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Title: Plasticity of photosynthetic heat tolerance in plants adapted to thermally contrasting biomes

Running head: Plasticity of photosynthetic heat tolerance

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ABSTRACT

In many biomes, plants are subject to heat-waves, potentially causing irreversible damage to the photosynthetic apparatus. Field surveys have documented global, temperature-dependent patterns in photosynthetic heat tolerance (P_{HT}); however, it remains unclear if these patterns reflect acclimation in P_{HT} or inherent differences among species adapted to contrasting habitats. To address these unknowns, we quantified seasonal variations in T_{crit} (high temperature where minimal chlorophyll-*a* fluorescence rises rapidly, reflecting disruption to photosystem II) in 62 species native to six sites from five thermally-contrasting biomes across Australia. T_{crit} and leaf fatty-acid (FA) composition (important for membrane-stability) were quantified in three temperature-controlled glasshouses in 20 of those species. T_{crit} was greatest at hot field sites, and acclimated seasonally (summer>winter, increasing on average 0.34 °C per °C increase in growth temperature). The glasshouse study showed that T_{crit} was inherently higher in species from warmer habitats (increasing 0.16 °C per °C increase in origin annual mean maximum temperature) and acclimated to increasing growth temperature (0.24 °C $°C^{-1}$). Variations in T_{crit} were positively correlated with the relative abundance of saturated FAs, with FAs accounting for 40% of T_{crit} variation. These results highlight the importance of both plastic adjustments and inherent differences determining contemporary continent-wide patterns in P_{HT}.

INTRODUCTION

Global climate change is warming many terrestrial ecosystems, with greater frequency, duration and intensity of heatwaves predicted worldwide (Meehl & Tebaldi 2004; Hansen *et al.* 2012). Extreme heat waves can irreversibly inhibit key plant metabolic processes including photosynthesis (Berry & Björkman 1980; Teskey *et al.* 2015) and reduce biomass accumulation (Ameye *et al.* 2012; Bauweraerts *et al.* 2013). Understanding the extent to which heat tolerance of metabolism differs inherently among species adapted to contrasting environments, and the degree of plasticity of metabolic heat tolerance, is therefore central to predicting global species distributions in a future, warmer world (Reyer *et al.* 2013).

In plants, photosynthesis has long been recognised as one of the most thermally sensitive metabolic processes (Schreiber *et al.* 1975; Berry & Björkman 1980; Seemann *et al.* 1984). During heat wave events, rising leaf temperatures often result in increased rates of respiratory and photorespiratory CO_2 release exceeding carboxylation rates, with the result that rates of net photosynthetic CO_2 uptake (A_{net}) decline beyond an optimal temperature to which net photosynthesis is acclimated (Dewar *et al*. 1999; Teskey *et al*. 2014). Heat-induced closure of stomata (in response to rising vapour pressure deficit of air) can result in photosynthesis being limited by CO_2 supply. In addition to these factors, two key components of photosynthetic machinery are highly susceptible to initial exposure to heat stress. Firstly, the active state of Rubisco (and thus carboxylation rates) declines with increasing leaf temperature, attributed to a decline in the activity of Rubisco's regulatory partner protein, Rubisco activase (Crafts-Brandner & Salvucci 2000; Allakhverdiev *et al.* 2008; Takahashi & Badger 2011). Secondly, photosystem II (PSII) is sensitive to high leaf temperatures, with heat stress resulting in the unfolding of protein complexes and loss of manganese from the oxygen-evolving complex (Schreiber & Berry 1977; Enami *et al.* 1994). Collectively, the above factors result in rates of A_{net} declining as leaves experience heat wave events.

A rapid and commonly used method to quantify photosynthetic heat tolerance is to measure the critical temperature (T_{crit}) at which minimal chlorophyll *a* fluorescence (F_o) increases sharply as leaves are heated (Schreiber & Bilger 1987). T_{crit} reflects the high temperature threshold at which PSII begins to be damaged. Quantification of the temperature response of F_o (i.e. F_o -T curves) – or similar methods that assess PSII functionality (e.g. temperature dependence of the maximum quantum yield of PSII of dark-adapted leaves) – therefore provides insights into the heat sensitivity of PSII (Schreiber *et al.* 1976; Schreiber & Berry 1977; Bilger *et al.* 1984; Krause & Weis 1984; Krause *et al.* 2010; Zhang *et al.* 2012; Curtis *et al.* 2014; Krause *et al.* 2015; Curtis *et al.* 2016). Using such approaches, advances have been made in our understanding of the physiological mechanisms (Yamane *et al.* 1998; Hüve *et al.* 2006; Hüve *et al.* 2011), broader ecological patterns and significance of photosynthetic heat tolerance (P_{HT}) (Downton *et al.* 1984; Seemann *et al.* 1986; Knight & Ackerly 2001; Knight & Ackerly 2002; Ghouil *et al.* 2003; Knight & Ackerly 2003; Krause *et al.* 2010; Zhang *et al.* 2012; Curtis *et al.* 2014; O'Sullivan *et al.* 2017). What is less clear, however, is the extent to which there are inherent differences in P_{HT} among species adapted to contrasting habitats. Doubt also remains about the extent to which T_{crit} acclimates to sustained changes in growth temperature, and seasonal changes in multiple abiotic factors in the natural environment. As a result, our ability to predict spatial and temporal variations in P_{HT} in a future warmer world remains limited.

To assess whether variations in P_{HT} in nature are due to inherent differences (that could result from adaptation to contrasting environments) *vs* plastic changes (i.e. acclimation) in heat tolerance, a combination of field and controlled environment studies is needed. In a survey of literature, we found only one study that combined field and glasshouse measurements of T_{crit} (Downton *et al.* 1984); in that study, seasonal variation (spring versus summer) in T_{crit} was reported for 13 perennial desert species growing at a single field site, Death Valley, California. For two of the 13 species, Downton *et al.* (1984) reported evidence of acclimation of T_{crit} plants grown in temperature-controlled glasshouses. Ghouil *et al.* (2003)

showed that *Quercus suber* seedlings subjected to a range of growth temperatures (10 to 40 °C) in a growth cabinet exhibited acclimation of P_{HT}, with T_{crit} acclimating *ca.* 0.3 °C per °C increase in growth temperature (Ghouil *et al.* 2003). More recently, O'Sullivan *et al.* (2017) reported seasonal variations in T_{crit} at two sites (temperate woodland and a tropical rainforest) in Australia (three species at each site) consistent with thermal acclimation patterns, with the seasonal adjustments being similar to the biome-to-biome patterns of T_{crit} values measured in summer at each site. Similar results were recently reported by Sastry and Barua (2017) assessing seasonal variations in P_{HT} at a dry tropical forest in India. By contrast, seasonal measurements of T_{crit} in leaves of a sub-alpine evergreen tree in Australia revealed that heat tolerance was higher in trees experiencing ice-encasement in winter than warmer conditions in summer (O'Sullivan *et al.* 2013). Thus, uncertainty remains in how seasonal changes in the environment affect T_{crit}, and whether spatial patterns can be used to predict how T_{crit} varies seasonally. To address these issues, more data are needed on seasonal variability in T_{crit} in a wider range of biomes.

As noted earlier, another unknown is whether species exhibit inherent differences in P_{HT}. In a glasshouse study, Knight and Ackerly (2003) compared four pairs of congeneric species from hot desert and cooler coastal regions of northern California but found no inherent differences in T_{crit}. By contrast, a common garden study on Australian desert plants by Curtis *et al.* (2016) found that species adapted to higher water availability experienced thermal damage at lower leaf temperatures than species adapted to low rainfall sites The contrasting nature of the above studies highlights the need for studies using a wider range of species adapted to several biomes before firm conclusions can be made on whether P_{HT} differs inherently among species adapted to contrasting environments.

