RESEARCH ARTICLE

An extreme heatwave enhanced the xanthophyll de-epoxidation state in leaves of *Eucalyptus* trees grown in the field

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Abstract Heatwaves are becoming more frequent with climate warming and can impact tree growth and reproduction. *Eucalyptus parramattensis* can cope with an extreme heatwave in the field via transpiratory cooling and enhanced leaf thermal tolerance that protected foliar tissues from photo-inhibition and photo-oxidation during natural midday irradiance. Here, we explored whether changes in foliar carotenoids and/or the xanthophyll cycle state can facilitate leaf acclimation to long-term warming and/or an extreme heatwave event. We found that leaves had similar carotenoid levels when grown for one year under ambient and experimental long-term warming (+ 3 °C) conditions in whole tree chambers. Exposure to a 4-day heatwave (> 43 °C) significantly altered the xanthophyll de-epoxidation state of carotenoids revealing one mechanism by which trees could minimise foliar photo-oxidative damage. The levels of zeaxanthin were significantly higher in both young and old leaves during the heatwave, revealing that violaxanthin de-epoxidation and perhaps de novo zeaxanthin synthesis contributed to enhancement of the xanthophyll cycle state. In a future climate of long-term warming and increased heatwave events, leaves of *E. parramattensis* will be able to utilise biochemical strategies to alter the xanthophyll cycle state and cope with extreme temperatures under natural solar irradiation.

Keywords Carotenoid · Xanthophyll de-epoxidation state · Zeaxanthin · Heat stress · Photosynthesis · *Eucalyptus*

Introduction

Heatwaves can consist of several consecutive days of extreme temperatures and are predicted to increase in intensity and frequency as the climate changes (Teskey et al. 2015). Indeed, several extreme heatwaves were recently observed in Europe, Australia, and China (Ciais et al. 2005; Schiermeier 2019; van Gorsel et al. 2016; Yuan et al. 2016). Heatwaves occur concomitantly with rising mean temperature (+ 0.85 °C globally from 1880 to 2012, + 1 °C for Australia from 1910 to 2016) that could rise as much as + 3 °C by 2100 (Australian Bureau of Meteorology; IPCC 2014). Heatwave events have detrimental effects on plant growth and physiological performance as well as ecosystem function. Trees can perceive and rapidly respond to heatwave events by maintaining transpiration to cool the leaves (Drake et al. 2018; Liu et al. 2015; Urban et al. 2017).

Higher air temperatures associated with long-term warming or heatwave events may exceed optimal temperatures for photosynthesis and reduce leaf photosynthesis because of increased mitochondrial respiration, photorespiration, stomatal closure and impairment to photosynthetic biochemistry (Lin et al. 2012; Teskey et al. 2015). Although the photosynthetic rate and stomatal conductance are often coupled, they decouple under extreme heatwave conditions, leading to high rates of transpiration that maintain latent cooling via transpiration thereby protecting the leaf against thermal damage (Drake et al. 2018; Urban et al. 2017; von Caemmerer and Evans

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2015). Net photosynthesis represents the sum of gross photosynthesis and the respiratory processes (Wohlfahrt and Gu 2015). A critical balance between photosynthesis and photorespiration is necessary to provide a substantial sink for electrons and reduce the risk of photo-inhibition. When unbalanced and or net photosynthesis approaches zero in plant foliage exposed to natural solar radiation, the dissipation of excess absorbed electron energy is necessary to avoid photo-inhibition as well as photo-oxidative damage (Foyer et al. 2009; Kato et al. 2003; Roach and Krieger-Liszkay 2014).

