the onset of the simulated springtime heatwave, *A* was positively correlated with TWM and annual temperature range (ATR) for plants grown in aCO₂ (Table S1, Fig. 2a). Stomatal conductance was negatively correlated with ETR in PSII. After 4 days of the heatwave, ETR was positively correlated with longitude, as well as with *A* before the heatwave. Four days after the heatwave ended, values for *g_s* were positively correlated with the *g_s* measured during the heatwave; similar relationships were observed for *A* during and after the heatwave.

The correlations between meteorology and physiology were different between plants grown in eCO₂ and aCO₂ (Table S1, Fig. 2b). Net photosynthetic CO₂ assimilation measured for plants in eCO₂ before the heatwave was positively correlated with mean annual precipitation and mean relative humidity,

and negatively correlated with mean annual temperature, mean maximum temperature, mean air temperature of the spring months and ATR, but not for plants in aCO₂. Notably, after the heatwave, the relationships for plants in eCO₂ were no longer significant. The ETR measured 4 days after the heatwave was negatively correlated with leaf-level gas exchange during the heatwave, but not after the heatwave. For plants in eCO₂, ETR was correlated with several meteorological variables, but only for values measured during the heatwave (Fig. 2b).

Genotypic variation in photosynthesis

There were no significant effects of genotype on any of the photosynthetic gas exchange or light harvesting processes. Likewise, there were no interactions of genotype with growth CO₂ concentration, heatwave exposure or time.

Discussion

The results of the simulated springtime heatwave experiment show that for plants of *E. camaldulensis* var. *camaldulensis* grown in eCO₂, (i) before the heatwave, *g_s* was lower than for plants in aCO₂, but the difference in *g_s* between aCO₂ and eCO₂ plants was lost during the heatwave; (ii) the only lasting effect of the heatwave was reduced ETR; (iii) there were multiple interactions among eCO₂, the heatwave treatment and time; (iv) correlations between photosynthesis and climate of origin differed for plants grown in aCO₂ and those grown in eCO₂ and (v) there was no variation in photosynthetic responses to the heatwave among genotypes.

Effects of elevated [CO₂] on photosynthesis

Growth of plants in eCO₂ for just over 2 months resulted in 55% higher *C_i*, 17% higher ETR, 20% higher *A* and 35% lower *g_s* than plants grown in aCO₂ (Fig. 1, Table 2). These results are consistent with Hypothesis 1 and previous findings for this and many other species (Tissue et al. 1997; Reddy et al. 2010; Blackman et al. 2016), and probably reflect higher [CO₂] at the carboxylation sites of Rubisco under eCO₂ growth conditions, as well as reduced photorespiration. Because photorespiration is generally higher in aCO₂ than in eCO₂ (Krall and Edwards 1992), the ETR:*A* ratio should be higher in aCO₂. However, for unknown reasons, this was not the case before the heatwave, but was possibly caused by our relatively small sample sizes. On the other hand, Blackman et al. (2016) found that specific leaf area and N content on an area basis were different for *E. camaldulensis* var. *camaldulensis* in aCO₂ vs. eCO₂, which may have affected light absorption by the leaves, or perhaps other optical properties that could affect the computation of ETR.

eCO₂ and heatwave responses

When we simulated a springtime heatwave in the glasshouse by increasing day/night air temperatures by 10°C above preheatwave air temperatures, we saw different responses of *E. camaldulensis* plants in terms of PSII and leaf gas exchange (Fig. 1 and Table 2). The patterns were complicated by growth in eCO₂, as evidenced by multiple significant interactions among [CO₂], heatwave exposure and time for both photosynthetic light processing (ETR) and leaf-level gas exchange (*g_s*, *A*). Four days into the heatwave treatment, the effect size

