

Functional Plant Biology

Consistently high heat tolerance acclimation in response to a simulated heatwave across species from the broadly distributed *Acacia* genus

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ABSTRACT

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Handling Editor: Suleyman Allakhverdiev When leaves exceed their thermal threshold during heatwaves, irreversible damage to the leaf can accumulate. However, few studies have explored short-term acclimation of leaves to heatwaves that could help plants to prevent heat damage with increasing heatwave intensity. Here, we studied the heat tolerance of PSII (PHT) in response to a heatwave in *Acacia* species from across a strong environmental gradient in Australia. We compared PHT metrics derived from temperature-dependent chlorophyll fluorescence response curves $(T-F_0)$ before and during a 4-day 38°C heatwave in a controlled glasshouse experiment. We found that the 15 *Acacia* species displayed surprisingly large and consistent PHT acclimation responses with a mean tolerance increase of 12°C (range, 7.7–19.1°C). Despite species originating from diverse climatic regions, neither maximum temperature of the warmest month nor mean annual precipitation at origin were clear predictors of PHT. To our knowledge, these are some of the largest measured acclimation responses of PHT from a controlled heatwave experiment. This remarkable capacity could partially explain why this genus has become more diverse and common as the Australian continent became more arid and suggests that the presence of *Acacia* in Australian ecosystems will remain ubiquitous with climate change.

Keywords: Acacia, Australian plants, climate change, heat tolerance, leaf physiology, photosystem II, plasticity, Tcrit, thermal safety margin.

Introduction

Heatwaves are prolonged periods of excessive heat (Perkins and Alexander 2013) that are increasing in duration, frequency, and intensity as the climate continues to change (Perkins-Kirkpatrick and Gibson 2017; Harris *et al.* 2018; Perkins-Kirkpatrick and Lewis 2020). For each degree of mean annual temperature increase, most regions on Earth are predicted to experience additional heatwave days at more extreme temperatures (Perkins-Kirkpatrick and Gibson 2017). The resilience of natural systems will be tested by heatwaves of increasing magnitude. However, despite model predictions for increasing prevalence and severity of heatwaves, relatively few studies have investigated the impacts that heatwaves have on plant heat tolerance (Geange *et al.* 2021), which is critical to understand plant vulnerabilities and tipping points (Breshears *et al.* 2021). Basal heat tolerance itself is not currently well known for many species of plants currently, let alone the capacity for a plastic response within the period of heatwaves. It is therefore essential that both heat tolerance and its plasticity be explored to better understand the potential for ecosystem composition to shift with climate change.

Photosynthesis has long been recognised as one of the most thermally sensitive metabolic processes (Schreiber and Berry 1977; Berry and Björkman 1980; Seemann *et al.* 1984). Two key components of the photosynthetic machinery are highly susceptible to changes in temperature. The active state of Rubisco declines with increasing leaf temperatures and PSII is very sensitive to high leaf temperatures, with heat stress

Received: 11 May 2022 Accepted: 18 September 2022 Published: 10 October 2022

Cite this:

Andrew SC et al. (2023) Functional Plant Biology, **50**(1), 71–83. doi:10.1071/FP22173

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Functional Plant Biology

causing unfolding of protein complexes and loss of manganese from the oxygen-evolving complex (Crafts-Brandner and Salvucci 2000; Takahashi and Badger 2011; Jajoo and Allakhverdiev 2017). A common method to quantify PSII Heat Tolerance (PHT) is to measure the critical temperature at which minimal chlorophyll *a* fluorescence (F_0) increases sharply as leaves are heated (Schreiber and Berry 1977; Smillie and Nott 1979; Seemann *et al.* 1984; Knight and Ackerly 2002; Knight and Ackerly 2003; Neuner and Pramsohler 2006; Hüve *et al.* 2011; Arnold *et al.* 2021; Coast *et al.* 2022; Posch *et al.* 2022). This critical temperature indicates a threshold beyond which physiological and photochemical systems have impaired function and where membrane integrity is reduced such that damage might occur if temperatures are sustained.

Differences among species in heat tolerance capacity may arise from adaptation to different local environmental conditions, differences in capacity for leaf temperature regulation or plastic changes due to environment. Changes in PHT from short-term physiological, structural, and biochemical adjustments in the chloroplast in response to heat stress are referred to here as plasticity, and more specifically acclimation, when heat tolerance improves with increasing temperature (Knight and Ackerly 2002; Wahid *et al.* 2007; Zhu *et al.* 2018). Numerous studies have demonstrated that leaf heat tolerance can increase during or by prior exposure to heat stress through acclimation (Downton *et al.* 1984; Buchner *et al.* 2017; O'Sullivan *et al.* 2017; Zhu *et al.* 2018), but few have investigated responses within the period of heatwaves specifically.