Carotenoid pigments play an important function in facilitating photoprotection and reducing photo-oxidation damage in leaves from plants exposed to certain stresses (Demmig-Adams et al. 2014). Carotenoid biosynthesis, degradation, and storage are concomitantly controlled in a tissue-specific manner to maintain an optimum balance matching the prevailing environmental conditions (Alagoz et al. 2018; Baranski and Cazzonelli 2016). In chloroplasts, the photosystems contain Chl a and β -carotene bound to plastid-encoded polypeptides, and the light harvesting complex (LHC), which is composed of nuclear-encoded light harvesting proteins that bind to Chl a and Chl b, and several xanthophylls (lutein, antheraxanthin, violaxanthin, and neoxanthin; as well as lutein epoxide in some species) (Caffarri et al. 2001; Morosinotto et al. 2003). Carotenoids are membrane-bound antioxidants and have an essential role in maintaining the functional stability of the photosynthetic apparatus and control the efficiency of excitation energy usage during fluctuations in the intensity and quality of light spectrum (Demmig-Adams and Adams 1996; Pogson et al. 2005). During excessive solar irradiation, carotenoids can accept excitation from chlorophylls, quench $^1\text{O}_2$, inhibit lipid peroxidation, stabilize membranes and dissipate the excessive energy as heat, thereby contributing to photoprotection (Demmig-Adams et al. 2014; Niyogi 1999).

The xanthophyll cycle carotenoid pigments facilitate the non-photochemical quenching of singlet chlorophyll ($^1\text{Chl}^*$) fluorescence that momentarily resides on the chlorophylls of the peripheral antenna complexes of plants, and serves to dissipate excess electronic excitation as thermal energy (Demmig-Adams et al. 2014; Niyogi 1999). Although usually reversible within seconds or minutes, if sustained photo-inhibition can reduce PSII activity (Niyogi 1999). The reversible de-epoxidation of violaxanthin via an antheraxanthin intermediate back to zeaxanthin determines the xanthophyll cycle state in photosynthetic tissues. Xanthophylls help facilitate the dissipation of excess light-induced excitation energy as heat and minimise photo-oxidative stress in leaves (Demmig-Adams and Adams 1996; Havaux et al. 2004; Jahns and Holzwarth 2012). The xanthophyll cycle state

varies over a timescale of days to seasons depending upon the intensity of light and temperature, as well as plant species (Buchner et al. 2015; Davison et al. 2002; García-Plazaola et al. 2008; Haldimann et al. 2008; Havaux et al. 1996; Hormaetxe et al. 2007; Lawanson et al. 1978; Logan et al. 1998, 2009, 2010; Yin et al. 2010). In leaves, zeaxanthin is the major thermo-stabilizer carotenoid because it has a longer conjugated double bond chain that can accept excitation from $^1\text{Chl}^*$ (Bukhov et al. 2001; Holt et al. 2005). Under elevated temperature, zeaxanthin could have a double role as a free compound in the thylakoids (antioxidant and thermal stabilizer of lipid membranes) and/or when bound to the light harvesting complex proteins, it can act as a signal transducer to quench conformational change (Havaux et al. 2007; Latowski et al. 2002; Strzałka et al. 2003).

There have been a few studies on the impact of long-term warming and an extreme heatwave event on the xanthophyll cycle state in trees grown in controlled environments. For example, in a glasshouse environment, leaves from seedlings of *E. macrorhyncha* and *E. rossii* exposed to short-term heatwave (45 °C) and elevated CO_2 , showed an enhanced xanthophyll pool size consisting of de-epoxidised carotenoids such as antheraxanthin and zeaxanthin (Roden and Ball 1996). In another glasshouse experiment, seedlings of *E. saligna* and *E. sideroxylon* were grown for a prolonged period in a warmed environment (+ 4 °C) generated variable effects in the foliar xanthophyll pool composition, depending upon the species (Logan et al. 2010). Studies undertaken on Mediterranean and Atlantic woody species trees in the field revealed that a combination of high light, heat and drought can affect photoprotective responses, through an associated increase in xanthophyll cycle pigments (García-Plazaola et al. 2008; Hormaetxe et al. 2007). Environmental warming and heatwaves are expected to increase with further climate change. Leaves from *E. parramattensis* trees can increase their thermal tolerance during a heatwave to maintain leaf temperature within the thermal limits of leaf function (Drake et al. 2018). We hypothesized that the xanthophyll cycle pigments played a role during the heatwave to limit photo-oxidation in leaves from trees exposed to natural irradiation. Carotenoids are considered as the main thermo-stabilisers in leaves (Bukhov et al. 2001; Holt et al. 2005), however, it is unknown whether the enhanced thermal tolerance of *E. parramattensis* leaves can correspond with the foliar xanthophyll pool. The objective of this study was to determine how an extreme heatwave event, in combination with long-term warming under natural solar irradiation, might affect the state of the xanthophyll cycle or zeaxanthin levels in field-grown *E. parramattensis* trees.

