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Responses to heatwaves of gas-exchange, chlorophyll fluorescence and antioxidants ascorbic acid and glutathione in congeneric pairs of *Acacia* and *Eucalyptus* species from relatively cooler and warmer climates.

Title Page

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- Title: Responses to heatwaves of gas-exchange, chlorophyll fluorescence and antioxidants ascorbic acid and glutathione in congeneric pairs of *Acacia* and *Eucalyptus* species from relatively cooler and warmer climates.

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- Abstract: According to climate change predictions, heatwaves will increase in frequency and intensity. This can threaten survival of sensitive tree species. Heatwaves affect photosynthetic capacity and cause an imbalance between light driven electron transport and carbon fixation. This can increase the concentration of reactive oxygen species and, can cause photo-oxidative stress. Heat dissipation and antioxidants are crucial in plant defence against photo-oxidative stress. We hypothesised that stomatal regulations, heat dissipation as measured by chlorophyll fluorescence and responses of major antioxidants ascorbic acid and glutathione to heatwaves will vary according to the climate at the site of origin of tree species. Tree seedlings from warmer (*Eucalyptus grandis, Acacia aneura*) and cooler climates (*Eucalyptus tricarpa, Acacia melanoxylon*) were exposed to air temperatures about 5 ºC above control levels for five days. The two species from warmer climates responded to heatwaves with stomatal closure restricting transpiration and carbon fixation, and *Eucalyptus grandis* also significantly increased heat dissipation (as judged by decreased efficiency of open reaction centres of PSII in the light). Species from cooler climates kept stomata open allowing continuing carbon assimilation and transpirational cooling. Heatwaves did not reduce maximum quantum efficiency of PSII in *Acacia* species, with insignificant decreases in *Eucalyptus* species. Glutathione concentrations increased significantly upon heatwave in *Eucalyptus tricarpa* (from cooler climates), showed a time dependent response to heatwave in *Eucalyptus grandis* (from warmer climates) with an increase at later stages, and showed a tendency to decrease upon heatwave in both *Acacia* species (non-significant only for *Acacia melanoxylon*). Ascorbic acid concentrations increased significantly in *Eucalyptus tricarpa* (cooler climates), and did not change significantly in other species. The redox state of ascorbic acid or glutathione only changed significantly and transiently in response to heatwave in *Acacia aneura* (warmer climates; *GSSG%* and *ASC%*) and *Acacia melanoxylon* (cooler climates; *GSSG%*), but not *Eucalyptus* species. Therefore, differences in stomatal regulations upon heatwaves aligned with the origin of species in warmer or cooler climates, whereas responses of ascorbic acid and glutathione to heatwaves were more aligned with the two genera.

- Keywords: *Acacia aneura* · *Acacia melanoxylon* ·*Eucalyptus grandis* ·*Eucalyptus tricarpa* · high temperature · ascorbic acid · glutathione

- Key message: Two species from warmer climates, but not the corresponding congeneric species from relatively cooler ones, decreased stomatal conductance upon heatwaves, and one of them showed significant decrease in the efficiency of open reaction centres of PSII in the light. In contrast, responses of major antioxidants ascorbic acid and glutathione to heatwaves were more similar between congeneric species than between species from similar climates.

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Abstract

According to climate change predictions, heatwaves will increase in frequency and intensity. This can threaten survival of sensitive tree species. Heatwaves affect photosynthetic capacity and cause an imbalance between light driven electron transport and carbon fixation. This can increase the concentration of reactive oxygen species and, can cause photo-oxidative stress. Heat dissipation and antioxidants are crucial in plant defence against photo-oxidative stress. We hypothesised that stomatal regulations, heat dissipation as measured by chlorophyll fluorescence and responses of major antioxidants ascorbic acid and glutathione to heatwaves will vary according to the climate at the site of origin of tree species. Tree seedlings from warmer (*Eucalyptus grandis, Acacia aneura*) and cooler climates (*Eucalyptus tricarpa, Acacia melanoxylon*) were exposed to air temperatures about 5 ºC above control levels for five days. The two species from warmer climates responded to heatwaves with stomatal closure restricting transpiration and carbon fixation, and *Eucalyptus grandis* also significantly increased heat dissipation (as judged by decreased efficiency of open reaction centres of PSII in the light). Species from cooler climates kept stomata open allowing continuing carbon assimilation and transpirational cooling. Heatwaves did not reduce maximum quantum efficiency of PSII in *Acacia* species, with insignificant decreases in *Eucalyptus* species. Glutathione concentrations increased significantly upon heatwave in *Eucalyptus tricarpa* (from cooler climates), showed a time dependent response to heatwave in *Eucalyptus grandis* (from warmer climates) with an increase at later stages, and showed a tendency to decrease upon heatwave in both *Acacia* species (non-significant only for *Acacia melanoxylon*). Ascorbic acid concentrations increased significantly in *Eucalyptus tricarpa* (cooler climates), and did not change significantly in other species. The redox state of ascorbic acid or glutathione only changed significantly and transiently in response to heatwave in *Acacia aneura* (warmer climates; *GSSG%* and *ASC%*) and *Acacia melanoxylon* (cooler climates; *GSSG%*), but not *Eucalyptus* species. Therefore, differences in stomatal regulations upon heatwaves aligned

with the origin of species in warmer or cooler climates, whereas responses of ascorbic acid and glutathione to heatwaves were more aligned with the two genera.

Keywords *Acacia aneura* · *Acacia melanoxylon* ·*Eucalyptus grandis* ·*Eucalyptus tricarpa* · high temperature · ascorbic acid · glutathione

Introduction

The World Meteorological Organization (WMO) defines heatwaves as episodes of five or more continuous days with air temperatures 5 °C above typical daily maximum temperatures (Frich et al. 2002). According to climate change predictions, heatwaves will become more frequent, prolonged and intense in many regions in the near future (Allen et al. 2010, IPCC 2014), which can result in an exceedance of the acclimation range and eventually even death of susceptible tree species (Rennenberg et al. 2006, Allen et al. 2010, IPCC 2014). Trees at the seedling stage are commonly most sensitive (Niinemets 2010, Johnson et al. 2011, Orsenigo et al. 2014), even though they can show greater flexiblity to a combination of stress factors than adult trees (Niinemets 2010). Seedling survival is an important part of forest ecosystems under future climate change.

Tree species differ in thermotolerance of photosynthesis (Berry and Björkman 1980), and this response depends on foliage type (Dreyer et al. 2001, Duan et al. 2014) or adaptation strategies to habitat conditions (Knight and Ackerly 2002, Cunningham and Read 2006, Weston and Bauerle 2007, Gunderson et al. 2010), even though species differences were not always found (Ghannoum et al. 2010) or the thermotolerance of species was unrelated to the temperature at their site of origin (Lin et al. 2013). Generally, most plant species including trees have temperature optimum for photosynthesis between 20 and 35 ºC (Rennenberg et al. 2006; Sage et al. 2008), and especially species from warmer climates can often continue carbon assimilation at much higher temperatures (Vargas and Cordero 2013). However, these species photosynthesise already at temperatures close to their optimum range which can be disadvantageous under future climate change predictions (Cunningham and Read 2003). In contrast, trees from cooler climates are usually adapted to fluctuations of temperatures and therefore may show greater flexibility in response to heatwaves than trees from warmer climates (Cunningham and Read 2003, Gunderson et al. 2010), or can be equally affected.

Under moderate heatwave conditions (most commonly reported at temperatures between 35 and 40 ºC) photosynthesis is reversibly reduced, but irreversible changes can occur when heat intensifies (Sharkey and Zhang 2010, Hüve et al 2011). Photosynthesis can be reduced directly (non-stomatal limitations) or indirectly (stomatal limitations) under heatwave conditions (Sage et al. 2008, Bussotti et al. 2014). Non-stomatal limitations are associated with decreasing Rubisco activity or reduced capacity to regenerate 1,5-bisphosphate

(RuBP), and reduced electron transport rate including down-regulation of photosystem II (PSII) (Rennenberg et al. 2006; Sage et al. 2008) and this can increase the concentration of carbon dioxide inside the leaf followed by stomatal closure (Teskey et al. 2014). In contrast, stomatal limitations (stomatal closure) reduce photosynthesis to counteract high evaporative demand which is a result of increasing air vapour pressure deficit (*VpdA*) driven by air temperatures (Sage et al. 2008, Will et al. 2013, Duursma et al. 2014).

Stomatal closure prevents excessive water loss and hydraulic failure, but also limits evaporative cooling and restricts CO_2 input into the leaf (McDowell et al. 2008). Generally, tree species tend to close stomata in response to high temperature, which is a strategy to save water before further increase in the temperature and/or drought stress occurs (Bauweraerts et al. 2014, Teskey et al. 2014). The restrictions on CO_2 input due to stomatal closure result in reduced carbon assimilation in the Calvin-Cycle (McDowell et al. 2008). This can cause an imbalance between consumption and production of metabolic energy carriers (Asada 2006, Sharkey and Zhang 2010, Foyer et al. 2012). As a result, excitation energy and electrons are not used for the production of metabolic energy carriers, despite continuous illumination driving the electron transport rate (Asada 2006, Foyer et al. 2012). This can lead to a situation of 'excess excitation energy' (EEE – energy absorbed in excess of the needs for CO_2 fixation), which can favour increased formation of reactive oxygen species (ROS) (Asada 2006, Foyer and Noctor 2009). In contrast to heat-induced stomatal closure, some trees keep stomata open under heatwave condition which enables effective transpirational cooling and continuing carbon assimilation (Feller 2006, Ameye et al. 2012, Teskey et al. 2014). Such a strategy prevents leaf from overheating (McDowell et al. 2008, Schymanski et al. 2013), but excessive water loss through transpiration can lead to hydraulic failure (McDowell et al. 2008).

Heatwave-driven stomatal closure can cause photo-oxidative stress, a situation where an imbalance between absorbed light energy and energy consumption in photosynthesis causes increased formation of ROS (Asada 2006). Defence mechanisms against photo-oxidative stress include flexible adjustment of PSII quantum efficiency, which leads to dissipation of absorbed light energy as heat and can be directly measured via chlorophyll fluorescence (e.g. F_v'/F_m'; Maxwell and Johnson 2000). In addition to heat dissipation, pathways such as photorespiration or the Mehler reaction can serve as alternative electron sinks and help utilise absorbed light energy when CO_2 fixation in the Calvin-Cycle is impaired (Asada 2006, Foyer and Noctor 2009, Sharkey and Zhang 2010, Demmig-Adams et al. 2012). Whilst these pathways are essential for the photoprotection of PSII while light energy driven electrons are in excess of the photosynthesis needs (Asada 2006), they are also a source of ROS promoting photo-oxidative stress. ROS can function as signal molecules in response to stress

conditions, but ROS need to be kept under control by the antioxidants ascorbic acid and glutathione (Suzuki and Mittler 2006, Foyer and Noctor 2009).