The few studies that have examined the heat tolerance limits of leaves, in response to heatwaves, using measures of chlorophyll fluorescence demonstrate that different species have rather varied responses. So far, it seems that some species can respond to heatwaves by slightly increasing their PHT (e.g. Drake et al. 2018; Karadar et al. 2018; French et al. 2019; Ahrens et al. 2021); however, it is unknown whether this adjustment in PHT is a general response or if PHT plasticity is species-specific or depends on factors such as species climatic origin. Furthermore, contrasting results between studies can also reflect differences in methodology rather than contrasting physiological responses (Rashid et al. 2020; Perez et al. 2021a). Comparing related species from varied climates within a single experiment under common conditions could help test the generality of the acclimation response. Focusing on congenerics could prove useful due to variation likely being more strongly linked to local adaptation rather than evolutionary distance as may be the case when comparing species from a range of genera (Lancaster and Humphreys 2020).

Climate and biogeography explain some of the variation in heat tolerance of plants. Globally, heat tolerance decreases slightly with absolute latitude and increases slightly with mean annual temperature (O'Sullivan *et al.* 2017; Lancaster and Humphreys 2020). Intuitively, the strongest climatic support for positive correlations between heat tolerance and maximum temperature of the warmest month (O'Sullivan et al. 2017; Perez and Feeley 2021) and annual mean maximum temperature (Zhu et al. 2018) of the origin (provenance) of individual species. Other water availability climate metrics, such as mean annual precipitation and precipitation of the warmest quarter have little support for driving differences in heat tolerance among species (Perez and Feeley 2021), despite transpirational cooling influencing both leaf temperature and heat tolerance (Drake et al. 2018). Beyond these macroclimatic and biogeographic drivers, local environmental conditions and microclimates that directly affect leaf temperatures may serve to explain additional variation in heat tolerance (Curtis et al. 2016). High PHT or high plasticity in PHT in response to heatwaves might mean higher resilience under future conditions with more intense and frequent heatwaves (Marchin et al. 2022). However, despite many studies on heat tolerance in wild plants, the extent to which heat tolerance relates to reproduction and survival remains to be seen. The measurement of PSII sensitivity to extreme temperatures in leaves using chlorophyll fluorescence is a widely used physiological measurement of heat tolerance, which is currently one of the best available metrics to partially represent overall heat tolerance (Geange et al. 2021). The Thermal Safety Margin (TSM) of leaves is the

drivers of heat tolerance in plants appear to be related to

maximum temperatures. Recent comparative studies found

difference between the plant's heat tolerance threshold and either their maximum leaf temperature or the maximum ambient air temperature. This margin is usually used to indicate how close plant leaves are to their thermal limits and therefore how vulnerable they may be to future warming and extreme events (O'Sullivan et al. 2017; Sastry and Barua 2017). Using leaf temperatures for calculating TSMs could provide more appropriate estimates of leaf vulnerability to extreme heat than estimates using air temperature due to leaf temperature often varying from air temperature (Leon-Garcia and Lasso 2019; Perez and Feeley 2020). Some studies using air temperatures to predict TSM have suggested that species with higher heat tolerance should have larger TSM (O'Sullivan et al. 2017; Sastry and Barua 2017). However, when using leaf temperatures instead, species that have high intrinsic heat tolerance also tended to have hotter leaves and therefore smaller TSM (Perez and Feeley 2020), indicating that these species might be more at risk. It is therefore essential to establish the contributions of leaf temperature, source climate, and species differences in PHT on changes in TSM in response to heatwaves.

To explore if PHT capacity and acclimation covaries between locally adapted species from diverse biomes, we grew a selection of native Australian *Acacia* species from seed in a common glasshouse environment before exposure to a controlled 4-day heatwave. *Acacia* is the largest plant genus in Australia and its diversification has been linked to