Materials and methods

Experimental site and long-term warming and heatwave treatments

The experimental site was in Richmond, NSW, Australia (33°36'40" S, 150°44'26.5" E) and used 12 whole tree chambers (WTCs) to manipulate air temperature (T_{air}), yet maintain vapour pressure deficit (VPD), relative humidity (RH) and atmospheric CO_2 concentration at ambient levels in the canopy air space of a local woodland tree (*E. parramattensis*) grown in field conditions. The experimental design and physiological response of *E. parramattensis* trees to long-term warming and a heatwave event are described in detail in Drake et al. (2018). The WTCs were large cylindrical structures topped with a cone (3.25 m in diameter, 9 m in height, volume of $\sim 53 \text{ m}^3$). One potted seedling (average height was 60 cm) was planted into each WTC on 23rd December 2015 and grown to a maximum height of the WTC (trees aged ~ 342 days). Six WTCs were used to track and simulate the natural variation in ambient T_{air} and RH at the site (i.e. ambient treatment). Another six WTCs were used to simulate a long-term warming treatment (ambient T_{air} plus 3 °C and ambient RH). Trees were irrigated every fortnight with half the mean monthly rainfall for the site until 1 month prior to the heatwave, when irrigation was suspended. An experimental heatwave with a maximum T_{air} of 43–44 °C (constant over day/night cycles) was imposed during the Austral Spring-Summer (Oct 31st–Nov 3rd, 2016) for four consecutive sunny days to three WTCs maintained at ambient T_{air} and three WTCs subjected to long-term warming. The heatwave and long-term warming was biologically relevant as the most significant heatwave recorded for this location over four consecutive days had a maximum T_{air} of 40–41 °C (heatwave from 5–8 Feb 2009, data from 1953–2016) and a + 3 °C increase in maximum temperature has been predicted for this region by 2100 (Cowan et al. 2014; Sillmann et al. 2013).

Pigment measurements

Three old (dark green and tough in texture) and three young (light green and tender in texture) leaves were collected of the same branch during natural midday irradiance from the upper third of the crown of each experimental tree on the 4th day of the heatwave between 14:30 and 15:30 h, when T_{air} exceeded 43 °C. The crown position of the leaves was similar to that sampled by Drake et al. (2018) for physiological measurements. Leaves were kept on ice (~ 1 h) and two fresh leaf discs (5 mm in diameter) were taken from the base of each leaf, avoiding the midrib, and frozen