Once produced, ROS concentration is controlled by antioxidants ascorbic acid and glutathione. These antioxidants are involved in complex pathways with other non-enzymatic and enzymatic antioxidants, which together regulate the concentration of ROS (Foyer and Shigeoka 2011). Stress conditions can affect the concentration of non-enzymatic antioxidants, such as the ubiquitous water-soluble compounds ascorbate and glutathione. The concentration of ascorbic acid and glutathione commonly increases under mild and moderate stress, which may reflect an acclimation response to stress conditions (Tausz et al. 2004). However, severe stress can cause an imbalance between the production of ROS and their removal by antioxidants (Tausz et al. 2004, Foyer and Noctor 2009). In addition to uncontrolled increase in the concentration of ROS, the probability of cellular damage also raises (e.g. can damage proteins, lipids, nucleic acids, and negatively affect cell membrane stability) in parallel with decreasing concentrations of antioxidants (Tausz et al. 2004, Foyer and Noctor 2009). The non-enzymatic antioxidants glutathione and ascorbic acid have also important roles in plant growth regulation and membrane stability, regeneration of other antioxidants or dissipation of absorbed light energy (Munné-Bosch and Alegre 2002, Foyer and Noctor 2009, Munné-Bosch et al. 2012).

Tree species exposed to high temperature showed initial increase in the concentration of non-enzymatic antioxidants followed by a decrease along with the duration of stress conditions (Ma et al. 2008) or increasing temperature level (Munné-Bosch et al. 2004). In several studies, generally conducted on one species, ascorbate and glutathione showed inconsistent responses to high temperature (Munné-Bosch et al. 2004, Ma et al. 2008, Silva et al. 2010, Contran et al 2013). Few studies examined non-enzymatic antioxidants in a closely related tree species or adapted to contrasting habitats, and the responses of antioxidants were inconsistent in terms of their adaptation to habitat conditions or congeneric species (Peltzer et al. 2002, García-Plazaola et al. 2008, Hu et al. 2013). In earlier work, we suggested that tree species from contrasting habitats differ in some antioxidative defence system responses to drought stress (Wujeska et al. 2013). In addition, two *Acacia* species adapted to humid and arid habitats differed in responses of ascorbate and glutathione to combined drought and heatwaves in our recent study (Wujeska-Klause et al. 2015). Here we therefore test how species from relatively warmer and cooler habitats respond to heatwaves under ample water supply. In the present study, we investigated the responses of two major antioxidants, ascorbic acid and glutathione.

Comparisons of genetically closely related tree species (from the same genus) from contrasting climates are well suited to investigate contrasting adaptations, because responses of tree species with

comparable foliage types can help to understand which species may cope more efficiently under future climate change predictions. Therefore we used two *Eucalyptus* species (*E. tricarpa* (L.A.S.Johnson) L.A.S.Johnson & K.D.Hill and *E. grandis* W.Hill) and two *Acacia* species (*A. melanoxylon* R. Br. and *A. aneura* F. Muell ex Benth), which are distributed along various temperature and rainfall gradients. We hypothesised that tree seedlings from warmer climates (*E. grandis* and *A. aneura;* as judged by the climate envelope of their distribution) will respond differently to heatwave conditions than tree seedlings from relatively cooler climates (*E. tricarpa* and *A. melanoxylon*). The hypothetical strategy of species from warmer climates will be (1) stomatal closure to reduce water loss through transpiration at high temperatures, (2) successful dissipation of excess energy as heat and therefore prevention of photodamage to counteract effects of stomatal closure, as well as (3) increases in the concentrations of ascorbic acid and glutathione to prevent photo-oxidative stress.

Materials and methods

Plant material and growth conditions

Two *Eucalyptus* and two *Acacia* species were chosen: *E. tricarpa* (L.A.S.Johnson) L.A.S.Johnson & K.D.Hill, *E. grandis* W.Hill, *A. melanoxylon* R. Br. and *A. aneura* F. Muell ex Benth (Table 1). Fourteen-month-old *Eucalyptus* seedlings were bought from Era Nurseries (Hamilton, Victoria, Australia), repotted to 1.5 L pots, and at the age of eighteen months transferred to 10 L pots and grown under natural weather conditions (Creswick Campus of the University of Melbourne, 143°53”E, 37°25”S). *Acacia* seedlings were raised from seeds in July 2012 (Australian Tree Seed Centre, CSIRO) and after two months repotted to 1.5 L pots. Nine-month-old *Acacia* seedlings were repotted to 10 L pots and grown under glasshouse conditions (Creswick Campus of the University of Melbourne, 143°53”E, 37°25”S). Three units of potting mix for native species including slow-release fertilizer (Native Mix Superior Potting & Planting Mix, Debco, Australia) were mixed with one unit of coarse grade sand (Propagating sand, Brunnings, Australia). Two year-old *Eucalyptus* seedlings and sixteen-month-old *Acacia* seedlings were used for the experiment conducted in the glasshouse. For each species 6 plants were used, which were then divided into two treatments: control (n = 3) and heat-stressed (n = 3). Repeated gas-exchange and pre-dawn chlorophyll fluorescence measurements were performed on the same phyllodes and leaves on days 0, 1, 3 and 5 of the experiment. Samples for antioxidant determination were collected on the same days. Approximately 5 to 20 % of the foliage has been removed at the end of the sampling campaign. Seedlings were approximately 38 cm (*E. tricarpa*), 40 cm (*E. grandis*), 45 cm (*A. aneura*) and 80 cm high (*A. melanoxylon*). All seedlings were equally well-watered to exclude drought as a limiting factor.

Experimental design and heatwave conditions

198 Three chambers constructed from wooden frames and covered with transparent plastic foil (approx. 90% of
199 glasshouse PPFD) were used for the heatwave treatment (width: 0.75 m, depth: 0.75 m, height: 1.60 m). All
200 chambers were equipped with the same type of external heat source supplied with a fan to evenly distribute air
201 throughout the chambers. A space of about 20 cm was left open at the bottom of each chamber where the heat
202 sources were placed. Heatwave treatment was performed over five consecutive days during a two week period
203 for each genus. Two seedlings (one of each species) were placed in each chamber and chambers were
204 distributed as follows: Week 1: one control and two heat chambers; week 2: two control and one heat chamber
205 (with new seedlings), resulting in n = 3 seedlings per treatment and species. This design was firstly used for
206 *Eucalyptus* seedlings followed by *Acacia* seedlings. Whilst the distribution of replicates to consecutive weeks
207 potentially introduced additional variability in our data, this potential loss of power would not affect differences
208 that were statistically significant. The design demands however even greater caution with the interpretation on
209 non-significant results.
210 Heat sources were targeted at reaching temperatures 5 °C above controls for heat chambers and switched off at
211 45 °C to avoid overheating. Actual temperature was dependent on air temperature (Supplemental material).
212 Control chambers were equipped with a fan, but without heat source. Heat sources were switched on between
213 5.00 – 7.00 am and switched off between 5.00 – 7.00 pm each day for five consecutive days. The range of time
214 when heat sources were switched on was dependent on the time when pre-dawn measurements of chlorophyll
215 fluorescence were finished and until heat sources were set up and running. As this was done manually, some
216 variation between days occurred. Experimental days were defined from 11.00 am to 10.59 am the next day. In
217 total, seedlings were exposed to 12 hours of elevated air temperature for each experimental day. To mimic
218 natural conditions, temperature was increased gradually during the day rather than in a step-increase. Air
219 temperature and relative humidity were recorded at canopy level using three mini data loggers (Microlite-PRO-
220 RH, Fourtec Fourier Technologies Ltd., Israel). Air vapour pressure deficit (*VpdA* (kPa)) was calculated using
221 relative humidity (*RH* (%)) from data loggers and actual vapour pressure at a given temperature (*SVP* (Pa))
222 according to the equation $VpdA = \frac{\left(\left(\frac{100 - \mathrm{RH}}{100}\right) * SVP\right)}{1000}$ (kPa) (Monteith and Unsworth 2008) (Supplemental
223 material).
224 All seedlings were well-watered maintaining soil water content above a minimum of 70% and up to
225 90% during the experimental period. Photosynthetically active radiation (PAR) in the glasshouse ranged from
226 70 to 800 µmol $m^{-2}s^{-1}$ PPFD for *Eucalyptus* species and 70 to 890 µmol $m^{-2}s^{-1}$ PPFD for *Acacia* species.
227 Photoperiod was approximately 14 h.

Gas-exchange and chlorophyll fluorescence

Gas-exchange and chlorophyll fluorescence were measured using a Li-Cor 6400 (Leaf fluorometer chamber 6400-40; Li-Cor, Lincoln, NE, USA) on the same phyllodes or leaves and the same seedlings between 9.30 and 11.00 am. Measurements were taken on the youngest fully expanded leaves or phyllodes from the upper one-third of the seedling height on days 0, 1, 3 and 5. On the same days as gas-exchange measurements, pre-dawn chlorophyll fluorescence measurements were performed using a portable fluorometer (os30p+ Opti-sciences, Inc., Hudson, NH, USA) and the maximum quantum efficiency of PSII was determined (F_v/F_m). For gas-exchange and simultaneous fluorescence measurements, Li-Cor chamber conditions were as follows: CO_2 concentration (reference) was set at 400 ppm, flow rate at 500 µmol $m^{-2}s^{-1}$ and light intensity at 1000 µmol $m^{-2}s^{-1}$ PPFD. Relative humidity in the chamber was 40 – 60 % and leaf temperature at measurement about 20 ºC for control plants and with the IRGA coolers off for heat-stressed seedlings. Gas-exchange measurements were recorded when the first steady-state was reached after chamber closure (~ 2-3 min). The following parameters were recorded: net CO_2 assimilation rate (A), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and the efficiency of open reaction centres of PSII in the light (F_v'/F_m').

Glutathione and ascorbic acid determination

Glutathione and ascorbic acid concentrations were measured on fully expanded phyllodes and leaves. Samples were frozen in liquid nitrogen immediately, and then freeze dried for 72 h. Afterwards, samples were weighed and aliquots (ca. 50 – 60 mg of plant material) ground to a fine powder using a pestle and mortar with equal amounts of polyvinyl-polypyrrolidone (PVPP), fine quartz sand and liquid nitrogen. Then 1.5 mL of 4.5 % (w/v) metaphosphoric acid was added and mixed by vortex. Samples were centrifuged for 2.5 min at 16,100 x g. Supernatants (extracts) were decanted and stored at -20 °C for later analysis.

A 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) assay was used to determine the total (*GSH* + *GSSG*) and oxidised glutathione (*GSSG*). Aliquots of the extracts were neutralised in potassium phosphate buffer (250 mM, pH 7) and to half of the aliquots 2-vinylpyridine was added to block the reduced form of glutathione and assess *GSSG* concentration. Excess 2-vinylpyridine was removed by adding triethanolamine to the extract. The other aliquot of the extracts did not receive 2-vinylpyridine and was used to determine *GSH* + *GSSG*. A working solution containing DTNB, dimethyl sulfoxide (DMSO) and glutathione reductase was mixed with the extracts and β-nicotinamide adenine dionucleotide phosphate reduced (NADPH) was used to start the reaction. Glutathione concentrations of both forms were determined by recording absorbance at 412 nm at room temperature. Concentrations of the total and oxidized forms were calculated according to Sgherri et al. (1994).

Reduced *GSH* was determined by calculating the difference between *GSH + GSSG* and *GSSG* based on the method described by Knörzer et al. (1996).