the exploration of novel climates as the Australian continent has become more arid since the Eocene (Renner et al. 2020). The group is prolific across a broad range of climates and many Acacia species have distributions that occur across multiple biomes (Dale et al. 2020). In addition, many species have become highly invasive outside their current range (Gallagher et al. 2011; Gallien et al. 2019). We ask whether Acacia species from a range of climatic origins differ in their PHT and TSM before and during a heatwave, which is indicative of local adaptation to their climate of origin. We expect species from warmer climatic origins to have higher PHT pre-heatwave, as well as a higher capacity for acclimating PHT during the heatwave, compared to species from colder origins. We also explore if PHT measurements for these species are above the current and expected future summer temperatures in their source location. In addition, we expect leaf temperature (T_{leaf}) to increase with $T_{\rm air}$, but $T_{\rm leaf}$ should not exceed PHT thresholds during a heatwave. We also expect species from warmer origins to have larger TSMs before and during the heatwave, compared to species from colder origins. Measuring species acclimation responses to heatwaves and changes in plant TSMs when under stress, could provide pivotal information for predicting which species will be most at risk across ecosystem under future climate scenarios.

Materials and methods

Study species and experimental design

The genus *Acacia* is one of the most diverse taxonomic groups in Australia with over 1000 accepted species and are prolific in many Australian vegetation communities and climates. The seed for the study species were sourced from the seed banks of the Australian Tree Seed Centre and the Australian National Botanical Gardens. The subset of available species that were selected to be grown were chosen to include collections from a range of source sites across Australia's climatic regions. There were 21 species grown but only 15 species (see Supplementary Table S1) that were sampled before and during the heatwave for PHT measurements. All 21 species are included in the Supplementary data but only the 15 species with data from both time points are included in the results presented here (see Supplementary R code).

Seeds were sown on 8 and 9 August 2019, immediately after activation to control seed germination date and therefore seedling age for this experiment. The seeds for some species could be activated with boiling water (2–3 min) while others with larger seeds needed scarification before being left to imbibe in water overnight after treatment. After imbibing, 3–5 seeds were sown per pot (15 cm diameter pots, volume \sim 3 L). The pots were laid out in a randomised block design with 15 blocks of 40 pots (600 pots in total) and with two pots per species in each block. The

blocks were laid out across six sets of benches $(\sim 3.5 \text{ m} \times 2 \text{ m})$ with two columns of three benches running down each side of the very large glasshouse, each row of two benches had three blocks with the middle block split across the right and left bench. The soil used was a custom-made eucalypt mix consisting of 1/3 potting mix, 1/3 river sand and 1/3 peat moss. The soil was kept moist during this germination period and pots were watered 2-3 times per week or as needed thereafter. Pots were fertilised with nodule stimulating nitrogen-free McKnight nutrient solution each week (McKnight 1949). Thus, plants were not water or nutrient limited. Extra seedlings or late emerging seedling were removed weekly or replanted to empty pots, replanting stopped after the first 6 weeks. In the growth glasshouse, the temperature was set to 24°C day (13 h) and 18°C night (11 h). This glasshouse had evaporative cooling and had under bench heating, but temperature control was not perfect. Three temperature loggers were kept in the glasshouse to take temperature measurements hourly, the mean maximum daily temperature was $24.4^{\circ}C$ (s.d. = $4.4^{\circ}C$) and the mean minimum daily temperature was 14.0°C (s.d. = 1.6° C), which is close to the expected values, but the hottest recorded temperature was 35.4°C and the minimum was 10.9°C. The plants also only received natural light and experienced natural changes in day length from the start of the experiment in winter to the end in summer. Despite this variation, all individuals were grown in a common environment from seed.

Seedlings were left to grow (size ranging from 12 to 265 mm) until 28 November 2019 when pots were moved to the Cropatron glasshouse (run by the Australian Plant Phenomics Facility, https://www.plantphenomics.org.au/ technologies/#greenhouses), which has a very high level of temperature control. From 28 November 2019 to 9 December 2019 plants were left to acclimatise after the move with temperature set to the same as previous (24°C day and 18°C night). On 10 December 2019, 4 months after planting, a 4-day heatwave treatment started, 38°C day (13 h) and 26°C night (11 h). The duration and magnitude of this heatwave follows the Australian Bureau of Meteorology definition of at least 3 days of significantly above average maximum and minimum temperatures (http://www.bom.gov.au/australia/ heatwave/knowledge-centre/). We took PHT measurements on the morning of the fourth day after the plants had completed three full days of maximum and minimum temperatures that were about 50% higher than growing conditions. The maximum temperature of 38°C selected here represents the upper range of mean maximum temperature experienced during the warmest month of the year at these species source location (Table S1), thus is a realistic and common temperature during summer in many parts of Australia. Plants were watered each morning during the heatwave so the effect of the temperature stimulus could be considered independently of droughting. Temperatures ramped up at 0800 hours and sampling was done between