Heat-wave events often occur during periods of drought, with the combination of high leaf temperature and reduced water availability increasing the severity of damage to leaf physiological functions (Teskey *et al.* 2015). Responses to heat and drought share many similar pathways such as the accumulation of organic solutes and volatile organic compounds

(Santariu.Ka 1973; Sharkey 2005; Hüve *et al.* 2006; Velikova *et al.* 2011; Rodríguez-Calcerrada *et al.* 2013). There is evidence from controlled-environment studies that drought conditions can increase P_{HT} in individual species, sometimes on a scale greater than that of elevated growth temperatures (Ladjal *et al.* 2000; Ghouil *et al.* 2003). Collectively, such findings point to drought-mediated heat tolerance and thus the need for aridity to be considered when assessing how T_{crit} values differ among wet *vs* dry biomes and/or vary seasonally.

An important factor influencing how high temperature events affect metabolic processes is the degree of saturation of fatty acid (FA) in the lipid bilayer of cell membranes, including thylakoid membranes in chloroplasts (Berry & Björkman 1980; Hochachka & Somero 2002). Rising temperatures increase fluidity of cell membranes (Los & Murata 2004), with the result that membranes may become leaky at high temperatures. However, lipid physical properties can acclimate to sustained increases in growth temperature via incorporation of FAs with a higher saturation level (e.g. increasing the proportion of saturated acyl chains). While relatively few studies have investigated variations in FA composition along environmental gradients in natural ecosystems, comparison of plant species from desert and coastal areas (Knight & Ackerly 2002; Knight & Ackerly 2003) revealed higher proportions of saturated FAs in plants grown under higher growth temperatures. Warm-adapted desert species also show intrinsically higher lipid phase transition temperatures than cool-adapted ones (Pike & Berry 1980); similarly, warm-acclimated plants exhibit higher levels of saturated FA than their cool-acclimated counterparts (Pearcy 1978; Larkindale & Huang 2004). Changes in FA composition such as these are likely to have important implications for chloroplast membrane stability and thermal stability of photosynthesis (Berry & Björkman 1980), and as such, to be linked to variations in T_{crit}. While there is evidence of the importance of membrane FAs for temperature responses in model and crop plant systems (Kodama *et al.* 1994; Murakami *et al.* 2000; Alfonso *et al.* 2001; Sung *et al.* 2003; Falcone *et al.* 2004; Yan *et al.* 2008; Hall *et al.* 2014), no studies have, to our knowledge, attempted to

directly link variations in P_{HT} with lipid physical properties for non-crop plants growing in unmanaged, contrasting environments in nature.

In our study, we quantified variations in T_{crit} in a wide range of plant species adapted to five thermally contrasting biomes across the Australian continent, with measurements being made under both field and glasshouse conditions. Australia is noted for its unique flora and range of contrasting thermal and water-supply environments (e.g. wet forests in tropical and temperate regions through to inland, arid and semi-arid woodlands); it is also experiencing rising air temperatures (Perkins & Alexander 2013; CSIRO & Bureau of Meterology 2016) and longer duration and severity of heat waves (Cowan *et al.* 2014; Lewis & King 2015; Steffen 2015). By measuring T_{crit} values at several field sites across Australia in two seasons (with a range of mean daily temperatures of 6.6 to 30.2 °C), and by growing plants in three different temperatures under controlled conditions, our study tested the following hypotheses:

(1) Photosynthetic heat tolerance (P_{HT}) quantified as T_{crit}: (a) varies seasonally in the field across biomes, being higher in summer than winter, (b) acclimates to sustained changes in growth temperature under controlled environment conditions;

(2) T_{crit} values are inherently higher in species adapted to hot/dry environments than their cool/moist-adapted counterparts, with inherent differences and acclimation of T_{crit} both contributing to reported biogeographic patterns in P_{HT} (O'Sullivan *et al.* 2017);

(3) Variations in T_{crit} are associated with variations in leaf lipid physical properties, with hot-adapted and/or warm-acclimated plants exhibiting higher saturation level of FAs than their cool-adapted/cool-acclimated counterparts.

MATERIALS AND METHODS

Field sites and species sampling

Six sites from thermally contrasting biomes across Australia were chosen. The sites are widely distributed geographically: two tropical rainforests, Cape Tribulation and Robson

Creek are located in Far North Queensland (FNQ) (CT_FNQ and RC_FNQ); tropical savanna, Alice Mulga is in the Northern Territory (AM_NT); mediterranean woodland, Great Western Woodland in Western Australia (GWW_WA); warm-temperate forest, Cumberland Plain in New South Wales (CP_NSW); and, cool-temperate rainforest, Warra in Tasmania (WAR_TAS). All sites belong to the Australian SuperSite Network (www.tern.org.au). Mean annual temperatures (MAT) range from 9.8 to 24.3 °C; annual precipitation ranges from 291 mm to 3671 mm (Table 1). Two seasonal campaigns took place at each site, with the exception of CT_FNQ where data were collected in the wet season only; the timing of campaigns was designed, within logistical constraints, to maximise environmental differences at each site (Table S1).

For each campaign, measurements were conducted over a one to two week period. At all locations other than the two FNQ sites, species considered the most abundant were chosen, with four to five replicate trees (one leaf per tree) of ca. 10 species typically sampled at each site; in total, 62 species were sampled. Species selection at the FNQ sites was made on the basis of availability of accessible trees dominating the upper canopy. For the repeat visits, sampling was made on the same trees for more than 80% of individuals in GWW_WA and CP_NSW. For other sites, identical trees could not be precisely located – nevertheless, trees sampled in the two seasons were in close proximity to each other, sharing similar microclimates. Upper canopy, sun-lit branches were excised and the stems immediately re-cut under water and stored in cool, moist dark conditions until measurements, which occurred within six hours of sampling. For all sites, branch sampling was done from mature plants either in the morning or early afternoon.

Controlled environment study

Following completion of field campaigns, seedlings of 25 species (Table S2) were studied in glasshouses during 2015 at the Australian National University in Canberra. All species had been included in the earlier field studies and most were obtained as seedlings from local nurseries near each field site (refer to Table S2 for provenances and climate details). For six

species, seedlings were not available and plants were raised in glasshouse using seeds purchased from a commercial supplier; these plants were cultivated four months prior to obtaining seedlings of remaining study species. The 25 selected species represent four different climatic origins: tropical FNQ, with seedlings purchased from Nuruga Native Plant Nursery, Walkamin, Qld; warm-temperate NSW, with seedlings purchased from Downes Wholesale Nursery, Stanhope Road, Theresa Park, NSW; cool-temperate TAS, with seedlings purchased from Habitat Plants, Jones Rd, Liffey, Tasmania; semi-arid WA, with seeds purchased from Nindethana Australian Seeds, Albany, WA. Provenances of all species were close to the field sites. Only one species *Acacia aneura* was available for NT; this species had two provenances, WA and NT, and NT was not included in data analysis (Table S2) as our study was not designed to assess variation within individual species. When first purchased, seedlings were 30–50 cm in height similar to the seedlings cultivated from seeds. They were then re-potted into 18 x 18 x 25 cm free-draining pots containing organic potting mix, enriched with Osmocote® OSEX34 EXACT standard slow-release fertiliser (Scotts Australia, Bella Vista, NSW) and 30% river sand. Plants were watered daily to field capacity.

The controlled environment study, conducted at RSB, The Australian National University in Canberra consisted of two stages. In Stage 1, all plants were grown under a single temperature treatment (25/20 °C day/night) for two months to assess whether there were inherent differences in P_{HT} of the 25 selected species. In Stage 2, plants initially grown in Stage 1 were separated into two groups to assess the capacity of individual species to thermally acclimate to lower and higher growth temperatures by exposing plants to two growth temperature treatments (20/15 °C and 30/25 °C day/night). Statistical analyses were conducted separately for the two experimental stages.