To determine total ascorbic acid (*ASC + DHA*) supernatant (extract) was neutralised in sodium phosphate buffer (150 mM, pH 7.4) containing triethanolamine (1.5 M v/v), and then a DL-Dithiothreitol (DTT, 20 mM) was used to reduce *DHA* to *ASC*. After incubation for 15 min, N-Ethylmaleimide (NEM, 0.5 % (w/v)) was added to remove excess DTT from the extract. To determine the reduced form of *ASC* replicate extracts were treated with de-ionised water. Ascorbic acid in the extracts was analysed by treatment with trichloroacetic acid (10 % w/v), orthophosphoric acid (40 % v/v), 2,2'-dipyridil (4 % in 70 % ethanol) and iron(III) chloride (15 % w/v). After incubation for 1 h at 37 °C, concentration of total and reduced ascorbic acid was measured using absorbance at 525 nm at room temperature based on a method described by Knörzer et al. (1996).

Statistical analysis

Statistical analysis and graphs were produced using R studio (version 0.98.1091; open source software; ©2009-2014 RStudio, Inc.). For the analysis of repeated measurements on the same individual seedlings and leaves or phyllodes for the entire 5 days, a linear mixed effects model was used ('lme' from R package 'nmle'). 'Day of the experiment' was used as within-subject factor and two 'treatment' factors ('control' and 'heat-stressed') were the between-subject factor for each species. Antioxidants at each sampling day were analysed using univariate general linear model with the factors 'day' and 'treatment' ('control' and 'heat-stressed') for each species separately ('aov' from R package 'stats'). To determine if data deviated from normality, Levene's test was used. Data were checked visually for correlations between variances and means.

Results

Gas-exchange and chlorophyll fluorescence

Heatwave treatment reduced net CO_2 assimilation rate (*A*; Fig. 1 a,c,e,g) and stomatal conductance (g_s; Fig. 1 b,d,f,h) in heat-stressed seedlings of both *Eucalyptus* species and *A. aneura*, but not in *A. melanoxylon*. Net CO_2 assimilation rate and g_s decreased significantly from day 1 onwards in heat-stressed seedlings of *A. aneura* and *E. grandis*. Stomatal conductance decreased between 59 and 69 % (*A. aneura*), 69 – 86 % (*E. grandis*) and 39 – 52 % (*E. tricarpa*) in heat-stressed compared to control seedlings. In heat-stressed seedlings, g_s ranged between 0.05 (*E. grandis*) and 0.20 - 0.25 mol H_2O $m^{-2}s^{-1}$ (*A. aneura*, *E. tricarpa*). There were no significant changes of *A* and g_s in control seedlings for all investigated species. There were also no significant differences in intercellular CO_2 concentration (C_i) between treatments for all species (Fig. 2 a-d). The concentration of C_i decreased in heat-stressed seedlings of *A. aneura*, *E. grandis* and *E. tricarpa* but these reductions were

statistically insignificant (Fig. 2 a-d). The efficiency of open reaction centres of PSII in the light (F_v'/F_m'; Fig. 3 a,c,e,g) and the maximum quantum efficiency of PSII (F_v/F_m; Fig. 3 b,d,f,h) decreased in heat-stressed seedlings of both *Eucalyptus* species and *A. aneura* (but treatment effect was significant only for *E. grandis* - F_v'/F_m'). In heat-stressed seedlings, F_v'/F_m' decreased between 14 and 21 % (*A. aneura*), 21 – 44 % (*E. grandis*) and 12 – 34 % (*E. tricarpa*) compared to control plants. The maximum quantum efficiency of PSII was reduced from 1 to 8 % (*A. aneura*), 9 – 18 % (*E. grandis*) and 3 – 19 % (*E. tricarpa*) in heat-stressed seedlings, but these reductions were statistically insignificant. *A. melanoxylon* did not show statistically significant changes in gas-exchange or chlorophyll fluorescence.

Concentration of non-enzymatic antioxidants in phyllodes and leaves

Glutathione

Total glutathione concentration (*GSH + GSSG*) was lower in heat-stressed seedlings of *Acacia* species than in control seedlings (Fig. 4 a,c,e,g). In *Acacia* species, *GSH + GSSG* concentration decreased from day 1 and the concentration remained lower until the end of the experiment (treatment effect highly significant at $P < 0.05$ for *A. aneura*, for *A. melanoxylon* $P = 0.07$). In *E. tricarpa*, *GSH + GSSG* concentration increased gradually from day 1 with a significant difference between treatments, whereas only inconsistent changes were observed in *E. grandis* during the whole experiment. The ratio of oxidized to total glutathione (*GSSG%*) was higher in heat-stressed seedlings of both *Acacia* species (Fig. 4 b,d,f,h). In phyllodes of *A. aneura* the *GSSG%* ratio increased significantly on day 5, while it reached a peak on day 1 in heat-stressed seedlings of *A. melanoxylon*. In both *Eucalyptus* species, *GSSG%* was low and constant throughout the experiment.

Ascorbic acid

Total ascorbic acid concentrations (*ASC + DHA*) decreased slightly in heat-stressed seedlings of both *Acacia* species (T: $P = 0.08$ for *A. aneura* but result was insignificant for *A. melanoxylon*) and *E. grandis* (T: $P = 0.053$) (Fig. 5 a,c,e,g). This was different from *E. tricarpa,* which had significantly higher concentrations of *ASC + DHA* in leaves of heat-stressed than control seedlings. The ratio of reduced to total ascorbic acid (*ASC%*) was around 80 % and decreased significantly only in heat-stressed seedlings of *A. aneura,* and only transiently on day 1 (dropped to 60 %) (Fig. 5 b,d,f,h). A slight but insignificant increase in this ratio was found for heat-stressed seedlings of *A. melanoxylon.*

Discussion

In our study, *A. melanoxylon*, a species adapted to cooler climates, did not show changes in stomatal conductance upon heat exposure. This species continued carbon assimilation and transpiration at high rates,

limiting phyllode temperatures by transpirational cooling. Similarly, smaller effects of heat on stomatal conductance in *E. tricarpa*, the species from cooler climates, were not significant, and stomata closed to a lesser extent than in species from warmer climates. This may suggest that *E. tricarpa* continued carbon assimilation and transpiration, but at lower rates than *A. melanoxylon*. However, if water would be a limiting factor then excessive water loss through transpiration could lead to hydraulic failure (McDowell et al. 2008). In contrast, the other two species closed stomata in response to heatwave conditions (*A. aneura*, *E. grandis*, from relatively warmer climates). Also, only small (and without significant treatment effect) changes of intercellular CO_2 concentration would suggest that CO_2 concentration had no effect on carbon assimilation, as observed previously in other *Eucalyptus* species (Roden and Ball 1996), and probably stomatal closure was unrelated to leaf water status in the investigated species (Thomas and Eamus 1999). In our study, stomatal closure occurred under ample water supply in the soil, but stomatal closure may be triggered by vapour pressure deficit rather than as a direct response to temperature (McDowell et al. 2008, Duursma et al. 2014), a trend also found in conifers adapted to warm and moist climates (Addington et al. 2004). In addition, stomatal closure in *A. aneura* and *E. grandis* could be also associated with smaller diameter of xylem vessels (Tissier et al. 2004, De Micco et al. 2008), a feature commonly found in species from drier climates. Smaller vessel sizes restrict water loss, decrease hydraulic conductance and therefore increase hydraulic safety under stressful conditions (Addington et al. 2004, Tissier et al. 2004, Venegas-González et al 2014). It seems likely that species from warmer climates reduced evaporative demand to save water under heatwave conditions, but we did not measure plant water potential or diameter of xylem cells, so our data provide no clear evidence to support this statement.

Stomatal closure and reduced CO_2 uptake can ultimately lead to the photo-oxidative stress, if absorbed light will not be dissipated efficiently as heat or used in the alternative pathways (Demmig-Adams et al. 2012, Foyer et al. 2012). Chlorophyll fluorescence measurements, such as the efficiency of open reaction centres of PSII in the light (F_v'/F_m') or the maximum quantum efficiency of PSII (F_v/F_m), provide useful information about the efficiency of PSII (i.e. heat dissipation or damaged photosystem II) under stress conditions (Maxwell and Johnson 2000, Demmig-Adams et al. 2012). In our study, *E. grandis* (and to lesser extent and without significant effect of heatwave in *E. tricarpa* and *A. aneura*) showed a reversible decrease in the efficiency of open reaction centres of PSII in the light, consistent with photoprotective mechanisms (heat dissipation) or, potential and relatively fast recovery from photodamage (Demmig-Adams et al. 2012, Bauweraerts et al. 2014). In seedlings of all species, the maximum quantum efficiency remained mostly unaffected, which indicates that photodamages were avoided or repaired overnight (Maxwell and Johnson 2000). The engagement of alternative

pathways such as photorespiration, along with flexible heat dissipation, would reduce the possibility of irreversibly decreased maximum quantum efficiency and damages to PSII (Maxwell and Johnson 2000, Demmig-Adams et al. 2012). Therefore, decreases in the maximum quantum efficiency of PSII (but without significant effect of heatwave) could result from reversible adjustments of the electron transport flow and heat dissipation, or if due to damages, these would be repaired overnight, especially in *E. grandis* and *E. tricarpa*. This is in agreement with previous findings showing undamaged PSII upon exposure to moderate temperature and well-watered conditions (Faria et al. 1999, Munné-Bosch et al. 2004, Sharkey 2005, Orsenigo et al. 2014, Teskey et al. 2014), also found for other *Eucalyptus* species (Roden and Ball 1996). It is possible that well-watered conditions and repeated episodes of gradually increasing temperature allowed seedlings to acclimate to high temperature and therefore avoid or repair damages to PSII (McDowell et al. 2008, Mittler et al. 2012, Teskey et al. 2014).

Reports about responses of *ASC + DHA* and *GSH + GSSG* to high temperature are mainly from single-species studies and revealed varied and inconsistent results (Munné-Bosch et al. 2004, Ma et al. 2008, Silva et al. 2010, 2012, Contran et al 2013). Studies which examined more than one species also showed inconsistent responses of non-enzymatic antioxidants to high temperature. For example, ascorbate and glutathione concentrations varied in response to temperature as well as in combination with drought stress among three oak species with different drought-tolerances (Hu et al. 2013). Similarly, two *Acacia* species adapted to humid and arid habitat differed in the responses of ascorbic acid and glutathione to combined drought and heatwaves (Wujeska-Klause et al. 2015). In contrast, trees from contrasting temperature climates (and different genera) all increased concentrations of non-enzymatic antioxidants in response to high temperature (Peltzer et al. 2002). In another study, the response was inconsistent among species from contrasting habitat conditions (García-Plazaola et al. 2008).

In our experiment, heatwaves decreased *ASC + DHA* (non-significant) concentrations slightly in all investigated species (except for *E. tricarpa*). Since ascorbic acid was in a highly reduced state (indicating healthy plant; Smirnoff 2011), this decrease may be associated with lower substrate availability for ascorbate synthesis (García-Plazaola et al. 2008) or involvement in photoprotection (Smirnoff 2011) rather than oxidative stress depleting the ascorbate pool. The ascorbate pool became more oxidised only in heat-stressed *A. aneura* at the beginning of the experiment, but this was transient and *ASC + DHA* concentrations did not change. Such a transient change in redox state may be an initial response and serve as a stress signal triggering acclimatory responses (Foyer and Noctor 2011, Smirnoff 2011). In contrast, the heatwave led to increased *ASC + DHA*

concentration in *E. tricarpa*, suggesting that this species allocated more carbon to *ASC + DHA* upon high temperatures, consistent with an acclimation reaction such as found recently for well-watered *A. melanoxylon* exposed to heatwave conditions (Wujeska-Klause et al. 2015), *Quercus* species (Hu et al. 2013) or *Jatropha curcas* (Silva et al. 2012).