1030 hours and 1200 hours with leaf temperature measurements taken at the end of this period, consistent for both pre-heatwave and Day 4 heatwave measurement days. Leaf temperature was measured using a FLIRi7 infra-red imaging gun (FLIR, Wilsonville, OR, USA). The infra-red images were taken from above and were analysed by taking five points per plant in the middle of leaves/phyllodes to acquire an average leaf temperature per plant using FLIR image processing software (FLIR 3.4 R&D version). Leaf size and shape varied greatly between these Acacia species and measurements were focused on the larger mature leaves/phyllodes or leaf clusters when leaves were very small. At this early stage of growth, leaf size and shape also varied greatly within species and even on a single plant, making it difficult to consider species differences due to leaf morphology. How seedlings grew also varied, seedling maximum height aboveground and also the length of the main stem were both measured before the heatwave, because some species tended to grow along the ground, the maximum of these two measurements was used to assess seedling size (Fig. S1).

Leaf PSII heat tolerance

We excised two leaves per plant on the day prior to the heatwave treatment and on the fourth day of the heatwave, which represented 4–25 samples per species per treatment (Table S1). Leaf samples were either an ~10 mm diameter leaf disk for large leaves, a leaf section for narrow leaves, or a whole leaf sample when leaves were <10 mm in diameter. Collected leaf samples were kept in bags with wet paper towel to avoid leaf dehydration prior to measurements, which were initiated within 2 h of harvest and completed within 5 h. To measure PHT of the *Acacia* species, we measured the temperature-dependent chlorophyll fluorescence response (*T*–*F*₀ curve) of leaf samples. Complete details of the system used are presented in Arnold *et al.* (2021).

Briefly, we attached leaf samples to white filter paper and placed on a Peltier plate (CP-121HT; TE-Technology, Inc., Michigan, USA). The thermoelectric modules of Peltier plate were controlled by a temperature controller (TC-36-25; TE-Technology, Inc.) and LabVIEW-based control software (National Instruments, Texas, USA) to heat the plate from 20°C to 70°C at a ramp rate of 60°C h^{-1} . This ramp rate is commonly used for most studies of PHT and supports comparability with existing data from the literature (e.g. Buchner et al. 2015; Zhu et al. 2018; Arnold et al. 2021). As temperatures across the Peltier plate surface that contained leaf samples are stable within $\pm 1^{\circ}$ C, we attached two type-T copper-constantan thermocouples (40-gauge, 2 m length; Omega Engineering Inc., NSW, Australia) to the underside of two randomly selected leaves on the plate as representative measures of leaf temperatures during the assay. Thermocouple temperatures were recorded every 10 s by a data logger (EL-GFX-DTC; Lascar Electronics Ltd., Salisbury,

UK) and the mean temperature of both thermocouples was used for thermal tolerance calculations. The Peltier plate assembly was fit within an aluminium frame at an ideal height below the fluorescence camera and double-glazed glass was used to compress samples against the plate surface to create a thermal buffer to ensure close matching of leaf and plate temperatures.

We used a Pulse Amplitude Modulated (PAM) chlorophyll fluorescence imaging system (MAXI-Imaging-PAM; Heinz Walz GmbH, Effeltrich, Germany) mounted above the Peltier plate to measure the basal chlorophyll fluorescence (F_0) after 30 min of dark adaptation, while the Peltier plate ramp the temperature up from 20°C to 70°C.

The $T-F_0$ curve produced by ramping the temperature of the leaf samples is characterised by a stable or slow-rise in F_0 values until a critical temperature threshold where there is a fast rise in F_0 . We normalised F_0 values for each leaf by scaling the minimum and maximum F_0 values between 0 and 1, so that relative F_0 values were used hereafter. We then calculated the inflection point of extrapolated regression lines for each of the slow and fast rise phases of the $T-F_0$ curve. This was achieved using break-point regression of the mean leaf temperature (from the two thermocouples) and relative F_0 values using the segmented package (Muggeo 2017) using the R Environment for Statistical Computing (R Core Team 2018). For each leaf sample, the inflection point referred to as the critical temperature, $T_{\rm crit}$, representing the onset of damage, the temperature at 50% of maximum F_0 (T_{50}) and the temperature at maximum F_0 (T_{max}), which corresponds to potential sustained damage to the photosynthetic apparatus, were extracted. There were 38 leaf samples where there was no clear inflection point in F_0 , from which $T_{\rm crit}$ could not be discerned, which were therefore excluded. T_{50} was highly correlated with T_{crit} (r = 0.92), therefore we used $T_{\rm crit}$ and $T_{\rm max}$ as measures of PSII heat tolerance hereafter. The $T-F_0$ curve to derive heat tolerance limits is a long-established and widely used technique across ecological and agricultural studies (e.g. Schreiber and Berry 1977; Seemann et al. 1984; Hüve et al. 2011; Arnold et al. 2022; Marchin et al. 2022; Posch et al. 2022). T_{crit} refers to the point of inactivation and potential damage to PSII, and T_{max} represents its complete disruption (Schreiber and Berry 1977; Smillie and Nott 1979; Terzaghi et al. 1989; Knight and Ackerly 2002; Ilík et al. 2003; Neuner and Pramsohler 2006; Frolec et al. 2008; Hüve et al. 2011). The values of T_{crit} and T_{max} may not necessarily correspond to permanent or irreversible damage (Hüve et al. 2011), but they define limits for structural thermal stability of PSII, a key component of the electron transport chain, beyond which function is impaired. These metrics are particularly useful for quantifying the capacity for species to respond to temperature change and allow us to directly compare acclimation responses among species.