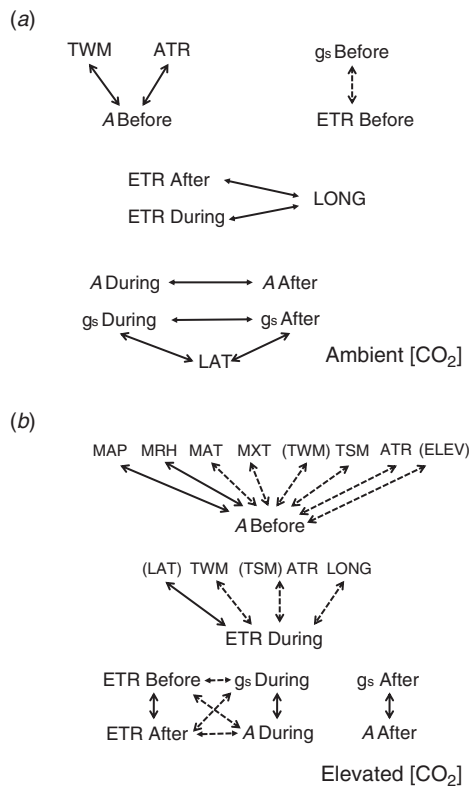


Fig. 2. Significant correlations between meteorological conditions (1971–2010) and photosynthesis for *Eucalyptus camaldulensis* var. *camaldulensis* grown in (a) ambient CO₂ concentration (aCO₂) or (b) elevated CO₂ (eCO₂), measured before, during and after a 4-day simulated springtime heatwave (+10°C above pretreatment day and night air temperatures) in the glasshouse. Positive and negative correlations are shown by solid or dashed arrows, respectively. Items in brackets are marginally significant (0.05 < *P* < 0.10). ATR, mean annual temperature range (°C); ELEV, elevation (M); LAT, latitude (degrees); LONG, longitude (degrees); MAP, mean annual precipitation (mm); MRH, mean annual relative humidity (%); MAT, mean annual air temperature (°C); MXT, mean annual maximum air temperature (°C); TWM, the mean temperature of the warmest month (January, °C); TSM, the mean air temperature of the spring months (October, November and December, °C); MMT, mean annual minimum air temperature (°C).

($e\text{CO}_2 : a\text{CO}_2$) was higher for C_i and g_s than before the heatwave, but A was unchanged and ETR was lower. Some portion of the increase in C_i may have been caused by higher rates of oxidative respiration in the warmer temperatures. ETR during the heatwave was higher in $a\text{CO}_2$ but lower in $e\text{CO}_2$ than before the heatwave, consistent with previous observations that the benefits of $e\text{CO}_2$ may be offset by high-temperature stress for some species (Hamilton *et al.* 2008; Wang *et al.* 2012). Likewise, there was no difference in g_s between $a\text{CO}_2$ and $e\text{CO}_2$ during the heatwave, in contrast to the usual reduced values in $e\text{CO}_2$ (Medlyn *et al.* 2001; Ainsworth *et al.* 2002). These results indicate that photochemistry and C metabolism do not change in parallel in response to the heatwave for plants grown in $e\text{CO}_2$. These results are partially consistent with Hypothesis 2, because only C_i and A were consistently higher in $e\text{CO}_2$ than in $a\text{CO}_2$ for all times of the heatwave. The $[\text{CO}_2]$ -dependent outcome of the heatwave exposure (i.e. 4 days after the cessation of the heatwave treatment in the glasshouse) is consistent with Hypothesis 3, as there appeared to be some recovery of photosynthetic processes. Specifically, C_i , A and g_s were not statistically different before vs. after the heatwave. These results are similar to previous studies of the effects of $e\text{CO}_2$ on photosynthesis for *Eucalyptus* spp. during or following exposure to high temperature events (Roden and Ball 1996a, 1996b), which can be driven, in some cases, by intraspecific variation within species (Drake *et al.* 2015) or by environmental variation across species collection sites (Lewis *et al.* 2011).