On the other hand, the heatwave led to slightly different responses of *GSH + GSSG* among investigated species. The concentration of *GSH + GSSG* decreased upon heat in both *Acacia* species (non-significantly only for *Acacia melanoxylon*), increased in *E. tricarpa* and remained unchanged in *E. grandis*. The response of *GSH + GSSG* in *Acacia melanoxylon* appears inconsistent with a recently published paper, which reported an increase in the concentration rather than reduction in response to heatwave conditions (Wujeska-Klause et al. 2015). This apparent discrepancy could be because of differences in the provenances used, different frequency of sample collection (weekly vs. every 2nd day) and therefore different time courses of results, and differences in duration and intensity of the heatwave (3 days followed by 2 days vs. 5 continuous days). In the recently published study, air temperatures were lower which may have allowed an acclimatory response including increased concentrations of *GSH + GSSG* in well-watered seedlings (Wujeska-Klause et al. 2015). In contrast, the higher air temperatures attained in the present study may have limited the capacity to maintain *GSH + GSSG* concentration. This would be in agreement with other studies, where moderate temperature (~ 30 – 35 ºC) led to increased concentrations of glutathione (Peltzer et al. 2002, Hu et al. 2013), while the concentration decreased at temperatures above 40 ºC (Ma et al. 2008). However, glutathione concentration increased in response to higher air temperatures in the leaves of *E. tricarpa*. This could suggest that the response is specific for the genus (or associated with different foliage types: phyllodes in *Acacia* versus leaves in *Eucalyptus*). In the present and previous experiment on *Acacia* species (Wujeska-Klause et al. 2015), the glutathione redox state in *A. melanoxylon* phyllodes responded sensitively, with transient oxidation to heat. Sometime after the heatwave, the glutathione pool reversed to a more reduced state in *A. melanoxylon* phyllodes, coinciding with a slightly more reduced redox state of ascorbate. In contrast, the concentration of ascorbic acid and glutathione was unaffected by heatwave treatment in *E. grandis.*

Conclusions

- In agreement with our first hypothesis, species from warmer climates (*A. aneura* and *E. grandis*) closed stomata in response to heatwave, leading to reduced transpiration (and therefore reduced transpirational cooling upon heatwave) and decreased carbon fixation. Whilst this appears somewhat counterintuitive, species from warmer climates will be more likely to encounter water deficit, and more sensitive

stomatal closure may be an adaptation to limit water loss under heatwave conditions. In contrast, species from cooler climates (*A. melanoxylon* and *E. tricarpa*) kept stomata open and maintained high transpiration rates which would limit temperature effects on the foliage. In our experiment with ample water supply this enabled continuing high rates of carbon fixation, but under increasing soil water deficit (which is likely to accompany heatwaves in the field) this strategy would not succeed.

- Only *E. grandis* engaged flexible heat dissipation in response to heatwaves (indicated by significantly decreased efficiency of open reaction centres of PSII in the light), and non-significant tendencies towards such response were seen for *E. tricarpa* and *A. aneura*. The observed changes are indicative of acclimatory (flexible) responses or repair processes rather than sustained damages to PSII, because the maximum quantum efficiency of PSII measured after recovery was not significantly affected by heatwave. In addition, responses of flexible heat dissipation and the maximum quantum efficiency of PSII do not support our second hypothesis since they were not well aligned with the origin of species in warmer or cooler climates.
- The observed responses of ascorbate and glutathione do not support our third hypothesis, because responses were only partially consistent with the temperature range at the site of origin of the investigated species, but were similar between congeneric species. Total ascorbic acid and glutathione decreased in both heat-stressed *Acacia* species in response to heatwaves, irrespective of carbon fixation changes. In *A. melanoxylon* from cooler climates, initial oxidation of the glutathione pool suggested a sensitive early redox signal. Heatwaves decreased total ascorbic acid concentration in *E. grandis* from warmer climates (but did not affect glutathione) and increased the concentration of both antioxidants in *E. tricarpa* (cooler climates), indicating an increase of ROS scavenging capacity as a potentially acclimatory response in this species.

Contribution statement:

A. W-K.: designing and conducting the experiment, writing the manuscript, running data analysis.

G. B.: supervising the work, detailed reading and revision of the manuscript, coordinating the research project.

M. T.: supervising the work, detailed reading and revision of the manuscript, coordinating the research project.

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Conflict of interests:

The authors declare that they have no conflict of interest.

References:

Addington, R. N., R. J. Mitchell, R. Oren and L. A. Donovan (2004). "Stomatal sensitivity to vapor pressure deficit and its relationship to hydraulic conductance in *Pinus palustris*." Tree Physiol. 24: 561-569.

Allen, C. D., A. K. Macalady, H. Chenchouni, D. Bachelet, N. McDowell, M. Vennetier, T. Kitzberger, A. Rigling, D. D. Breshears, E. H. T. Hogg, P. Gonzalez, R. Fensham, Z. Zhang, J. Castro, N. Demidova, J.-H. Lim, G. Allard, S. W. Running, A. Semerci and N. Cobb (2010). "A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests." Forest Ecol. Manag. 259: 660-684.

Ameye, M., T. M. Wertin, I. Bauweraerts, M. A. McGuire, R. O. Teskey and K. Steppe (2012). "The effect of induced heat waves on *Pinus taeda* and *Quercus rubra* seedlings in ambient and elevated CO_2 atmospheres." New Phytol. 196(2): 448-461.

Asada, K. (2006). "Production and Scavenging of Reactive Oxygen Species in Chloroplasts and Their Functions." Plant Physiol. 141(2): 391-396.

Bauweraerts, I., M. Ameye, T. M. Wertin, M. A. McGuire, R. O. Teskey and K. Steppe (2014). "Acclimation effects of heat waves and elevated [CO2] on gas exchange and chlorophyll fluorescence of northern red oak (*Quercus rubra* L.) seedlings." Plant Eco. 215(7): 733-746.

Berry, J. and O. Bjorkman (1980). "Photosynthetic response and adaptation to temperature in higher plants." Ann. Rev. Plant Physiol. 31: 491-543.

Bussotti, F., F. Ferrini, M. Pollastrini and A. Fini (2014). "The challenge of Mediterranean sclerophyllous vegetation under climate change: From acclimation to adaptation." Environ. Exp. Bot. 103: 80-98.

Contran, N., M. S. Gunthardt-Goerg, T. M. Kuster, R. Cerana, P. Crosti and E. Paoletti (2013). "Physiological and biochemical responses of *Quercus pubescens* to air warming and drought on acidic and calcareous soils." Plant Biol. (Stuttg) 15 Suppl 1: 157-168.

Cunningham, S. C. and J. Read (2003). "Do temperate rainforest trees have a greater ability to acclimate to changing temperatures than tropical rainforest trees." New Phytol. 157: 55-64.

Cunningham, S. C. and J. Read (2006). "Foliar temperature tolerance of temperate and tropical evergreen rain forest trees of Australia." Tree Physiol. 26: 1435-1443.

De Micco, V., G. Aronne and P. Baas (2008). "Wood anatomy and hydraulic architecture of stems and twigs of some Mediterranean trees and shrubs along a mesic-xeric gradient." Trees 22(5): 643-655.

Demmig-Adams, B., C. M. Cohu, O. Muller and W. W. Adams (2012). "Modulation of photosynthetic energy conversion efficiency in nature: from seconds to seasons." Photosynth. Res. 113(1-3): 75-88.

Dreyer, E., X. L. Roux, P. Montpied, F. Alain, Daudet and F. Masson (2001). "Temperature response of leaf photosynthetic capacity in seedlings from seven temperate tree species." Tree Physiol. 21: 223-232.

Duan, B., T. Dong, X. Zhang, Y. Zhang and J. Chen (2014). "Ecophysiological responses of two dominant subalpine tree species *Betula albo-sinensis* and *Abies faxoniana* to intra- and interspecific competition under elevated temperature." Forest Ecol. Manag. 323: 20-27.

Duursma, R. A., C. V. M. Barton, Y.-S. Lin, B. E. Medlyn, D. Eamus, D. T. Tissue, D. S. Ellsworth and R. E. McMurtrie (2014). "The peaked response of transpiration rate to vapour pressure deficit in field conditions can be explained by the temperature optimum of photosynthesis." Agr. Forest Meteorol. 189-190: 2-10.

Faria, T., M.Vaz, P. Schwanz, A. Polle, J. S. Pereira and M. M. Chaves (1999). "Responses of Photosynthetic and Defence Systems to High Temperature Stress in *Quercus suber* L. Seedlings Grown under Elevated CO_2." Plant Biol. 1: 365-371.

Feller, U. (2006). "Stomatal opening at elevated temperature: An underestimated regulatory mechanim? ." Gen. Appl. Plant Physiol.: 19-31.

FloraBank. (2014). "Greening Australia, http://www.florabank.org.au, Accessed 07 January 2015.".

Foyer, C. H., J. Neukermans, G. Queval, G. Noctor and J. Harbinson (2012). "Photosynthetic control of electron transport and the regulation of gene expression." J. Exp. Bot. 63(4): 1637-1661.

Foyer, C. H. and G. Noctor (2009). "Redox Regulation in Photosynthetic Organisms: Signaling, Acclimation, and Practical Implications." Antioxid. Redox Sign. 11(4).

Foyer, C. H. and G. Noctor (2011). "Ascorbate and Glutathione: The Heart of the Redox Hub." Plant Physiol. 155(1): 2-18.

Foyer, C. H. and S. Shigeoka (2011). "Understanding Oxidative Stress and Antioxidant Functions to Enhance Photosynthesis." Plant Physiol. 155(1): 93-100.

Frich, P., L. V. Alexander, P. Della-Marta, B. Gleason, M. Haylock, A. M. G. K. Tank and T. Peterson (2002). "Observed coherent changes in climatic extremes during the second half of the twentieth century." Clim. Res. 19: 193–212.

García-Plazaola, J. I., R. Esteban, K. Hormaetxe, B. Fernández-Marín and J. M. Becerril (2008). "Photoprotective responses of Mediterranean and Atlantic trees to the extreme heat-wave of summer 2003 in Southwestern Europe." Trees 22(3): 385-392.

Ghannoum, O., N. G. Phillips, M. A. Sears, B. A. Logan, J. D. Lewis, J. P. Conroy and D. T. Tissue (2010). "Photosynthetic responses of two eucalypts to industrial-age changes in atmospheric [CO_2] and temperature." Plant Cell Environ. 33(10): 1671-1681.

Gunderson, C. A., K. H. O'Hara, C. M. Campion, A. V. Walker and N. T. Edwards (2010). "Thermal plasticity of photosynthesis: the role of acclimation in forest responses to a warming climate." Glob. Chang. Biol. 16(8): 2272-2286.

Hu, B., J. Simon and H. Rennenberg (2013). "Drought and air warming affect the species-specific levels of stress-related foliar metabolites of three oak species on acidic and calcareous soil." Tree Physiol. 33(5): 489-504.

Hüve, K., I. Bichele, B. Rasulov and Ü. Niinemets (2011). "When it is too hot for photosynthesis: heat-induced instability of photosynthesis in relation to respiratory burst, cell permeability changes and H_2O_2 formation." Plant Cell Environ. 34(1): 113-126.