immediately in liquid nitrogen. Leaf samples and discs were collected as quickly as possible over a similar short period of time, ensuring that leaves from trees grown in the control WTC could be used to determine a baseline of the xanthophyll cycle state without influence from temperature changes during harvesting. The combined weight of two leaf discs was similar ($\sim 16 \text{ mg} \pm 0.3 \text{ mg}$ of standard error) regardless of leaf age and the WTC environmental treatment. Frozen leaf tissues were homogenised to a fine powder in TissueLyser® (QIAGEN) for 2 min at 20 Hz speed using stainless steel beads (~ 3 mm diameter) in 2 mL clear microtubes. Finely powdered leaf tissues were extracted with 1 mL extraction buffer containing acetone and ethyl acetate (60:40 v/v) with 0.1% butylated hydroxytoluene (BHT, w/v; an antioxidant that prevents photooxidation of carotenoids) under dim light conditions. Next, 0.8 mL of water was added and mixed gently followed by centrifugation for 5 min at 15,000 rpm at 4 °C to separate phases. The upper phase, containing 20 μL of ethyl acetate pigment was injected into a HPLC (Agilent 1260 Infinity) equipped with YMC-C30 (250 $\times$ 4.6 mm, S-5 μm) column and Diode Array Detector (DAD). Individual carotenoids were separated using a reverse phase gradient of methanol with 0.1% (v/v) triethylamine (solvent A) and methyl tert-butyl ether (solvent B). Initially, solvent composition was maintained at 85% solvent A and 15% solvent B. The reverse phase solvent gradient started at 1.0 min which was reached to 35% of B at 11.0 min. The second gradient started at 11.1 min and the ratio of B was increased to 65% at 15.0 min. The ratio of B was changed to 100% at 15.1 min stabilized until 17.0 min. The solvent flow rate was maintained 1 mL/min until 17.0 min, after which the solvent composition returned to 15% of B and was subsequently equilibrated for 8-min with a flow rate of 2.0 mL/min. The DAD signals were recorded at 440 nm and carotenoid species were identified by retention time, spectral fine structure and the ratios of maximum absorption peaks. The amount of carotenoids on a fresh weight basis ($\mu\text{g/gfw}$) was derived by integrating peak area from standard curves as previously described (Alagoz et al. 2020; Cazzonelli et al. 2010; Dhami et al. 2018; Pogson et al. 1996). The de-epoxidation of the pigment interconversion within the xanthophyll cycle (violaxanthin;V, antheraxanthin;A, zeaxanthin;Z) can be described by the ratio of $(A + Z)/(V + A + Z)$, which we herein refer to as the de-epoxidation state.

Data analysis

The experiment consisted of a factorial combination of temperature treatment, heatwave treatment, and leaf age cohort. The temperature treatment levels were ambient and

warmed (+ 3 °C) and constituted the growth temperatures of the trees prior to imposition of the heatwave and not during the heatwave. The 4-day heatwave treatment levels were control (peak daily temperature of 28–29 °C) and heatwave (peak daily temperature of 43–44 °C) and were randomly assigned to trees of both growth temperatures ($n = 3$). The leaf age cohorts were young and old. Three young leaves and three old leaves were sampled from each tree in each of the 12 WTCs and thus constituted subsamples for each cohort and tree. Carotenoid pigment data were presented in terms of the treatment mean $\pm$ standard error ($n = 3$).

The experimental design was a split-plot with temperature (1 degree of freedom; d.f.) and heatwave (1 d.f.) applied in factorial combination to individual tree chambers as the whole plot factor in a completely randomised design. Tree chamber constituted a random factor. Using analysis of variance, temperature and heatwave effects were tested against the whole-plot error term (Tree [temperature, heatwave], 8 d.f.). Leaf age (young and old) constituted the nested sub-plot factor. The main effect of leaf age (1 d.f.) and interactive effects of leaf age with temperature (i.e. leaf age $\times$ temperature) and heatwave (i.e. leaf age $\times$ heatwave) treatments were analysed using the sub-plot error term (Tree $\times$ Leaf age [temperature, heatwave], 8 d.f.).

Results

Leaf maturation and long-term warming does not affect zeaxanthin content or the xanthophyll de-epoxidation state

We first assessed the impact and interaction of leaf age and long-term climate warming on carotenoid content in leaves from trees grown in the WTCs. Long-term temperature warming had no significant effect on pigment levels, and nor

was there an apparent interaction between leaf age and temperature on pigment levels (Table 1). There was a significant effect by leaf age, with older leaves having up to 1.8-fold higher levels of carotenoid and chlorophyll pigments in comparison to younger leaves (Table 2). While the xanthophyll cycle pigments, violaxanthin and antheraxanthin were significantly enriched during leaf aging, zeaxanthin content appeared unchanged (Table 1). The xanthophyll de-epoxidation state remained unaffected by leaf age or long-term warming (Tables 1, 2). In conclusion, long-term warming had no significant effect on pigmentation regardless of leaf age. While most pigments (e.g. neoxanthin, violaxanthin, antheraxanthin, lutein, β -carotene, chlorophyll a and chlorophyll b) were enriched during leaf aging, the xanthophyll de-epoxidation state remained similar.