In Stage 1, seedlings were arranged using a split-block design in three glasshouses. A total of 260 plants (25 species (+*A. anuera* from NT) × 5 replicates × 2 adjacent plants) were located in five replicate blocks; within each block, species from the same origins were randomly nested within sub-blocks randomly positioned within each block. To facilitate

subsequent separation of plants into cooler and warmer growth temperatures (i.e. Stage 2), Stage 1 included two adjacent plants of each species. Sampling for Stage 1 measurements started after two months of growth in the 25/20 °C treatment, using newly developed foliage. One of the two adjacent plants from each block was used for sampling for Stage 1 measurements, thus totally 130 measurements (i.e. 25 species (+*A. anuera* from NT) x 5 blocks) were made. The timing of sampling of each species × replicate combination was randomized (both within and among days), with measurements of P_{HT} (quantified as T_{crit}) and associated traits being made during daylight hours, at least two hours after sunrise and one hour before sunset. In another study, we monitored the temporal changes of T_{crit} of one species, *Polycias elegans* over a period of 19 days: no significant diurnal or day-to-day variation in T_{crit} was observed (Zhu *et al*., data not shown). For the current study, no significant differences in T_{crit} values were found among the five blocks distributed among the three glasshouses (F = 0.24, P = 0.849), indicating a lack of block effect on T_{crit}. Thus, while we cannot rule out diurnal fluctuations in T_{crit} for all of the selected species (e.g. in response to diurnal fluctuations of osmolytes such as sugars or other factors; Hüve *et al.* 2006), for the current study we assumed that sampling time did not influence T_{crit} of the selected species. Measurements were made over a 20-day period in winter (June) 2015 when day-length was ca. 10 hours (14 h night).

Stage 2 provided an opportunity to assess whether there were inherent differences in T_{crit}, using newly formed leaves developed under warmer (30/25 °C) and colder (20/15 °C) growth conditions. Stage 2 also provided an opportunity to evaluate the potential of T_{crit} to acclimate to contrasting growth temperatures. Based on the low variability in T_{crit} values among replicate blocks observed in Stage 1, time constraints, and the need to improve speed of sampling of the larger number of treatments, a smaller number of species (20 + *A. anuera* from NT) and replicates (four) were sampled in Stage 2; in total, 168 of plants (i.e. 20 species (+*A. anuera* from NT) x two treatments x four replicates) were measured in Stage 2, using plants sourced from Stage 1. Two of the original three glasshouses were used for Stage 2

(with temperatures adjusted to the new treatment requirements), with plants randomly arranged within four blocks. Measurements of T_{crit} commenced 20 days after temperatures were adjusted in the glasshouses. In most cases, newly-developed, mature leaves that formed under the new growth conditions were used for T_{crit} measurements; the exceptions were two *A. anuera* populations, as well as *A. burkitti* and *A. hemetelis*, where T_{crit} was measured using pre-existing mature foliage. As was the case with Stage 1, sampling sequence of each treatment/species/block was randomized. Measurements took place in spring (October) 2015 over a 15-day period when average day-length was ca. 13 hours.

In both experimental stages, whole-leaves or shoots (for small-leaf species) were detached in the glasshouses and put in plastic seal bags with moist paper. Bags with samples were stored in the dark in a cool box. Samples were then transported to an adjacent lab for measurements. All samples were taken from sun-exposed, fully expanded foliage.

Determination of photosynthetic heat tolerance

Whole detached leaves were placed in a Peltier temperature-controlled, well-mixed chamber (3010-GWK1 Gas-Exchange Chamber, Walz, Heinz Walz GmbH, Effeltrich, Germany) and kept in the dark for 30 min before data recording. The gas flow in the chamber was controlled by a LiCor 6400XT portable gas exchange system (LiCor Inc., Lincoln, NE, USA). Fluorescence signals were recorded every 30 s (i.e. at ca. 0.5 °C intervals) using a MiniPAM portable chlorophyll fluorometer (HeinzWalz, Effeltrich, Germany) by positioning the fibre-optic sensor on the surface of the glass lid of the chamber. The chamber was cooled to 10 °C during the dark-adaption period and F_o was observed to be stable, after which leaves were heated at a rate of 1 °C min^{-1} toward 60-70 °C. Leaf temperature was recorded every second using a small-gauge wire copper constantan thermocouple pressed against the underside of the leaf, with the thermocouple being attached to a LI-6400 external thermocouple adaptor (LI6400-13, LiCor Inc., Lincoln, NE, USA), with temperatures recorded by the both LI-6400XT and WALZ chamber. T_{crit} was calculated as the intersection of two regression lines, representing the flat and steep sections of the F_o-T response curve; see Fig. S1 for an example.

Fatty acid sampling and analysis

The method of quantification of fatty acid composition was modified from James *et al.* (2011). Fatty acid sampling followed Stage 2 of the glasshouse study. Totally 17 species were sampled for FA analysis. Fresh leaves were frozen in liquid N_2 and stored at -80 °C, and then freeze-dried (Virtis, Sentry 2.0, SP Scientific). Freeze-dried samples were ground using a ball mill; thereafter, ca. 5 mg samples were extractively methylated with 3 M methanolic hydrochloric acid/chloroform (1mL, 10:1 v/v) into fatty acid methyl ester (FAME). Heptadecanoic acid was used as internal standard. The methylation was quenched with 1ml water and the FAMES were extracted using 4:1 v/v hexane:chloroform. The extract was concentrated under N_2 gas stream and transferred to auto-sampler vials for GC/MS (Gas Chromatography Mass Spectrometry) analysis. Each FA species composition was expressed as the mole percentage of total FA. Double bond index (DBI) of FA composition was calculated as: $1\times\sum C_i{:}1 + 2\times\sum C_i{:}2 + 3\times\sum C_i{:}3$, where i represents the number of carbon atoms (chain length) and the subsequent number represents the number of double bonds.

Climate data

Field real-time (30 min resolution) climate data were obtained from the eddy covariance flux tower at each site, except for the two earliest campaigns, which preceded tower construction (dry season at RC_FNQ and summer at WAR_TAS in 2012) where we obtained climate data (temperature and precipitation) using ANUCLIM (Table S1). Thus, for the field data, we were able to calculate historic climatic variables based on the dates each plant was measured. For the common environment study, long-term climate data of species origins was obtained from the *Atlas of Living Australia* (ALA) (www.ala.org.au, sourced March, 2016) based on the provenances of each species provided by the nurseries or seed supplier. Climate data from ALA records were extracted using ANUCLIM V6 (Xu & Hutchinson 2011). A similar climate-sourcing approach can be found in Curtis *et al.* (2016).

Data analysis

Linear mixed models were used to conduct two-way analyses of variance (ANOVA) for field data to assess whether there were differences in T_{crit} values among sites and seasons. Site and season were set as fixed effects, while species and replicates (tree individuals) were included in the random term. For ANOVAs assessing the effect of season within individual sites, season and species were set as fixed effects, with replicates included in the random term. For the glasshouse work, a split-block design ANOVA was used for the stage 1 experiment to test for differences in T_{crit} among origins. For the Stage 2 experiment, a two-way ANOVA was used to identify differences in T_{crit} and FA composition among origins and between the two temperature treatments. Statistics were performed using GenStat (16^{th} edition SP1).

Pearson correlations were used to quantify correlations between paired variables (trait-to-trait or trait-to-climate). Linear regressions were performed to assess relationships between T_{crit} and acclimated temperature and FA species composition. Stepwise linear regression with forward selection was performed to assess relationships between T_{crit} and potential explanatory climate variables and leaf traits, and relationships between FA species composition and climate variables. Linear regressions were performed in R (R Development Core Team 2013).