IPCC, R. (2014). Climate Change 2014: Impacts, Adaptation, and Vulnerability. Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. C. B. Field, V.R. Barros, D.J. Dokken, K.J. Mach, M.D. Mastrandrea, T.E. Bilir, M. Chatterjee, K.L. Ebi, Y.O. Estrada, R.C. Genova, B. Girma, E.S. Kissel, A.N. Levy, S. MacCracken, P.R. Mastrandrea, and L.L. White. Cambridge, United Kingdom and New York, Intergovernmental Panel on Climate Change. Part A.

Johnson, D. M., K. A. McCulloh and K. Reinhardt (2011). The Earliest Stages of Tree Growth: Development, Physiology and Impacts of Microclimate. Size- and Age-Related Changes in Tree Structure and Function. F. C. Meinzer, B. Lachenbruch and T. E. Dawson. Dordrecht Heidelberg London New York, Springer Science+Business Media B.V.

Knight, C. and D. Ackerly (2002). "An ecological and evolutionary analysis of photosynthetic thermotolerance using the temperature-dependent increase in fluorescence." Oecologia 130(4): 505-514.

Knörzer, O. C., J. Burner and P. Boger (1996). "Alterations in the antioxidative system of suspension-cultured soybean cells (*Glycine max*) induced by oxidative stress." Physiol. Plantarum 97: 388-396.

Lin, Y. S., B. E. Medlyn, M. G. De Kauwe and D. S. Ellsworth (2013). "Biochemical photosynthetic responses to temperature: how do interspecific differences compare with seasonal shifts?" Tree Physiol. 33(8): 793-806.

Ma, Y.-H., F.-W. Ma, J.-K. Zhang, M.-J. Li, Y.-H. Wang and D. Liang (2008). "Effects of high temperature on activities and gene expression of enzymes involved in ascorbate–glutathione cycle in apple leaves." Plant Sci. 175(6): 761-766.

Maxwell, K. and G. N. Johnson (2000). "Chlorophyll fluorescence-a practical guide." J. Exp. Bot. 51(345): 659-668.

McDowell, N., W. T. Pockman, C. D. Allen, D. D. Breshears, N. Cobb, T. Kolb, J. Plaut, J. Sperry, A. West, D. G. Williams and E. A. Yepez (2008). "Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought?" New Phytol. 178(4): 719-739.

Mittler, R., A. Finka and P. Goloubinoff (2012). "How do plants feel the heat?" Trends Biochem. Sci. 37(3): 118-125.

Monteith, J. L. and M. H. Unsworth, Eds. (2008). Principles of Environmental Physics. USA, Elsevier.

Munné-Bosch, S. and L. Alegre (2002). "The Function of Tocopherols and Tocotrienols in Plants." Crit. Rev. Plant Sci. 21(1): 31-57.

Munné-Bosch, S., J. Peñuelas, D. Asensio and J. Llusiá (2004). "Airborne Ethylene May Alter Antioxidant Protection and Reduce Tolerance of Holm Oak to Heat and Drought Stress." Plant Physiol. 136(2): 2937-2947.

Munné-Bosch, S., G. Queval and C. H. Foyer (2012). "The Impact of Global Change Factors on Redox Signaling Underpinning Stress Tolerance." Plant Physiol. 161(1): 5-19.

Niinemets, Ü. (2010). "Responses of forest trees to single and multiple environmental stresses from seedlings to mature plants: Past stress history, stress interactions, tolerance and acclimation." Forest Ecol. Manag. 260(10): 1623-1639.

557 Orsenigo, S., A. Mondoni, G. Rossi and T. Abeli (2014). "Some like it hot and some like it cold, but not too
558 much: plant responses to climate extremes." Plant Ecol. 215(7): 677-688.
559 Peltzer, D., E. Dreyer and A. Polle (2002). "Differential temperature dependencies of antioxidative enzymes in
560 two contrasting species: *Fagus sylvatica* and *Coleus blumei*." Plant Physiol. Biochem. 40: 141-150.
561 Rennenberg, H., F. Loreto, A. Polle, F. Brilli, S. Fares, R. S. Beniwal and A. Gessler (2006). "Physiological
562 Responses of Forest Trees to Heat and Drought." Plant Biol. 8(5): 556-571.
563 Roden, J. S. and M. C. Ball (1996). "Growth and photosynthesis of two eucalypt species during high
564 temperature stress under ambient and elevated [CO_2]." Glob. Chang. Biol. 2(2): 115-128.
565 Sage, R. F., D. A. Way and D. S. Kubien (2008). "Rubisco, Rubisco activase, and global climate change." J.
566 Exp. Bot. 59(7): 1581-1595.
567 Schymanski, S. J., D. Or and M. Zwieniecki (2013). "Stomatal Control and Leaf Thermal and Hydraulic
568 Capacitances under Rapid Environmental Fluctuations." PLoS ONE 8(1): e54231.
569 Sgherri, C. L. M., B. Loggini, S. Puligaa and F. Navari-Izzo (1994). "Antioxidant system in *Sporobolus*
570 *stapfianus*: Changes in response to desiccation and rehydration." Phytochemistry 35(3): 561-565.
571 Sharkey, T. D. (2005). "Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions,
572 rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene." Plant Cell
573 Environ. 28: 269–277.
574 Sharkey, T. D. and R. Zhang (2010). "High temperature effects on electron and proton circuits of
575 photosynthesis." J. Integr. Plant Biol. 52(8): 712-722.
576 Silva, E. N., S. L. Ferreira-Silva, A. d. V. Fontenele, R. V. Ribeiro, R. A. Viégas and J. A. G. Silveira (2010).
577 "Photosynthetic changes and protective mechanisms against oxidative damage subjected to isolated and
578 combined drought and heat stresses in *Jatropha curcas* plants." J. Plant Physiol. 167(14): 1157-1164.
579 Silva, E. N., S. A. Vieira, R. V. Ribeiro, L. F. A. Ponte, S. L. Ferreira-Silva and J. A. G. Silveira (2012).
580 "Contrasting Physiological Responses of *Jatropha curcas* Plants to Single and Combined Stresses of
581 Salinity and Heat." J. Plant Growth Regul. 32(1): 159-169.
582 Smirnoff, N. (2011). "Vitamin C: The metabolism and functions of ascorbic acid in plants." Adv. Bot. Res. 59:
583 107-177.
584 SpeciesBank (2014). Australian Biological Resources Study, Canberra.
585 http://www.environment.gov.au/biodiversity/abrs/online-resources/species-bank/index.html. Accessed
586 03 April 2014.
587 Suzuki, N. and R. Mittler (2006). "Reactive oxygen species and temperature stresses: A delicate balance
588 between signaling and destruction." Physiol. Plantarum 126: 45–51.
589 Tausz, M., H. Šircelj and D. Grill (2004). "The glutathione system as a stress marker in plant ecophysiology: is
590 a stress-response concept valid?" J. Exp. Bot. 55(404): 1955-1962.
591 Teskey, R., T. Wertin, I. Bauweraerts, M. Ameye, M. A. McGuire and K. Steppe (2014). "Responses of tree
592 species to heat waves and extreme heat events." Plant Cell Environ. doi: 10.1111/pce.12417
593 Thomas, D. S. and D. Eamus (1999). "The influence of predawn leaf water potential on stomatal responses to
594 atmospheric water content at constant C_i and on stem hydraulic conductance and foliar ABA
595 concentrations " J. Exp. Bot. 50: 243-251.

Tissier, J., L. Lambs, J. P.Peltier and G. Marigo (2004). "Relationships between hydraulic traits and habitat preference for six *Acer* species occurring in the French Alps." Ann. Forest Sci. 61(1): 81-86.

Vargas G, G. and R. A. Cordero S (2013). "Photosynthetic responses to temperature of two tropical rainforest tree species from Costa Rica." Trees 27(5): 1261-1270.

Venegas-González, A., G. von Arx, M. P. Chagas and M. T. Filho (2014). "Plasticity in xylem anatomical traits of two tropical species in response to intra-seasonal climate variability." Trees 29(2): 423-435.

Weston, D. J. and W. L. Bauerle (2007). "Inhibition and acclimation of C_3 photosynthesis to moderate heat: a perspective from thermally contrasting genotypes of *Acer rubrum* (red maple)." Tree Physiol. 27: 1083-1092.

Will, R. E., S. M. Wilson, C. B. Zou and T. C. Hennessey (2013). "Increased vapor pressure deficit due to higher temperature leads to greater transpiration and faster mortality during drought for tree seedlings common to the forest-grassland ecotone." New Phytol. 200(2): 366-374.

Wujeska-Klause, A., G. Bossinger and M. Tausz (2015). "Seedlings of two *Acacia* species from contrasting habitats show different photoprotective and antioxidative responses to drought and heatwaves." Ann. Forest Sci. 72(4): 403-414

Wujeska, A., G. Bossinger and M. Tausz (2013). "Responses of foliar antioxidative and photoprotective defence systems of trees to drought: a meta-analysis." Tree Physiol. 33(10): 1018-1029.

List of table captions:

Table 1. Characteristics of species used in this study (FloraBank 2014, SpeciesBank 2014)

List of figures captions:

Fig.1 Net CO_2 assimilation rate (*A*; a, c, e, g) and stomatal conductance (g_s; b, d, f, h) of two *Acacia* (*A. aneura* and *A. melanoxylon*) and two *Eucalyptus* species (*E. grandis* and *E. tricarpa*) under control (open circle) and heat-stressed conditions (closed circle). Values are means (±SE) of n = 3. *P*-values indicate significance of effects of treatment (T), day (D) as well as their interaction (TxD).

Fig.2 Intercellular CO_2 concentration (*Ci*; a-d) of two *Acacia* (*A. aneura* and *A. melanoxylon*) and two *Eucalyptus* species (*E. grandis* and *E. tricarpa*) under control (open circle) and heat-stressed conditions (closed circle). Values are means (±SE) of n = 3.