An extreme heatwave enhanced zeaxanthin content and the xanthophyll de-epoxidation state

We observed a significant impact of the heatwave on individual carotenoid content, although there were no significant interactive effects between the heatwave and leaf age or long-term warming treatments (Table 1). The heatwave caused an increase in antheraxanthin content ($p < 0.07$), yet did not significantly alter violaxanthin levels (Fig. 1, Table 2). Zeaxanthin was the only carotenoid significantly affected by the heatwave (Table 1), increasing 1.5 to 2.5-fold across all treatment combinations (Fig. 1).

We examined the xanthophyll cycle state in response to the heatwave in different leaf types from trees grown under ambient and/or long-term warming environments. The de-epoxidation state was significantly enhanced by 1.2 to 1.4-fold during the heatwave (Table 3). The chlorophyll a/b ratio was not significantly affected by the heatwave. These results demonstrate that the extreme heatwave enhanced not only zeaxanthin content, but also the xanthophyll de-epoxidation state during natural midday solar irradiation.

Table 1 Split-plot ANOVA with temperature, heatwave and leaf type applied in factorial combination to individual trees

Source of variation	Carotenoid content							Xan ratio (A + Z)/ (V + A + Z)	Chlorophyll content			Chl ratio (a/b)
	Neo	Vio	Ant	Lut	Zea	β -c	Total		Chl a	Chl b	Total	
Temperature	0.28	0.57	0.67	0.27	0.32	0.98	0.98	0.20	0.35	0.54	0.35	0.39
Heatwave	0.46	0.25	0.07	0.48	0.00	0.53	0.53	0.00	0.54	0.67	0.69	0.16
Temperature * heatwave	0.29	0.77	0.86	0.27	0.66	0.23	0.23	0.68	0.85	0.28	0.66	0.34
Leaf age	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.58	0.00	0.00	0.00	0.69
Temperature * leaf age	0.34	0.81	0.51	0.08	0.79	0.99	0.99	0.97	0.87	0.50	0.88	0.15
Heatwave * leaf age	0.63	0.82	0.41	0.97	0.13	0.15	0.15	0.58	0.47	0.95	0.57	0.45
Temperature * heatwave * leaf age	0.76	0.23	0.70	0.78	0.51	0.89	0.89	0.32	0.78	0.61	0.68	0.53

Italicized values; ($p < 0.05$), *Neo* Neoxanthin, *Vio/V* Violaxanthin, *Ant/A* Antheraxanthin, *Lut* Lutein, *Zea/Z* Zeaxanthin, β -C β -carotene, *Chl* Chlorophyll, *Xan* Xanthophyll

Table 2 Effects of warming on carotenoid and chlorophyll content in leaves from field-grown *E. parramattensis* trees

Leaf age	Climate factor (temperature)	Carotenoid content (µg/gfw)							Xan ratio (A + Z)/(V + A + Z)	Chlorophyll content (µg/gfw)		
		Neo	Vio	Ant	Lut	Zea	β-C	Total		Total	Chl a	Chl b
Young	Ambient	21	23	17	121	25	74	280	0.63	805	526	280
	Warmed	27	24	17	146	24	92	330	0.64	1090	740	350
	SE	2	4	2	12	4	8	26	0.0	162	134	34
Old	Ambient	37	35	23	189	22	118	424	0.56	1361	910	452
	Warmed	48	30	24	254	30	135	521	0.63	1720	1135	585
	SE	4	4	2	17	5	9	34	0.0	195	178	44
Leaf age FC (old/ yng)-amb		1.8	1.5	1.4	1.6	0.9	1.6	1.5	0.9	1.7	1.7	1.6
Leaf age FC (old/ yng)-warm		1.8	1.2	1.4	1.7	1.2	1.5	1.6	1.0	1.6	1.5	1.7
Temperature FC (warm/amb)-yng		1.3	1.1	1.0	1.2	1.0	1.2	1.2	1.0	1.4	1.4	1.3
Temperature FC (warm/amb)-old		1.3	0.9	1.1	1.3	1.3	1.1	1.2	1.1	1.3	1.2	1.3

Neo Neoxanthin, Vio/V Violaxanthin, Ant/A Antheraxanthin, Lut Lutein, Zea/Z Zexanthin, P-C P-carotene, Chl Chlorophyll, yng Young, amb ambient, warm warmed, FC Fold change, SE Maximum standard error