RESULTS

Seasonal acclimation

A linear mixed model ANOVA that combined different species at each site revealed that there was a significant interaction between site and season when assessing variability in high temperature tolerance of photosynthesis (i.e. T_{crit}) values (Table 2). Because of this, seasonal variations in T_{crit} (Table S3, Fig. 1) were analysed separately for each site. This analysis showed seasonal differences in T_{crit} that were significant for four of the five sites that were visited twice [RC_FNQ, AM_NT, GWW_WA and CP_NSW (Table S3)], with P_{HT} being higher in the warmer season. There was no seasonal variation in T_{crit} at the most southerly site

(WAR_TAS), despite a 10 °C difference in mean daily temperature between the two seasons (Table S1). Importantly, significant differences in T_{crit} were also found among species (P < 0.001) at all sites except RC_FNQ (Table S3).

Based on measurements made at a single time point during the year (mostly in the warmest season), a recent global survey (O'Sullivan *et al.* 2017) reported a positive linear relationship between T_{crit} and mean maximum T of the warmest month (MTWM). The current study shows that - notwithstanding the lack of seasonal adjustments at WAR_TAS - those patterns hold when including measurements made in cooler seasons. By using mean maximum temperature (MMT) of 30 days prior to date of measurement (PDM) as a measure of recent thermal history, a similar linear pattern was found (Fig. 1). We decided to use 30 days PDM as this period is likely to be sufficient for full acclimation for leaf metabolic processes (Cunningham & Read 2003b; Reich *et al.* 2016). In addition, we also tested linear models using a continuous series of number of days ranging from 1 day to 30 days; using this approach, we found that the R^2 values were maximal after 30 days, albeit with relatively similar R^2 values and statistical significance values when using shorter periods (data not shown). Using this approach, we found that T_{crit} increased 0.28 °C per °C rise in MMT of 30 days PDM (T_{crit} = 40.56 °C + 0.28*MMT; R^2 = 0.364, P < 0.001) when including data from all six sites (Fig. 1, Table S5). We found no association between T_{crit} and precipitation (PPT) of 30 days PDM (R = 0.169, P = 0.075).

When combining all species at a site, plants at GWW_WA and RC_FNQ exhibited the highest seasonal variations in T_{crit} (increasing 0.52 °C and 0.51°C per °C rise in MMT of the previous 30 days), while WAR_TAS showed nearly no seasonal variation (0.04 °C per °C); the remaining two sites showed seasonal acclimation adjustments of ca. 0.2 °C per °C. Across all five sites where seasons were compared (i.e. excluding RC_FNQ where only one season of data was available), the average acclimation in T_{crit} was 0.34 °C per °C rise in MMT of 30 days PDM (Table 2).

Controlled environment study

In Stage 1 of the common environment study, all plants were subject to only one temperature treatment (25/20 °C day/night) and 25 species were surveyed for inherent differences in T_{crit}. We found significant differences of T_{crit} among plant origins (i.e. provenance) (Table S4). The highest T_{crit} values were found in plants sourced from tropical Queensland (FNQ, T_{crit} = 46.1 °C) and semi-arid regions of Western Australia (WA, T_{crit} = 46.1 °C), with plants from temperate ecosystems of Tasmania (TAS, T_{crit} = 43.9 °C) exhibiting the lowest T_{crit} values. For plants grown under a single favourable temperature regime, a positive linear relationship was found between T_{crit} and annual mean maximum temperature (AMMT) of plant origins, with high temperature tolerance being greatest in plants sourced from the hottest sites (Table S5). Thus, the results of Stage 1 suggest that high temperature tolerance of photosynthesis is inherently higher in plants adapted to hotter sites than their cold-adapted counterparts, increasing by 0.16 °C per °C increase in AMMT.

In Stage 2, we assessed the impact of colder (20/15 °C) and warmer (30/25 °C) growth temperatures on T_{crit}. Leaves from all origins showed consistently and significantly higher T_{crit} when developed under temperatures of 30/25 °C than when developed at 20/15 °C (Fig. 2, Table 3). Acclimation degree was 0.24 °C per °C averaged across all species and provenances (Table S7). As was the case in Stage 1 where leaves developed at 25 °C, in Stage 2, species from warm-climate origins also showed inherently higher T_{crit} than species from cool-climates when grown at 30/25 °C and 20/15 °C (F = 13.72, P < 0.001; Table 3). Combining Stage 1 and 2 of the glasshouse experiment when plants were assessed under three common temperatures, T_{crit} increased by 0.16 °C per °C increase in origin AMMT (T_{crit} = 40.56 °C + 0.16*AMMT; R^2 = 0.49, P < 0.001; Fig. 3, Table S5), suggesting that adaptation to warmer sites is linked to inherently higher P_{HT}. No significant correlation was found between T_{crit} and annual precipitation of species origin (R = -0.130, P = 0.290), suggesting that variations in inherent T_{crit} are not linked to precipitation of a species origin.

Compared at the origin-mean level, significant differences in T_{crit} were found among origins (Table 3). Within each environmental origin, substantial variability was observed in

the degree of thermal acclimation of T_{crit}, ranging from more than 0.30 °C per °C in some cases, whereas others exhibited acclimation less than 0.1°C per °C for a given provenance (Table S7). Interestingly, some species showed similar degrees of seasonal acclimation in the field and thermal acclimation in the glasshouse (e.g. *E. lucida*, *N. cunninghamii*, *P. elegans*), while others exhibited higher (e.g. *A parramattensis*, *A. burkitii*, *A. hemiteles*, *E. fibrosa*) or lower (e.g. *A. melanoxylon*, *M. squarrosa*, *P. apetala*) acclimation in the field than in the glasshouse. Thus, in addition to T_{crit} values differing markedly among co-existing species at each field site, the ability of some species to thermally acclimate appears to be highly variable in the field from controlled environment. Species from NSW exhibited the highest degree of acclimation (0.34 °C per °C increase in growth temperature) while species from other origins, including the thermally stable tropical wet forest region, exhibited slightly less acclimation, with T_{crit} increasing by 0.20–0.22 °C for each 1 °C increase in growth temperature (Table S7, Fig. 2). Importantly, there was no evidence of species from the different origins systematically differing in acclimation capacity, with a linear mixed ANOVA (with site of origin as a fixed effect and species as a random term) showing no significant differences in acclimation capacity among origins ($F = 0.81$, $P = 0.507$).

A previous global survey across seven biomes (O'Sullivan *et al.* 2017) suggested no relationships in T_{crit} with leaf mass per unit area (LMA), foliar concentrations of total nitrogen ([N]) or total phosphorus ([P]). Similar results were found in our current study ([N] and [P] data not shown). To investigate whether other traits might contribute to adaptive and acclimation-dependent variations in T_{crit}, we quantified membrane FA composition in the Stage 2 grown plants. Fatty acids detected and present in all plant species were: C16:0, C16:1, C18:0, C18:1, C18:2, C18:3. FAs with high abundance were C18:3 (ca. 50% of the total), C18:2 (ca. 17%), and C16:0 (ca. 15%). Relative abundance of two saturated FAs, C16:0 and C18:0, and the mono-unsaturated FA C18:1 were generally higher in plants acclimated to the warmer growth temperature of 30/25 °C than in plants acclimated to 20/15 °C (Fig. 4, Table 3). The relative amounts of the saturated FA C16:0 and the unsaturated FAs C18:2 and C18:3

differed significantly among the four origins from which the species were sourced ($P < 0.001$, Fig. 5, Table 3). C16:0 and C18:2 also exhibited significant linear relationships with AMMT of plant origins (Fig. 5, Table 3 & S5). Thus, C16:0 was not only more abundant in warm-adapted species it also increased when individual plants were grown under warmer conditions. Interestingly, C18:3 which is the most abundant FA in membrane and is generally thought to be more abundant in plants from cooler growth conditions, did not exhibit consistently lower abundance when plants were grown at 30/25 °C (compared to 20/15 °C grown plants). Therefore, there was an overall pattern of both acclimation and inherent differences in FA composition, with some types of FAs showing consistent adjustments from cool to warm growth conditions, and with plants from the warmest origins (FNQ and WA) exhibiting inherently higher saturated FA composition than their cool-adapted counterparts, when grown under common conditions in the glasshouse.