Fig.3 Efficiency of open reaction centres in PS II in the light (F_v'/F_m'; a, c, e, g) and maximum quantum efficiency of PS II (F_v/F_m; b, d, f, h) of two *Acacia* (*A. aneura* and *A. melanoxylon*) and two *Eucalyptus* species (*E. grandis* and *E. tricarpa*) under control (open circle) and heatwave conditions (closed circle). Values are means (±SE) of n = 3. Description of significance levels as in Fig. 1

Fig.4 Total concentration of glutathione (*GSH + GSSG*; a, c, e, g) and the ratio of oxidized to total glutathione (*GSSG%*; b, d, f, h) in phyllodes of two *Acacia* (*A. aneura* and *A. melanoxylon*) and leaves of two *Eucalyptus* species (*E. grandis* and *E. tricarpa*) under control (open circle) and heatwave conditions (closed circle). Values are means (±SE) of n = 3. Description of significance levels as in Fig. 1

Fig.5 Total concentration of ascorbic acid (*ASC + DHA*; a, c, e, g) and the ratio of reduced to total ascorbic acid (*ASC%*; b, d, f, h) in phyllodes of two *Acacia* (*A. aneura* and *A. melanoxylon*) and leaves of two *Eucalyptus* species (*E. grandis* and *E. tricarpa*) under control (open circle) and heatwave conditions (closed circle). Values are means (±SE) of n = 3. Description of significance levels as in Fig. 1

639 **List of tables**

Species name	*Eucalyptus tricarpa* (L.A.S.Johnson) L.A.S.Johnson & K.D.Hill	*Eucalyptus grandis* W.Hill	*Acacia melanoxylon* R. Br.	*Acacia aneura* F. Muell ex Benth
Provenance	exact provenance unknown		Wodonga 146°54"E, 36°12"S	Cobar 145°45"E, 31°31"S
Mean annual rainfall	550-1000 mm	725-3500 mm	750- 1500 mm	200-400 mm
Mean annual temperature	8 - 19 °C (max. 28 °C)	12 - 25 °C (max. 34 °C)	9 - 18 °C (max. 27 °C)	-6 - 46 °C
Soils	shallow	deep	deep	shallow
Growth rate	moderate	fast	fast	slow
Drought tolerance	tolerant	sensitive	sensitive	tolerant
Foliage type	leaves		phyllodes	

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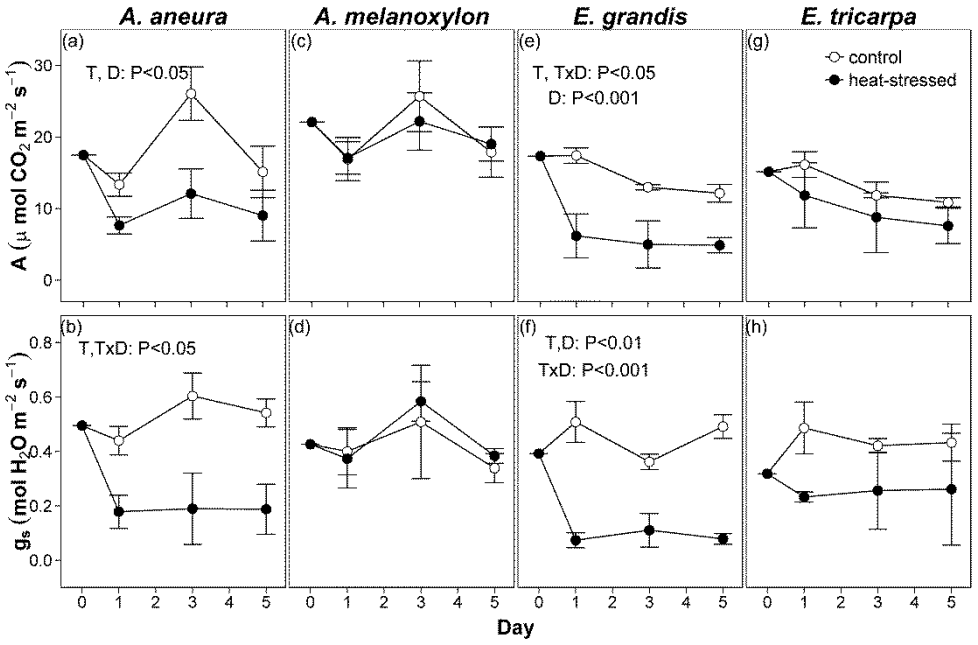
641 **List of figures**



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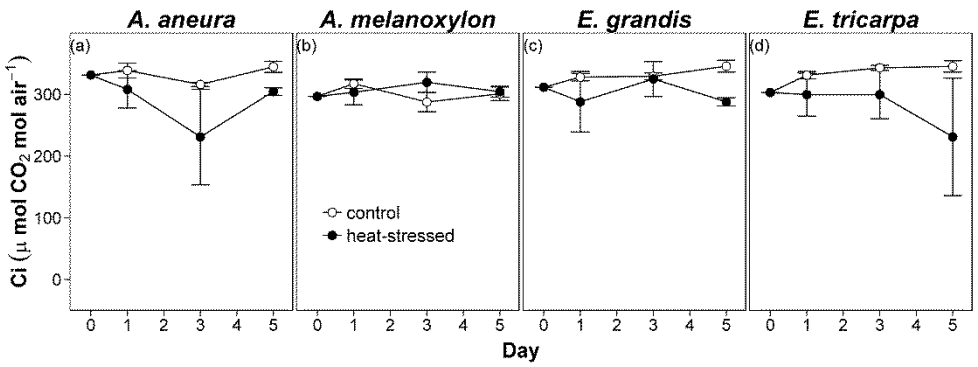

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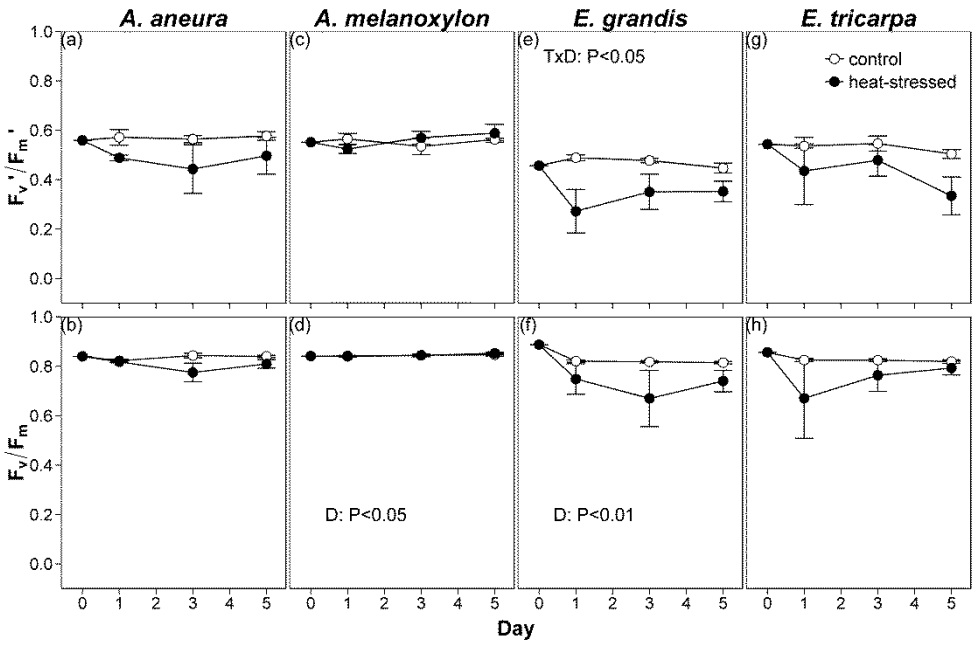
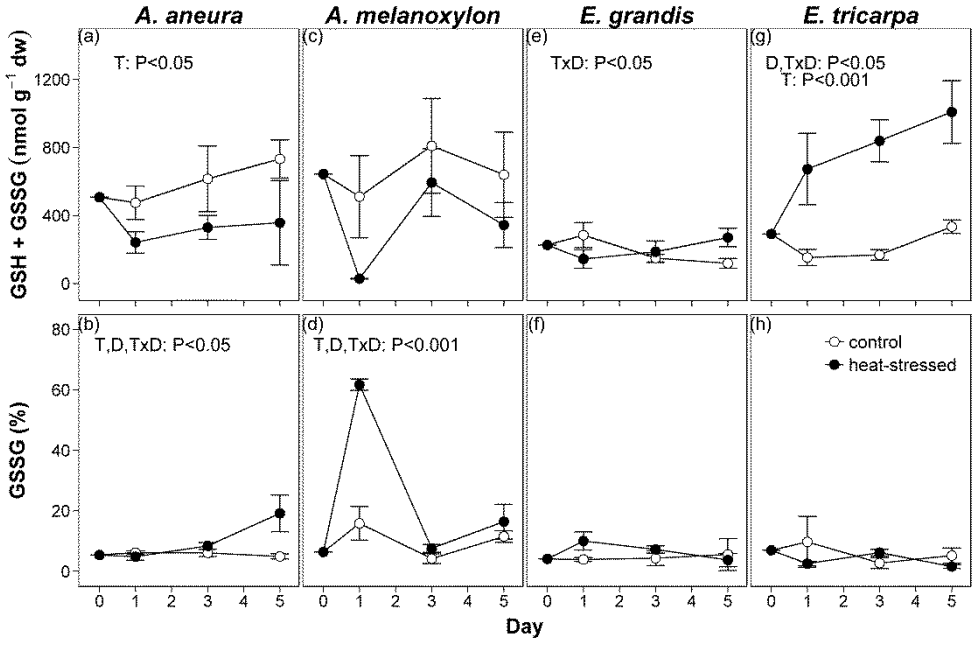
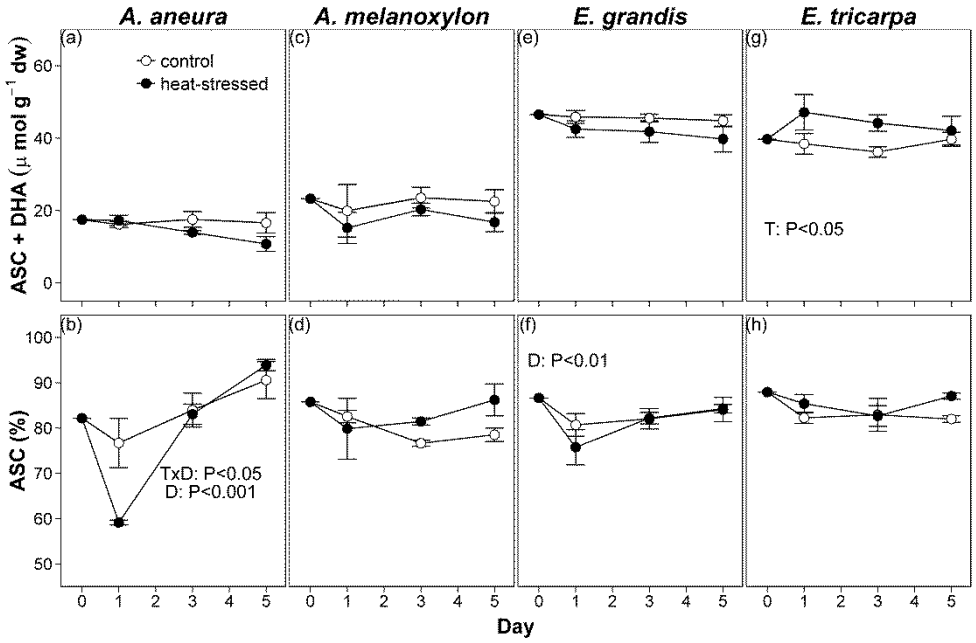

A. aneura
A. melanoxylon
E. grandis
E. tricarpa
(a)
(c)
(e)
(g)
(b)
(d)
(f)
(h)
TxD: P<0.05
control
heat-stressed
D: P<0.05
D: P<0.01
F_v'/F_m'
F_v/F_m
Day