Fig. 1 The effects of an extreme heatwave on carotenoid and chlorophyll content. A heatwave ratio (heatwave/control) was calculated for two leaf types (young and old) from trees grown under two temperature treatments (ambient and warm). Ratio of neoxanthin (Neo), violaxanthin (Vio), antheraxanthin (Anth), lutein (Lut),

zeaxanthin (Zea), β-carotene (β-car) and total carotenoid content. Plots represent sample mean and error bars display standard error (n = 3). Star represents statistically significant differences as analysed by a *post-hoc* Tukey ANOVA ($p < 0.05$)

Table 3 A heatwave enhances xanthophyll de-epoxidation state

Leaf age	Climate factor	Xanthophyll ratio (A + Z)/(V + A + Z)		Chlorophyll ratio (chl a/chl b)	
		Ambient	Warm	Ambient	Warm
Young	Control	0.63 ± 0.04	0.64 ± 0.06	1.94 ± 0.22	2.01 ± 0.40
	Heat wave	<i>0.73 ± 0.04</i>	<i>0.84 ± 0.04</i>	2.12 ± 0.10	2.96 ± 0.20
	Fold change (heat wave/control)	1.2	1.3	1.1	1.5
Old	Control	0.56 ± 0.01	0.63 ± 0.04	2.08 ± 0.17	195 ± 0.50
	Heat wave	<i>0.76 ± 0.04</i>	<i>0.80 ± 0.05</i>	2.23 ± 0.07	2.59 ± 0.16
	Fold change (heat wave/control)	1.4	1.3	1.1	1.3

Ratios represent a mean ± standard error (n = 3). A significant difference is highlighted by italicized values ($p < 0.05$). V Violaxanthin; A Antheraxanthin; Z Zexanthin ; Chl Chlorophyll

Discussion

Carotenoid and chlorophyll pigmentation increased during leaf aging, which is consistent with a previous report demonstrating an increase in chlorophyll content during the aging of foliar tissues in *Eucalyptus* species (Datt 1998). There is limited evidence to support what effects a future climate change in growth temperature (ambient $T_{\text{air}} + 3\text{ }^{\circ}\text{C}$) could have on carotenoid or chlorophyll pigmentation in trees. Our prolonged growth-temperature warming treatment did not significantly affect carotenoid or chlorophyll content in leaves from *E. parramattensis* trees grown in the field. These data are consistent with that reported by Drake et al. (2018), in that leaf physiological parameters such as dark-adapted variable fluorescence relative to maximal fluorescence (F_v/F_m), and net photosynthesis was unaffected by $+3\text{ }^{\circ}\text{C}$ warming. Therefore, climate warming by up to $+3\text{ }^{\circ}\text{C}$ does not affect the photoprotective acclimation response in leaves from trees subjected to a four-day heatwave.

It was previously shown that photosynthetic pigments do not change in response to a short-term heat treatment (Buchner et al. 2015). However, prolonged and higher fluctuations in air temperature can influence photosynthetic pigment metabolism in leaves (Lawanson et al. 1978; Logan et al. 2010; Tran and Raymundo 1999). The xanthophyll de-epoxidation state has been shown to change in high alpine shrub species, Mediterranean and Atlantic trees, and rice subjected to heat stress (Buchner et al. 2015; García-Plazaola et al. 2008; Hormaetxe et al. 2007; Yin et al. 2010). In agreement, we observed a significant increase in the xanthophyll de-epoxidation state in leaf tissues harvested from *E. parramattensis* trees grown under natural solar irradiance and exposed to a 4-day heatwave event consisting of very high air and leaf temperatures. The increased xanthophyll state was mostly due to the significant increase in zeaxanthin, and to a lesser extent a subtle increase in antheraxanthin.