Pearson correlations revealed significant correlations between T_{crit} and the percentage of C16:0 ($R = 0.332$, $P < 0.001$), C16:1 ($R = -0.246$, $P = 0.\ 0.009$), C18:1 ($R = 0.355$, $P < 0.001$), C18:3 ($R = -0.368$, $P < 0.001$), and DBI ($R = -0.373$, $P < 0.001$). To assess whether FAs could be used to predict variations in T_{crit}, we used a stepwise regression approach using inputs of composition of C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3, and a combination of forward and backward selection within the stepwise approach, the best equation was: $T_{crit} = 46.58 + (0.865*\text{C18:0}) + (0.289*\text{C18:1}) - (0.080*\text{C18:3})$ ($R^2 = 0.385$, $P = 0.001$). Thus, the relative abundance of certain saturated and unsaturated FAs accounts for ca. 40% of the variation in high temperature tolerance of photosynthesis.

Discussion

Our study assessed the degree of plasticity and evolutionary adaptation in photosynthetic heat tolerance (P_{HT}) in native vegetation of several thermally contrasting biomes by combining both field surveys and controlled environment studies. The study also investigated whether variations in P_{HT} (as quantified by T_{crit} measurements) are linked to variations in the composition of membrane fatty acids; temperature dependent changes in FAs are known to

affect membrane fluidity but have not previously been linked to variations in P_{HT}. Our results provide strong support for hypothesis 1, showing that photosynthetic heat tolerance varied seasonally in the field, and is capable of acclimating to sustained changes in growth temperatures under controlled environment conditions. The controlled environment study also provided strong evidence of inherent differences in T_{crit} (hypothesis 2), with P_{HT} being inherently higher in species adapted to hot environments compared to their cooler-adapted counterparts. Importantly, we found no evidence that variations in P_{HT} in the field or controlled environment were linked to precipitation at each species origin. Such findings suggest that evolutionary adaptation history and plastic responses to the contemporary thermal environment both contribute to global patterns in P_{HT} (O'Sullivan *et al.* 2017), and that the lipid composition of cellular membranes (hypothesis 3) may be a crucial factor determining the level of P_{HT} observed in nature.

Acclimation and inherent differences of photosynthetic heat tolerance across biomes

In our study we extended the surveys on temporal patterns of P_{HT} to a wider range and greater number of biomes than in previous studies, and surveyed a broader range of species. Our study revealed that – with the exception of the wet forest site in Tasmania - T_{crit} acclimated in a consistent manner to both seasonal temperature variations and to sustained changes in glasshouses, suggesting T_{crit} is highly temperature dependent.

Given the mounting evidence that T_{crit} acclimates to growth temperature, we also need to consider the upper thermal limits of acclimation process that is, what is the maximum temperature that photosynthetic metabolism can cope with in nature? In our study, we surveyed T_{crit} values in field-grown plants growing at some of the hottest and driest sites in Australia, with summer maximum air temperatures near 40 °C in the period before measurements at three of the six sites (Table S1). Under these heat-wave and drought conditions, where adaptive and acclimation dependent changes would be expected to maximise heat tolerance, T_{crit} rarely exceeded 55 °C, with most species exhibiting maximum T_{crit} values near 50 °C. Two other studies on Australian desert plant species during summer

(Curtis *et al.* 2014; Curtis *et al.* 2016) also found the highest thermal tolerance indicated by T_{50} of photosynthetic efficiency (50% decline in the maximum quantum yield of PSII of dark-adapted leaves) - was below 55 °C. Similar results were also reported in studies of 35 desert species of P_{HT} in the USA (Downton *et al.* 1984) and 24 savanna woody species in China (Zhang *et al.* 2012). Moreover, Krause et al. (2010) found that for two late successional tropical tree species, the upper thermal limit of T_{50} was between 50 and 52 °C, while in our study T_{crit} of the tropical species rarely exceeded 50 °C. In the global survey, only a few species in the tropical rainforests of Peru exceeded this range (O'Sullivan *et al.* 2017). Thus, the available evidence strongly suggests that 50-55 °C is the upper limit by which acclimation/adaptation can increase heat tolerance of photosynthetic light reactions. If true, this suggests that even in species that can rapidly acclimate and which have inherent traits that maximize heat tolerance, leaf temperatures greater than 55 °C may be lethal to the photosynthetic electron transport processes that influence carbon uptake, and thus growth. This finding has particular relevance for predictions of how future heat waves will impact on the functioning of high temperature ecosystems around the world. Further work is needed to establish if the upper limit of P_{HT} differs among plants adapted to thermally contrasting environments and how quickly heat tolerance near the upper limit can be achieved. Future work is also needed to determine what factors control the upper limit of photosystem II.

A further question that arises from studies assessing plasticity of T_{crit} is whether there is a lower limit to T_{crit} values. In the recent global survey of P_{HT} by O'Sullivan *et al.* (2017), measurements were made in summer at a site in the Alaskan tundra where MTWM is 16.7 °C; at that site, T_{crit} was 41.5 °C. The overall relationship reported by O'Sullivan *et al.* (2017) suggests that at lower MTWM, T_{crit} values would decline further (e.g. at a MTWM of 10 °C, T_{crit} is predicted to be 40 °C). In our current study, we made measurements at a cold site in southern Tasmania (WAR_TAS) where mean maximum temperature of the measuring month was near 10°C (Table S1) and T_{crit} was 44 °C. Further, measurements of sub-alpine evergreen trees in Australia showed that T_{crit} values did not fall below 41 °C, even in winter when

leaves experienced ice-encasement (O'Sullivan *et al.* 2013). Thus, while there is an overall agreement between our seasonal-derived measurements (Fig. 1b) and the spatially-derived measurements of O'Sullivan *et al.* (2017), the available data suggests that T_{crit} does not fall below 40 °C. Taken together with the above discussion on maximum values, it is tempting to suggest that the operating range of T_{crit} values may be 40 °C (minimum) to 55 °C (maximum).

A further issue is how acclimation might affect the thermal safety margin (TSM) of T_{crit} – calculated as the difference between P_{HT} and maximum habitat temperatures (O'Sullivan *et al.* 2017; Sastry & Barua 2017). Based on both field and glasshouse study, we found the acclimation pattern to rising growth temperatures results in increases in P_{HT} that are below unity (~ 0.3 °C per °C), rising air temperatures (e.g. due to global warming) are likely to reduce the TSM, with the impact of this reduction on leaf function being greatest in summer than winter as leaves experience temperatures that approach the maximal values of P_{HT}.

Our results suggest that inherent differences of P_{HT} may contribute to about one third of the global patterns. In the O' Sullivan *et al*. (2017) study, T_{crit} increased 0.38 °C per °C rise in the MMT of the growth environment. In Stage 1 of our glasshouse experiment, we found that T_{crit} varied among species adapted to thermally contrasting biomes, rising 0.16°C per °C in MMT of origin (Fig. 3). Thus, PSII is inherently more heat tolerant in species adapted to hot biomes. However, we do not totally exclude the possibility of adaptation to water limitations because water availability may also play an important role in influencing P_{HT} in some species (Ghouil *et al.* 2003; Curtis *et al.* 2016). Species might vary in T_{crit} in the cooling ability of leaves which can be influenced by factors such as leaf thickness, shape and size (Vogel 2009; Leigh *et al.* 2012).

The finding of inherent differences in our study was not based solely on measurements of T_{crit} but also by quantification of membrane lipid composition, with the results supporting the hypothesis of 'membrane adaptation' (Hochachka & Somero 2002) whereby species adapted to hot biomes through genetic modifications that result in high

content of saturated FAs (i.e. C16:0) to maintain higher membrane stability when leaves are hot [note: such changes, while beneficial in hot climates, could however result in a penalty in cold biomes if membranes are too rigid]. Other biochemical adaptations that increase protein heat stability, for example induction of heat shock proteins (HSPs) (Vierling 1991; Coleman *et al.* 1995) or the accumulation of thermo-protectant osmolytes (Jiang & Huang 2001; Hüve *et al.* 2006) might also play a role in increasing inherent heat tolerance of species adapted to hot climates.