644

645

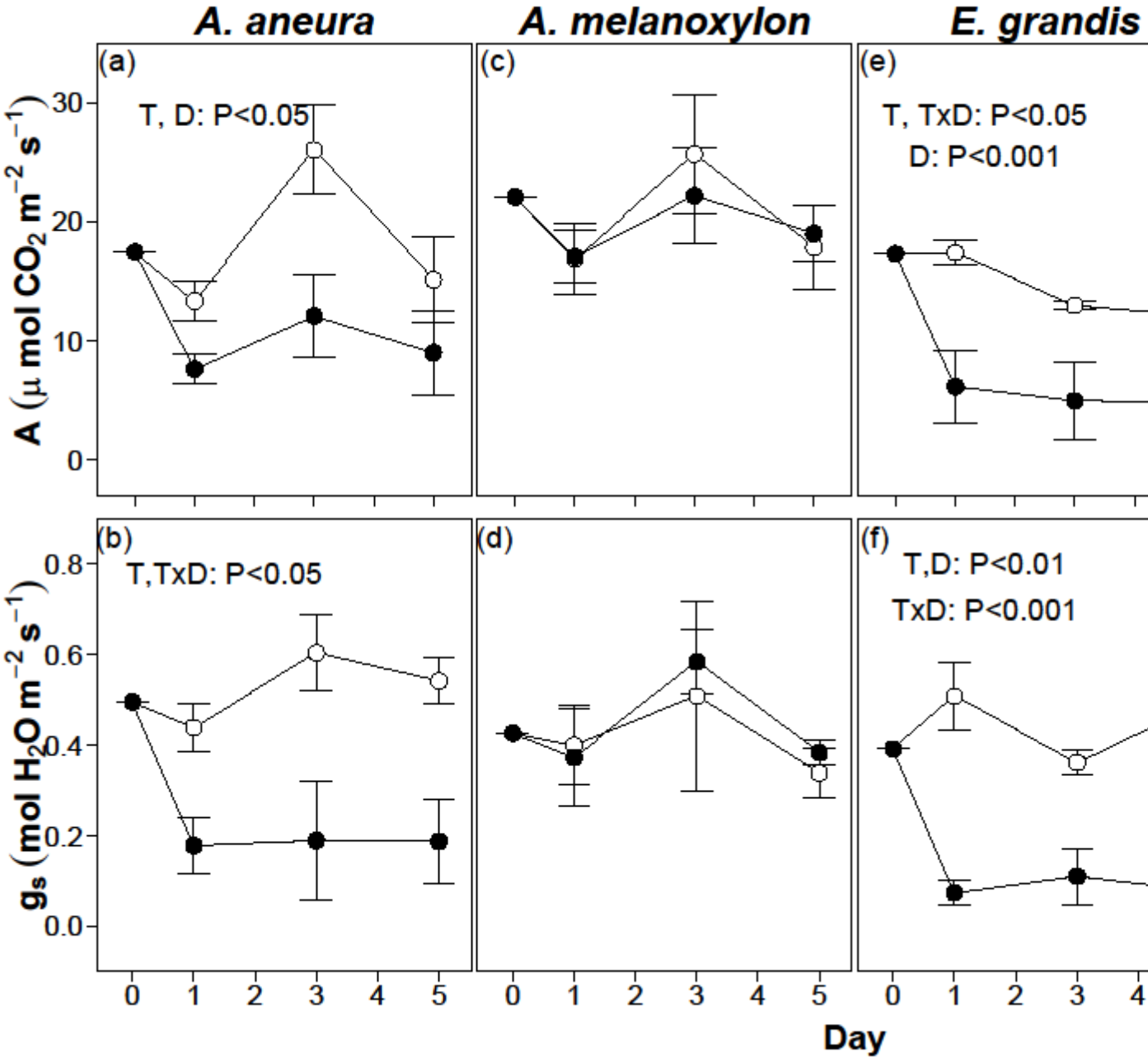

A. aneura
A. melanoxylon
E. grandis
E. tricarpa
(a)
(c)
(e)
(g)
(b)
(d)
(f)
(h)
T: P<0.05
TxD: P<0.05
D,TxD: P<0.05
T: P<0.001
T,D,TxD: P<0.05
T,D,TxD: P<0.001
control
heat-stressed
GSH + GSSG (nmol g^{-1} dw)
GSSG (%)
Day

646

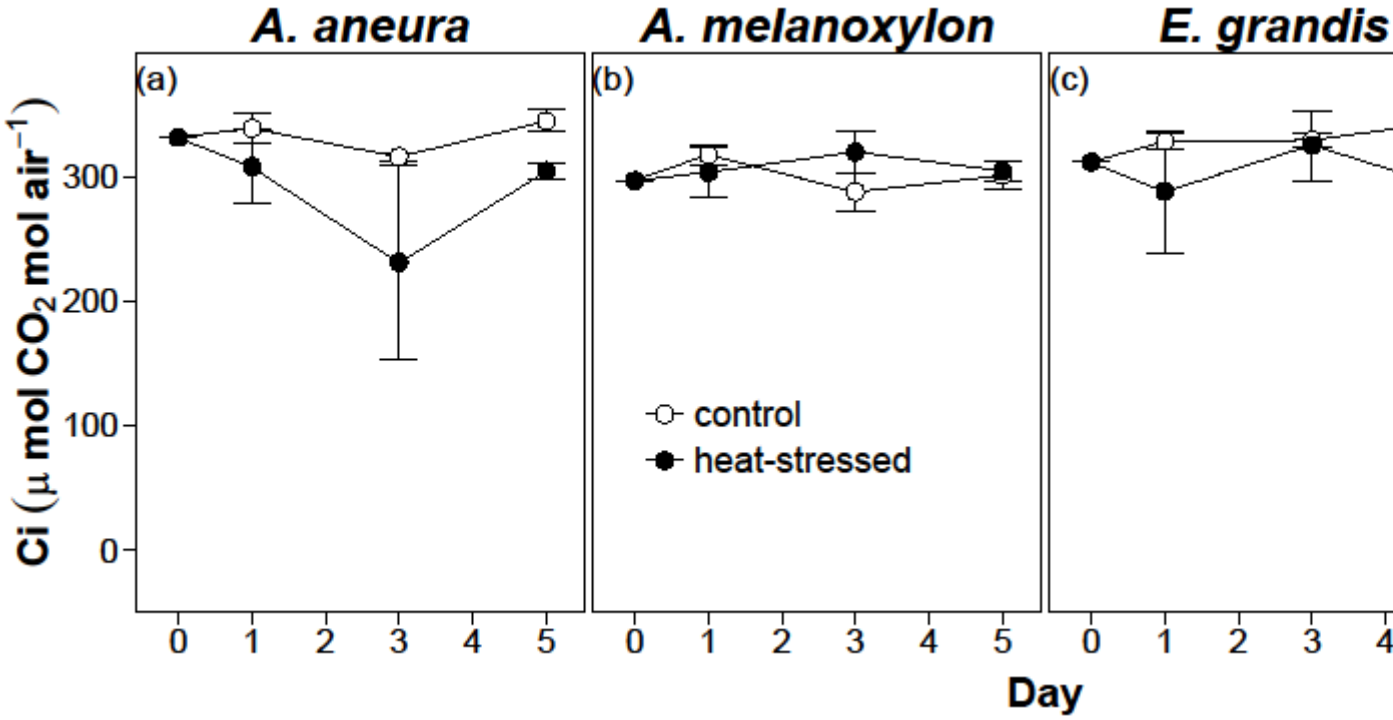


Supplemental material. Averaged daytime air temperature (Air temp.) and air vapour pressure deficit (*VpdA*) in the control and heat chambers. Numbers in brackets indicate the averaged maximum values for Air temp and *VpdA*. Values are means (± SE) of n = 3 replicate chambers

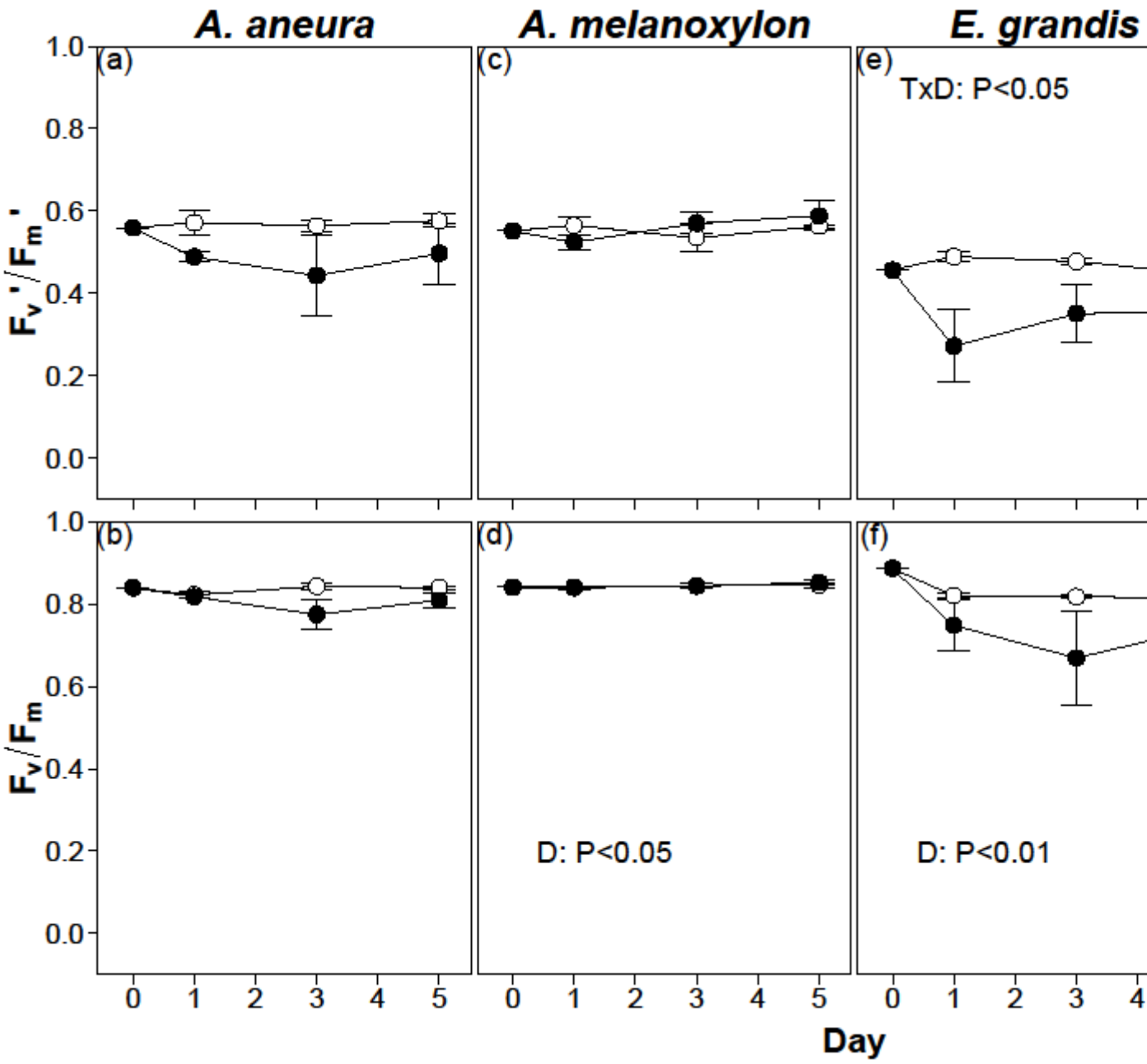
Day of heatwave treatment	Parameter	*Eucalyptus spp.*		*Acacia spp.*	
		control chamber	heat chamber	control chamber	heat chamber
0	Air temp. (°C)	19.5 ± 0.5 (33.7)	19.7 ± 0.8 (37.2)	22.8 ± 0.3 (31.4)	24.4 ± 0.3 (35.3)
	VpdA (kPa)	1.2 ± 0.1 (3.8)	1.4 ± 0.2 (5.1)	1.7 ± 0.0 (3.2)	2.0 ± 0.1 (4.4)
1	Air temp. (°C)	26.9 ± 0.3 (33.3)	38.7 ± 0.4 (48.2)	25.2 ± 0.4 (34.2)	30.9 ± 0.3 (39.4)
	VpdA (kPa)	2.5 ± 0.1 (3.8)	6.2 ± 0.2 (10.6)	2.0 ± 0.1 (3.7)	3.3 ± 0.1 (5.4)
2	Air temp. (°C)	26.8 ± 0.4 (35.2)	31.0 ± 0.5 (40.3)	26.4 ± 0.3 (35.7)	30.9 ± 0.4 (40.1)
	VpdA (kPa)	2.7 ± 0.1 (4.7)	3.9 ± 0.1 (6.2)	2.1 ± 0.1 (4.1)	3.2 ± 0.1 (5.0)
3	Air temp. (°C)	26.1 ± 0.4 (37.8)	36.7 ± 0.3 (43.9)	30.4 ± 0.3 (38.9)	35.4 ± 0.4 (44.0)
	VpdA (kPa)	2.6 ± 0.1 (5.5)	5.3 ± 0.1 (7.8)	3.0 ± 0.1 (5.1)	4.2 ± 0.1 (7.0)
4	Air temp. (°C)	22.2 ± 0.3 (26.3)	30.6 ± 0.5 (39.4)	30.6 ± 0.4 (39.1)	35.2 ± 0.5 (44.8)
	VpdA (kPa)	1.4 ± 0.1 (2.1)	3.7 ± 0.1 (6.2)	3.0 ± 0.1 (5.1)	4.4 ± 0.1 (6.8)
5	Air temp. (°C)	24.3 ± 0.3 (28.6)	35.3 ± 0.3 (40.9)	27.6 ± 0.3 (36.9)	33.0 ± 0.2 (38.0)
	VpdA (kPa)	1.6 ± 0.1 (2.3)	4.6 ± 0.1 (6.5)	2.4 ± 0.1 (4.6)	3.6 ± 0.1 (4.8)