The effects of $e\text{CO}_2$ on the heat or high-temperature stress tolerance of plants can act on several sites or processes within cells (Weis and Berry 1988; Murata *et al.* 2007), which may help determine major distribution patterns (Osmond *et al.* 1987; O'Sullivan *et al.* 2017). Changes in A in response to $e\text{CO}_2$ can be related to membrane fatty acid saturation (Gombos *et al.* 1994) or the activity of Rubisco (Law and Crafts-Brandner 1999; Salvucci and Crafts-Brandner 2004). Moreover, episodic high-temperature stress can lead to altered patterns of gene expression, the products of which can affect photosynthesis directly, but may also be involved in cellular detoxification, energy metabolism and protein biosynthesis (Allakhverdiev and Murata 2004; Wang *et al.* 2008; Rollins *et al.* 2013). The photosynthetic tolerance of high temperature stress events is complicated by growth in $e\text{CO}_2$ (Wang *et al.* 2012), and can be dependent upon other resources and conditions such as water availability and N content (Hamerlynck *et al.* 2000; Wang *et al.* 2014). Importantly, the effects of growth in $e\text{CO}_2$ result in enhancements of A during acute heat stress in some species (Shanmugam *et al.* 2013; Sun *et al.* 2013), similar to our observations for *E. camaldulensis* var. *camaldulensis*, yet lead to reductions in A or growth as a consequence of high-temperature stress in other species (Hamilton *et al.* 2008; Wang *et al.* 2008). Photosynthetic sensitivity to thermal stress may result from loss of the light processing capacity within chloroplasts, the effects of reactive oxygen species, impaired protein synthesis or downregulated C metabolism (Nishiyama *et al.* 2006; Allakhverdiev *et al.* 2008). Growth in $e\text{CO}_2$ did not upregulate electron transport or energy dissipation to compensate for downregulated C metabolism in loblolly pine (*Pinus taeda* L.) after several years of exposure (Logan *et al.* 2009). On the other hand, certain *Eucalyptus* species adjust their

energy partitioning between electron transport and dissipation when grown in $e\text{CO}_2$ and $+4^\circ\text{C}$ (Logan *et al.* 2010). Growth in $e\text{CO}_2$ can affect thylakoid composition, which may underlie adjustments in PSII function (Ekman *et al.* 2007). Photosynthesis can also be constrained by structure, such as vein density, which affects sugar export (Adams *et al.* 2007; Adams *et al.* 2013); however, in a prior study vein density was not affected by $e\text{CO}_2$ for *E. camaldulensis* var. *camaldulensis* (Blackman *et al.* 2016).

Photosynthesis and climate of origin

The complex relationships between the meteorological history at the genotypes' climate of origin and A (Table S1 and Fig. 2) are different between plants grown in $a\text{CO}_2$ and those grown in $e\text{CO}_2$. Only TWM and ATR were correlated with A for plants in $a\text{CO}_2$ before the heatwave (Fig. 2a). By contrast, mean annual precipitation and mean relative humidity were positively correlated with A for plants in $e\text{CO}_2$, whereas mean annual temperature, mean maximum temperature and temperature of the spring months were negatively correlated with A for plants in $e\text{CO}_2$ (Fig. 2b). We note that ATR was positively correlated with A under $a\text{CO}_2$ and negatively correlated with A for plants grown in $e\text{CO}_2$. These results indicate that A for *E. camaldulensis* leaves was higher for genotypes from cooler climates of origin under $a\text{CO}_2$, but this relationship was very different in $e\text{CO}_2$. The relationship between TWM and ATR and A before the heatwave was surprising, because higher ATR is usually associated with cooler locations. This may have been caused by having a relatively small number of climates of origin or a limited geographic range. For the widespread species *Eucalyptus grandis* W.Hill and *Eucalyptus tereticornis* Sm., intraspecific variation in A and growth showed that the capacity to cope with 3.5°C warming is higher in cooler sites, suggesting that trees from warmer sites are near a thermal limit (Drake *et al.* 2015). Our results for photosynthetic tolerance of a springtime heatwave are somewhat similar for plants in $a\text{CO}_2$ but not $e\text{CO}_2$. We hypothesise that correlations of A in plants grown in $e\text{CO}_2$ in climatic conditions representative of the year 2100 would yield similar patterns for A in plants grown in $a\text{CO}_2$ and the climate conditions for 1971–2010, which were used in this study.