Role of membrane lipid composition

Our results suggest that adjustments of membrane lipid composition not only influence thermal acclimation of T_{crit} but also impact on inherent differences in P_{HT}. All the three major FA (C16:0, C18:2, C18:3) in our analysis showed patterns consistent with adaptation to the original habitats, with C18:2 exhibiting the strongest relationships with origins' long-term thermal history (Fig. 5). Given these observations, we suggest that the major FAs contributing to inherent differences in membrane properties for these natural ecosystem species may be C16:0 and C18:2, rather than C18:3 which has been studied extensively in model plants (Murakami *et al.* 2000; Matsuda *et al.* 2005).

The large variations of T_{crit} among co-existing species at individual sites in the global survey could not be adequately explained by leaf structure (i.e. LMA) or chemistry (i.e. [N] and [P]) (O'Sullivan *et al.* 2017). Our current study, building on that earlier work by assessing seasonal variations in T_{crit} at each site, also found high variability in T_{crit} among species, both in the field and in the glasshouse study. By investigating membrane lipid composition, we found that ca. 40% of the variation in T_{crit} of the glasshouse grown plants could be explained by FA composition alone. Hence, much of the variability in T_{crit} among species in the field is likely to be due to inherent differences in membrane lipid composition. Importantly, we now need to determine what other factors account for the remaining variation in T_{crit} among species. Here, studies on HSPs (Vierling 1991; Wang *et al.* 2004), osmolyte

adjustments (McNeil *et al.* 1999; Jiang & Huang 2001; Hüve *et al.* 2006)and volatile organic compounds (Sharkey *et al.* 2001; Rasulov *et al.* 2015) are likely to be informative.

Concluding comments

Photosynthetic heat tolerance, quantified as T_{crit}, reflects the upper temperature threshold above which normal functions of photosystem II are severely interrupted. Our study has provided strong evidence of the ability of P_{HT} of species from a wide range of habitats to acclimate to seasonal changes of temperature in the field and also the capacity to acclimate to sustained changes of temperature in controlled environment conditions. With this acclimation, plants are likely to have higher survival chances in facing the increase of intensity and duration of heat waves occurring along with warming, provided that sustained leaf temperatures remain below the upper limit of that acclimation. Above those thresholds, climate warming may finally cause significant negative effects on plant performance, leading to long-term changes in ecosystem function and species composition. Given that tropical and mid-latitude forests and woodlands are likely to approach the boundary of upper thermal limits earlier than equatorial biomes (Doughty & Goulden 2008; O'Sullivan *et al.* 2017), it seems likely that negative effects of heat waves will be seen in those ecosystems first.

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Table 1 List of the field sites surveyed in this study, including location, biome, vegetation types and climate data are annual long-term averages of interpolated data obtained from the Ecosystem Modelling and Scaling Infrastructure Facility (eMAST; www.emast.org.au). Moisture index is shown as the ratio of precipitation to potential evapotranspiration. Additional details for each site may be found on the Australian Supersite Network website (www.supersites.net.au).

Site	Latitude	Longitude	Biome	Vegetation	Mean annual Temp. (°C)	Annual PPT (mm)	Moisture index
Cape Tribulation, Far North Queensland (CT_FNQ)	16°60' S	145°27' E	Tropical rainforest	Closed forest	24.3	3671	3.36
Robson Creek, Far North Queensland (RC_FNQ)	17°7' S	145°38' E	Tropical rainforest	Closed forest	20.4	1813	1.65
Alice Mulga, Northern Territory (AM_NT)	22°17' S	133°15' E	Tropical savanna	Low, open woodland	22.5	357	0.22
Great Western Woodlands, Western Australian (GWW_WA)	30°16' S	120°42' E	Mediterranean woodland	Semi-arid woodland	18.9	291	0.31
Cumberland Plain, New South Wales (CP_NSW)	33°37' S	150°44' E	Temperate forest	Semi-humid woodland	17.7	788	0.83
Warra, Tasmania (WAR_TAS)	43°5' S	146°39' E	Temperate rainforest	Tall, wet forest	9.8	1591	4.41

Table 2 Seasonal variations of T_{crit} (°C) and linear mixed model ANOVA results (*F* and *P* values) for five sites where measurements were made in both seasons in the field study (note: T_{crit} of CT site was only quantified in one season). For the two tropical rainforest sites, seasons are distinguished more by variations in rainfall (wet or dry) than temperature. Thus, wet and dry seasons are used, with the wet season being slightly warmer than the dry season. 'Acclimation degree' was calculated as the seasonal change in T_{crit} using species mean values, expressed per °C change in the mean maximum temperature (MMT) of the 30 days prior to the date of measurement. Values shown are the site/season mean (standard error, number of observations), with means of each site/season combination calculated using species mean values.

Site	T_{crit} (°C)		
	Summer / Wet	Winter / Dry	Acclimation degree
CT_FNQ	48.35 (0.58, 43)	-	-
RC_FNQ	49.17 (0.28, 55)	46.79 (0.53, 24)	0.51 (0.13, 11)
AM_NT	50.11 (1.56, 10)	46.82(1.07, 21)	0.22 (0.08, 5)
GWW_WA	50.58 (0.58, 25)	46.01 (0.67, 40)	0.52 (0.09, 15)
CP_NSW	47.42 (0.41, 51)	44.97 (0.57, 53)	0.20 (0.06, 11)
WAR_TAS	44.55 (0.67, 15)	44.01 (0.66, 34)	0.04 (0.02, 8)
Average	48.52 (0.34, 199)	45.71 (0.32, 172)	0.34 (0.05, 50)
Source of variation (Linear mixed ANOVA)			
	F	P	
Site	10.9	< 0.001	
Season	111.47	< 0.001	
Site×Season	6.84	< 0.001	

Table 3 Two-way ANOVA for impact of growth temperature (20: 20/15°C; 30: 30/25°C) and species origin on high temperature tolerance of photosynthesis (T_{crit}), and fatty acid composition: C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, and double bond index (DBI) expressed as a percentage of total FA content of plants sourced from four regions across Australia.

	Origin		Treatment		Origin × treatment	
	F	*P*	*F*	*P*	*F*	*P*
T_{crit}	13.03	< 0.001	46.25	< 0.001	0.39	0.760
C16:0	19.17	< 0.001	4.67	0.033	0.20	0.894
C16:1	4.34	0.006	0.32	0.572	0.42	0.741
C18:0	9.91	< 0.001	7.44	0.001	0.09	0.965
C18:1	4.12	0.008	5.56	0.002	0.69	0.563
C18:2	21.60	< 0.001	2.95	0.089	2.84	0.041
C18:3	13.28	< 0.001	0.49	0.486	1.30	0.279
DBI	6.03	< 0.001	1.41	0.237	0.44	0.728