A. aneura
A. melanoxylon
E. grandis
(a)
T, D: P<0.05
(c)
(e)
T, TxD: P<0.05
D: P<0.001
A (μ mol CO2 m-2 s-1)
(b)
T,TxD: P<0.05
(d)
(f)
T,D: P<0.01
TxD: P<0.001
gs (mol H2O m-2 s-1)
Day

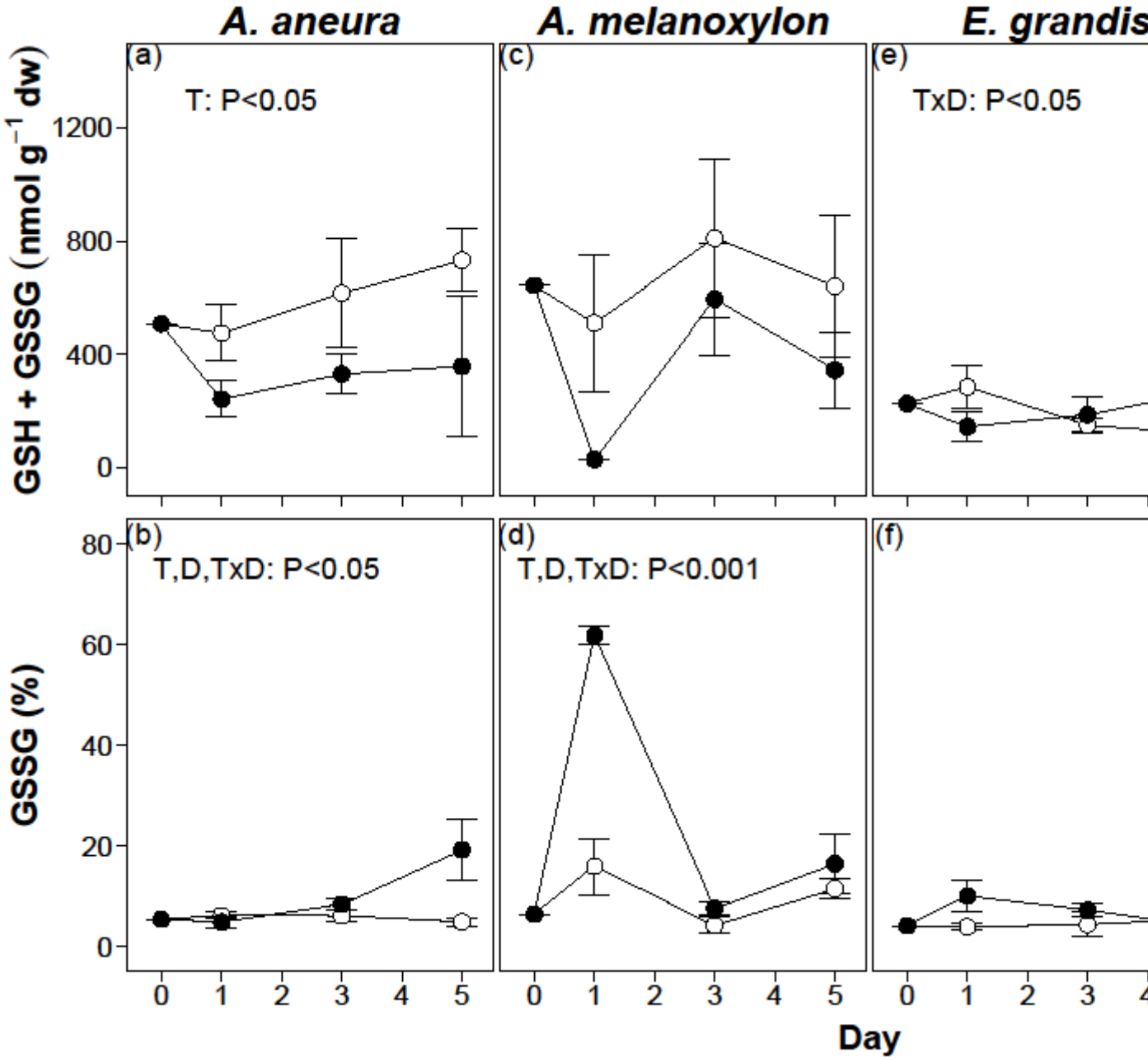
653
654


A. aneura
A. melanoxylon
E. grandis
(a)
(b)
(c)
Ci (μ mol CO2 mol air−1)
0
100
200
300
control
heat-stressed
0 1 2 3 4 5
Day

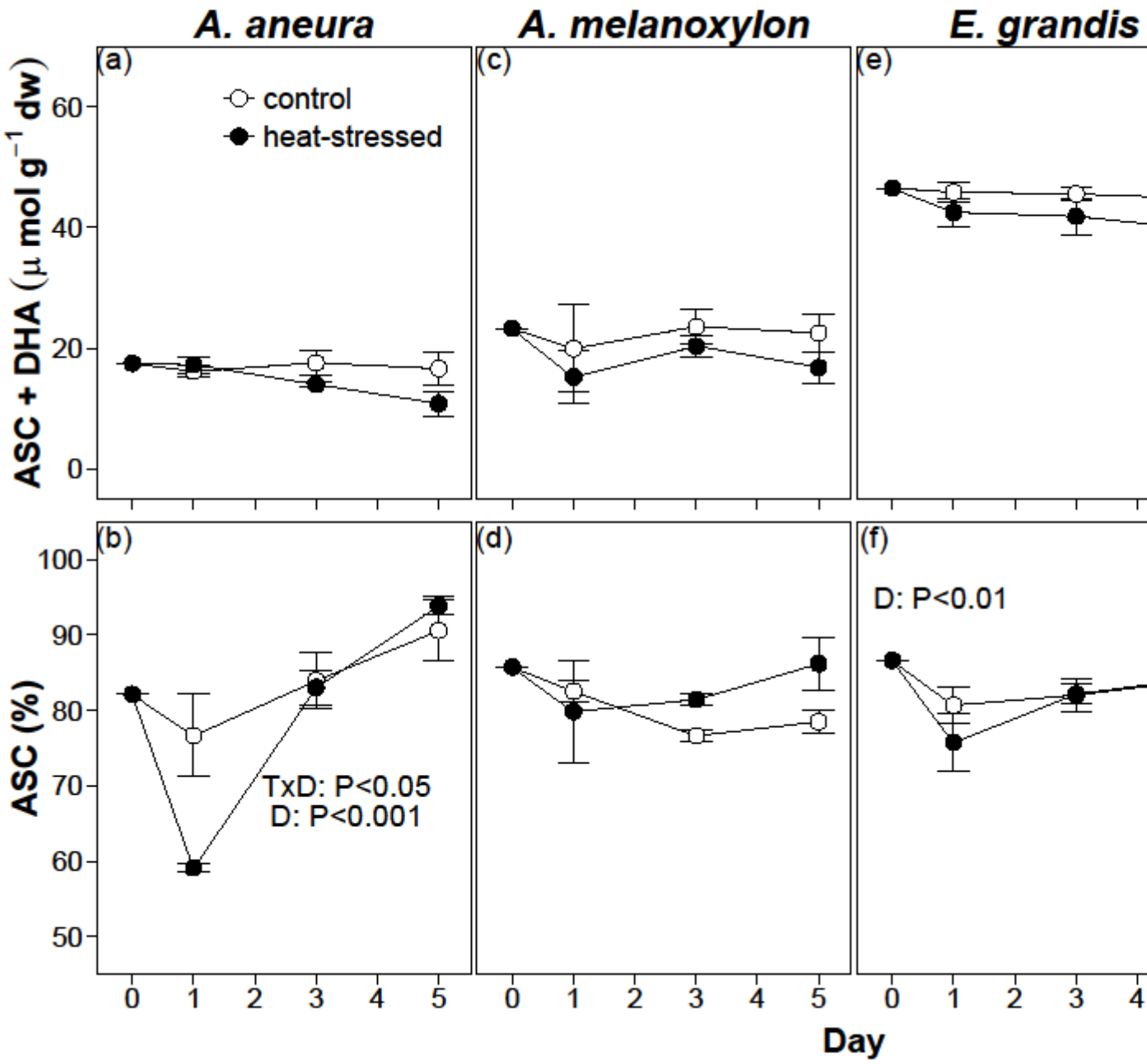
655
656


A. aneura
A. melanoxylon
E. grandis
(a)
(b)
(c)
(d)
(e)
(f)
TxD: P<0.05
D: P<0.05
D: P<0.01
Fv'/Fm'
Fv/Fm
Day
0.0
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4
5

657
658


A. aneura
A. melanoxylon
E. grandis
(a)
T: P<0.05
(c)
(e)
TxD: P<0.05
GSH + GSSG (nmol g⁻¹ dw)
1200
800
400
0
(b)
T,D,TxD: P<0.05
(d)
T,D,TxD: P<0.001
(f)
GSSG (%)
80
60
40
20
0
0 1 2 3 4 5
Day

659
660


A. aneura
A. melanoxylon
E. grandis
(a)
(c)
(e)
control
heat-stressed
ASC + DHA (μ mol g⁻¹ dw)
(b)
(d)
(f)
ASC (%)
TxD: P<0.05
D: P<0.001
D: P<0.01
Day

676
677

Species name	***Eucalyptus tricarpa*** (L.A.S.Johnson) L.A.S.Johnson & K.D.Hill	***Eucalyptus grandis*** W.Hill	***Acacia melanoxylon*** R. Br.	***Acacia aneura*** F. Muell ex Benth
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Foliage type	leaves		phyllodes	

678

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