Growth in $e\text{CO}_2$ resulted in significant correlations between electron transport rate during the heatwave, and TWM and annual temperature range (ATR) that were not significant for plants in $a\text{CO}_2$. Some of the $[\text{CO}_2]$ -dependent differences occurred 4 days into the heatwave and persisted 4 days after cessation of the heatwave. These results suggest that physiological pathways that contribute to the relationships with meteorological variables were set in place in part by growth in $e\text{CO}_2$ before the experimental heatwave and that responses were also influenced during the heatwave by higher C_i under $e\text{CO}_2$. Moreover, at all phases of the experimental heatwave (i.e. before, during and after the 4-day heatwave event in the glasshouse), the relationships between the light harvesting responses of ETR with the leaf-level gas exchange responses of g_s and A were quite different for plants in $e\text{CO}_2$ compared with $a\text{CO}_2$. A shift in correlations was also found for leaf economics, hydraulics and photosynthetic traits in

E. camaldulensis var. *camaldulensis* plants grown in aCO₂ compared with eCO₂ (Blackman *et al.* 2016). These results suggest that some functional components of photosynthesis are differently sensitive to eCO₂, which results in complex responses to an episodic stress. These results also highlight the importance of measuring multiple aspects of complex photosynthetic processes, which can provide more detailed insights into the effects of eCO₂ on plant responses to stress.

Variation in heat stress tolerance

It has been recognised for some time that several species of *Eucalyptus* exhibit morphological and physiological variation across spatial gradients, particularly in terms of temperature stress tolerance (Ashton 1958; Pryor 1959). A comparison of *E. camaldulensis* leaf tolerance of high temperatures from 11 widespread seed source locations showed heat tolerance between 47 and 50°C, but this was not related to the temperature of the hottest month (Karschon and Pinchas 1971). For *Eucalyptus obliqua* L'Hér., short-term heat stress at 40°C did not cause damage to leaves, but 50% of leaves were killed after about 10 min at 60°C (Moore *et al.* 1977). Photosynthesis for *Eucalyptus pauciflora* Sieber ex Spreng. from lower, warmer elevations had a higher temperature optimum and rates of net photosynthesis at the highest growth temperature than plants from higher elevations (Slatyer and Ferrar 1977). Although the magnitude of thermal acclimation was the same for plants from both elevations, the temperature optimum was ~5°C higher for plants from the lower elevation (Slatyer 1977). Comparisons of growth and extreme temperature responses of *E. camaldulensis*, *E. pauciflora* and *Eucalyptus viminalis* Labill. suggest that their distributions are related to tolerance of thermal extremes, rather than their growth rate responses to typical sublethal conditions (Paton 1980). These prior results show that responses to both episodic temperature extremes and conditions that mimic slow, chronic warming are important for photosynthetic physiology of some *Eucalyptus* species, and can help explain their geographic distribution (Drake *et al.* 2015). Previous research has observed genotypic variation in physiological response to eCO₂ within *E. camaldulensis* var. *camaldulensis* (Dillon *et al.* 2015; Blackman *et al.* 2016) but this may not be universal across all tree species (Resco de Dios *et al.* 2016b). Here, we did not detect significant effects of genotype in our overall statistical model. It is possible that we did not include enough genotypes or that we did not sample from locations where genotypic variation is greater, compared with those studies. Our plants were well-watered and fertilised to minimise interactions from drought or nutrient limitations, which may not be the case for plants experiencing an *in situ* heatwave. The degree of evidence for genotypic variation might also depend on the parameter of interest (Aspinwall *et al.* 2014; Resco de Dios *et al.* 2016a).

Conclusions

Our results show that for the widespread Australian tree *E. camaldulensis* var. *camaldulensis* heatwaves increase g_s in eCO₂, ETR was still affected 4 days after the heatwave had ended, and correlations of photosynthetic traits with climate of origin are dependent upon growth [CO₂]. Growth in eCO₂ will

affect some (but not all) high-temperature stress responses for components of photosynthetic electron transport in PSII, leaf-level g_s and photosynthetic CO₂ uptake for this species. Survival and growth of this species may be minimally affected by more frequent off-season springtime heatwaves in Australia (Cowan *et al.* 2014; Purich *et al.* 2014). However, outcomes for fitness and potential changes at the plant community level may be dependent on whether specific aspects of photosynthetic C uptake are affected by a heatwave when plants are grown in eCO₂, as well as interactions with other stresses, such as drought or nutrient limitations.

Conflicts of interest

The authors declare no conflicts of interest.

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