Leaves from the trees subjected to 4 days of an extreme heatwave may have benefited from the enhanced xanthophyll de-epoxidation state by avoiding photosystem and photo-oxidative damage. Here, we correlate the increased xanthophyll de-epoxidation state to the integrative physiological response of the crown of the whole tree canopy that displayed a net photosynthetic rate of approximately zero under the heatwave conditions. Transpiration persisted to maintain canopy cooling and leaf thermal tolerance within the limits of leaf function (Drake et al. 2018). The photosynthetic rate was established by a leaf-level photosynthetic model that defines expectations for the responses of photosynthesis and transpiration (Duursma 2015; Medlyn et al. 2011). The heatwave did not cause widespread

crown damage or growth reductions; however, there were some leaf browning symptoms that affected 1.1% of the leaf area of heatwave trees compared with 0.3% for control trees (Drake et al. 2018). While other electron scavenging mechanisms are likely to exist, the enhanced xanthophyll de-epoxidation state may have contributed to photoprotection in *E. parramattensis* leaves during the extreme heatwave event, evidenced by a lack of significant leaf browning, bleaching or decay, typical symptoms of photo-oxidative damage.

There are strong connections between the photoprotection of PSII and high irradiation-induced intra-thylakoid acidification that stabilizes the thylakoid membrane and PSII reaction centres. It was reported that the acidification of the thylakoid lumen can activate violaxanthin de-epoxidase, and hence the xanthophyll cycle (Havaux and Niyogi 1999). Given that the levels of violaxanthin did not significantly change in response to the heatwave, we suggest there might be some degree of de novo synthesis of zeaxanthin that contributed to the higher xanthophyll de-epoxidation state. Alternatively, a constant period of environmental stress such as a cold winter and/or nocturnal retention may be able to cause a higher level of zeaxanthin and antheraxanthin to persist due to incomplete re-epoxidation in some species (Barker et al. 2002; Demmig-Adams and Adams 1992, 1996). The higher zeaxanthin levels could help protect the thylakoid membranes from lipid peroxidation, by quenching $^1\text{Chl}^*$ as zeaxanthin can act as a free compound in the thylakoids (having a double role as an antioxidant and thermal stabilizer) or be bound to LHC proteins (Niyogi 1999). Whether the extreme heatwave enhanced de novo biosynthesis of zeaxanthin or impaired re-epoxidation in leaves from *Eucalyptus* trees requires further validation.

It is well known that higher light intensities can enhance the xanthophyll de-epoxidation state (Niyogi 1999). There is a strong interactive effect between light and temperature in maintaining photo-protection and thermal acclimation (Yin et al. 2010). For example, xanthophyll de-epoxidation activity was reported to be higher in *Ranunculus glacialis* leaves subjected to $38\text{ }^{\circ}\text{C}$ temperatures under strong irradiation (Streb et al. 2003). In our experiment, the higher xanthophyll de-epoxidation state exemplified by the accumulation of antheraxanthin and zeaxanthin in *Eucalyptus* leaves occurred under conditions of natural solar irradiance. Our findings are in concert with previous findings where rice (Yin et al. 2010), alpine shrubs (Buchner et al. 2015), and *Eucalyptus* (Logan et al. 2010; Roden and Ball 1996) grown under controlled lighting environments with additional heat stress resulted in an enhanced de-epoxidation state. The xanthophyll cycle has been reported to be activated upon heat stress in the dark, although to a much lesser extent (Fernández-Marín et al. 2010; 2011). Similarly, the de-

epoxidation of violaxanthin was induced under a lower irradiation in response to heat stress in wheat seedlings (Ilik et al. 2010). Exposure to a heatwave can enhance transpiration cooling and leaf thermal tolerance (Drake et al. 2018). Here we revealed that an extreme heatwave event under natural solar irradiance can enhance zeaxanthin content and the xanthophyll de-epoxidation state in both young and old leaves from *Eucalyptus* trees grown in the field.

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Author contributions CIC and ND conceived idea to link xanthophyll de-epoxidation to extreme heatwave. ND performed sample collection and pigment measurements. JED, MGT and DTT developed and implemented the experimental design of the long-term warming treatment and heatwave event in the whole tree chambers. MGT conducted the statistical analyses. CIC and ND analysed data and wrote the manuscript with critical input from all authors.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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