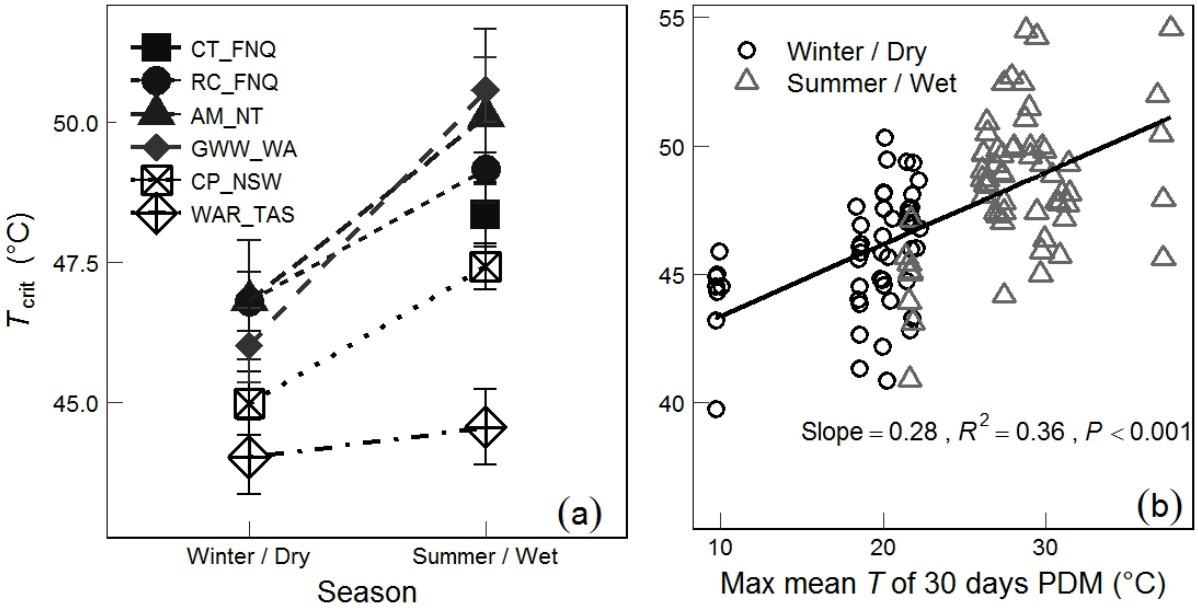

Figure 1 Variations of field measured T_{crit} between two seasons (a) and the linear relationship (b) between T_{crit} and mean maximum temperature (MMT) of 30 days prior to the date of measurements (PDM). (a) Data shown for six sites distributed across the Australian continent: CT_FNQ, Cape Tribulation in tropical wet forest Far North Queensland; RC_FNQ, Robson Creek in tropical wet forest Far North Queensland; AM_NT, Alice Mulga in an arid woodland of Northern Territory; GWW_WA, Greater Western Woodland in semi-arid woodland, Western Australia; CP_NSW, Cumberland Plain in temperate woodland of New South Wales; WAR_TAS, Warra in a cool-temperate wet forest in Tasmania. For all sites other than CT_FNQ, measurements were made in the cool and warm seasons. (b) Linear models found the slope and intercept of the fitted regression line under two seasons did not differ. Thus, only one regression was used by combining data from both seasons. Details of the statistical analysis can be found in Table S5.

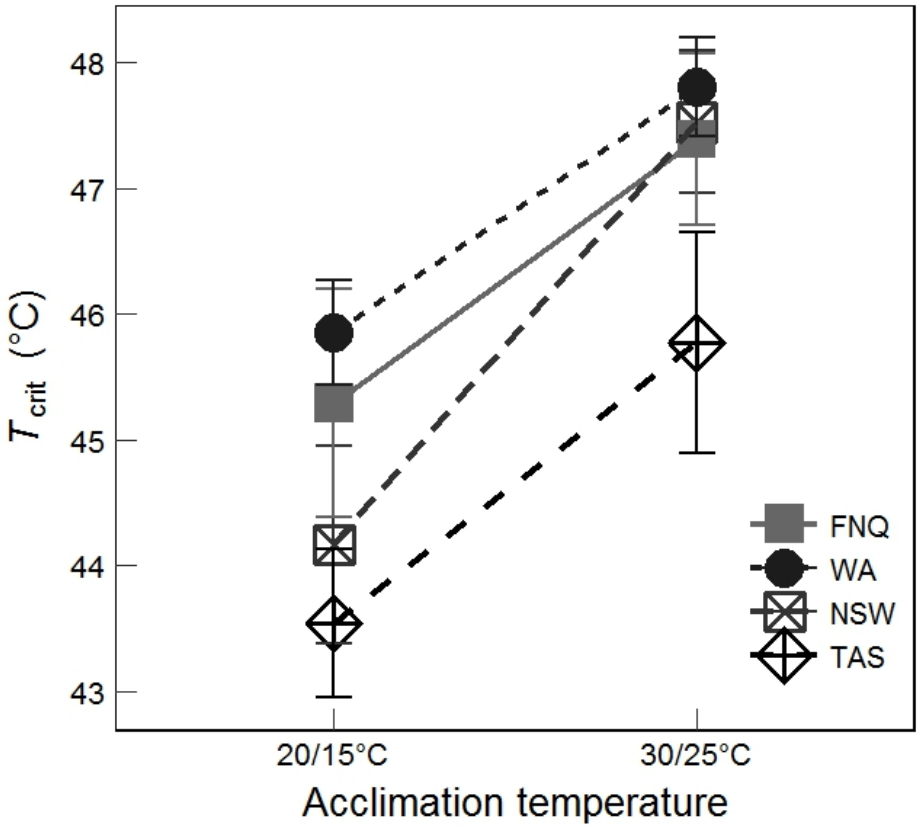

Figure 2 Response of T_{crit} to two growth temperature treatments (20°C day /15°C night and 30°C day /25°C night) in plants from different origins grown in Stage 2 of the glasshouse study. Origins are ordered from warm to cool biomes. FNQ represents tropical rainforest, WA for Mediterranean woodland, NSW for temperate forest and TAS for temperate rainforest.

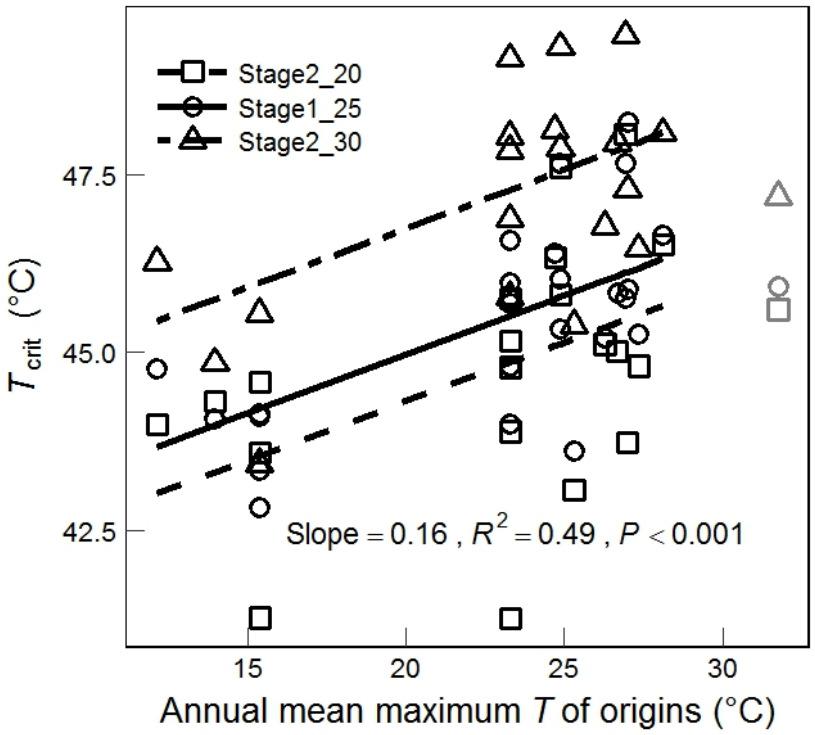

Figure 3 Relationships between high temperature tolerance of photosynthesis (T_{crit}) and annual mean maximum temperature (AMMT) of the origin (i.e. provenance) of individual species (note: *A. aneura* from NT (AMMT > 30 °C) is showing in grey symbols and was not included in data analysis). Points show species means and different symbols represent different growth temperature treatments: 25/20°C (day/night) in the glasshouse Stage 1 experiment (Stage1_25); 20/15°C (Stage2_20) and 30/25°C (Stage2_30) in the Stage 2 experiment. Models found the slope under three temperature treatments did not differ but intercept differed. Lines show linear regressions of T_{crit} and AMMT for plants grown under three common temperatures. Details of linear regressions can be found in Table S5.

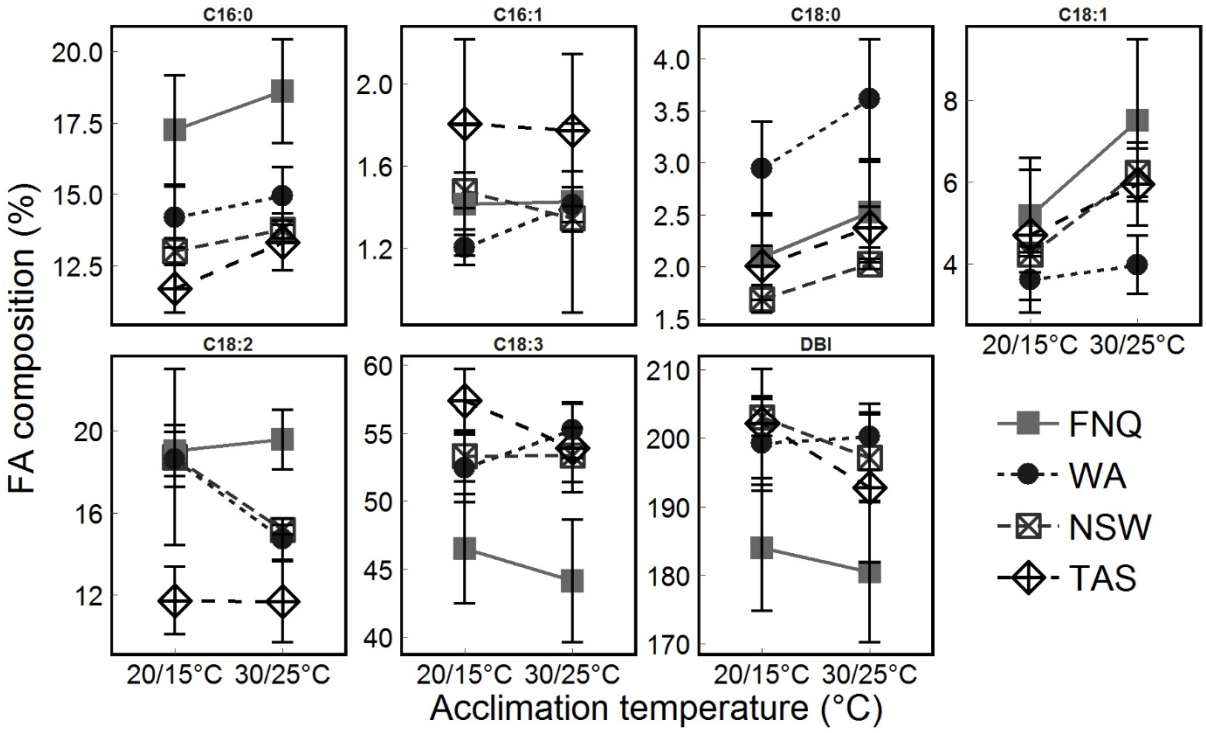

Figure 4 The responses of fatty acid (FA) composition including double bond index (DBI) to two temperature treatments (20°C day /15°C night and 30°C day /25°C night) in plants from different origins grown in Stage 2 of the glasshouse study. Origins are ordered from warm to cool biomes. FNQ represents tropical rainforest, WA for arid Mediterranean woodland, NSW for warm-temperate forest and TAS for cool-temperate rainforest.

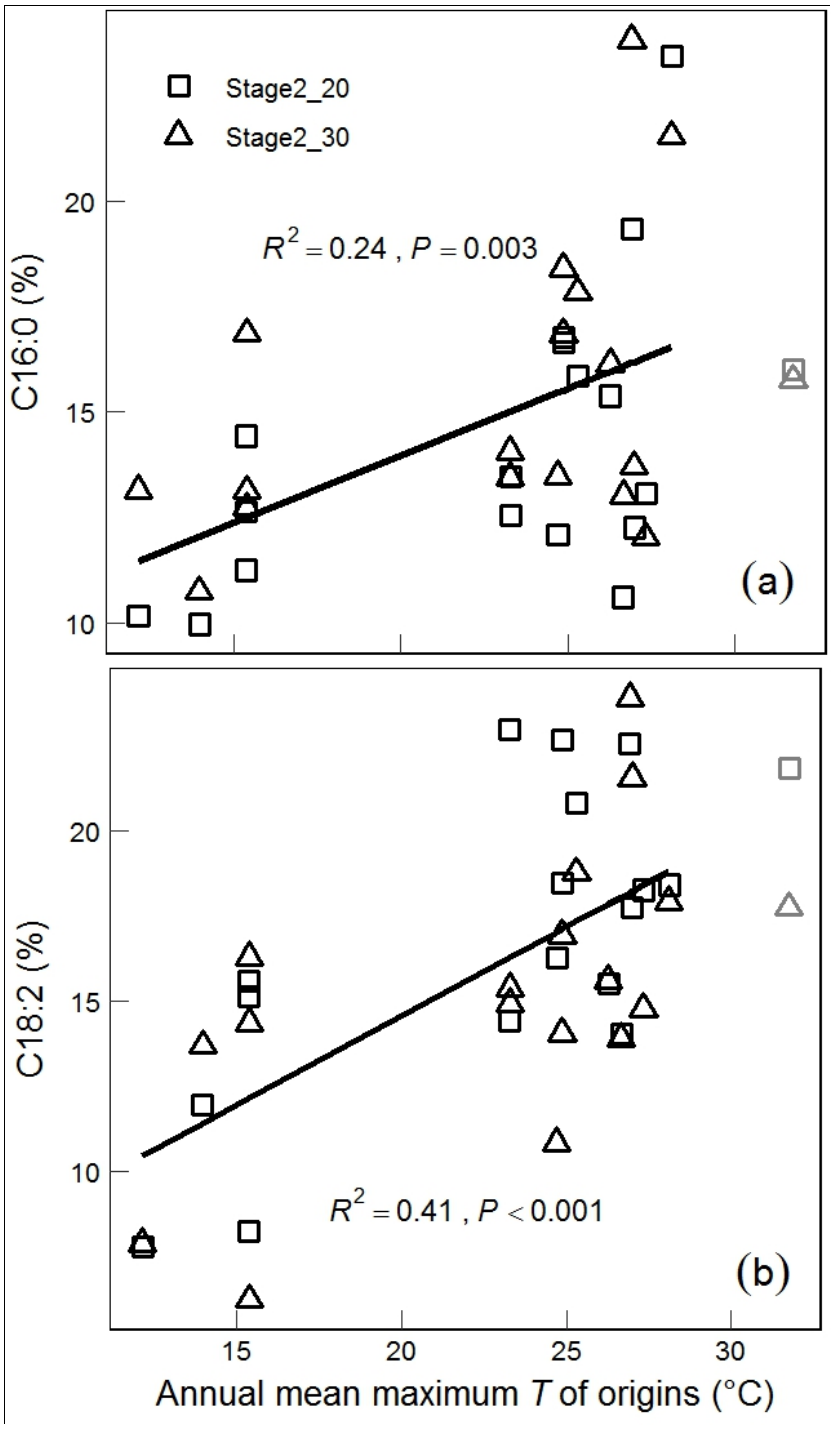

Figure 5 Relationships between fatty acid composition and annual mean maximum temperature (AMMT) of the origin (i.e. provenance) of individual species (note: *A. aneura* from NT (AMMT > 30 °C) is showing in grey symbols and was not included in data analysis) for plants grown in Stage 2 experiments, either at temperature treatment 20°C day /15°C night (square) or 30°C day /25°C night (triangle) day time temperature. Panels show

the percentage of total fatty acid (FA) composition present as (a) C16:0, (b) C18:2. Models found the slope and intercept under two temperature treatments did not differ. Thus, only one regression was used by combining data from both treatments. The relationship between T_{crit} and FA composition (composition of all individual FAs) was investigated by performing stepwise regressions (see main text in result section). All regressions were performed using species mean data.