Because the same individuals were not sampled at the preheatwave and Day 4 time points, species means were used to calculate the acclimation of PHT between the pre- and during heatwave time points. PHT acclimation was derived as the difference in mean PHT from pre-heatwave to mean PHT measured on Day 4 of the heatwave ($\Delta T_{\rm crit}$ and $\Delta T_{\rm max}$). We also calculated thermal safety margins (TSM) for each species as the differences between mean leaf temperature ($T_{\rm leaf}$) and mean $T_{\rm crit}$ or $T_{\rm max}$. Therefore, we have estimates for the TSM of inactivation of PSII ($T_{\rm leaf} - T_{\rm crit}$) and for complete disruption and potential damage of PSII ($T_{\rm leaf} - T_{\rm max}$). Post-heatwave recovery could not be measured due to root sampling and plant harvesting at the end of the heatwave for a related project.

Statistical analysis

All analyses and visualisations were carried out using R ver. 3.6.1 (R Core Team 2018). The *lme4* package was used for Linear Mixed Models (LMMs, Bates *et al.* 2015) and the package *lmerTest* (Kuznetsova *et al.* 2017) was used with *lme4* to calculate degrees of freedom and *P* values for the LMM outputs (we report significant trends when P < 0.05). For LMMs the *MuMIn* package from (Johnson 2014) was used to calculate the variance explained by fixed effects, known as marginal R^2 (mR^2) and the variance explained by both fixed and random effects is reported as the conditional R^2 (cR^2). The Inter-class Correlation Coefficient (ICC) describes the proportion of the total variance in the data that is explained by the random effects alone.

For LMM response, predictor variables were scaled. Semi-partial correlations (here after, 'semi-partial r') scale the response and predictor variables so the mean is 0 and the standard deviation is 1 (Schielzeth 2010). This scaling results in estimates that are similar to a Pearson's correlation coefficient and are able to be related to estimates of other response variables within models and between similarly structured models with the same predictors, with higher slopes indicating stronger relationships. However, all *P*-values and *t*-values remain unchanged due to scaling. Scaling also allows for binary variables, to be coded as -1 and 1, which allows these binary factors to be directly compared with continuous variables (Schielzeth 2010). All R code for our main analyses is reported in the Supplementary material.

To examine the effect of the heatwave on $T_{\rm crit}$, $T_{\rm max}$, and $T_{\rm leaf}$, LMMs were run with a similar structure. Temperature treatment was a binary fixed effect (coded as -1 and 1) and species was included as a random factor with an interaction with temperature treatment so random factor levels could have independent intercepts and slopes. To examine variation between species within time points for $T_{\rm crit}$, $T_{\rm max}$, and $T_{\rm leaf}$, a second set of LMMs was used. These models used source location maximum temperature of the warmest month (MTWM) and annual precipitation (from WorldClim variables BIO5 and BIO12 respectively, Fick and Hijmans 2017) along with mean species $T_{\rm leaf}$ measured on the day as fixed effects and species as a random factor.

The maximum temperature of the warmest month (MTWM) for source locations were averaged for five Global Climate Models (GCMs): ACCESS1.0, CESM1-CAM5, GFDL-ESM2M, HadGEM2-CC and MIROC5 to explore how far the measures of PHT pre- and during heatwave are from current and future projected MTWM. All GCMs used RCP 8.5 for years 2061-2080 and were accessed via the CHELSA climate data repository (Karger et al. 2017). These five models were selected based on recommendations from the Climate Change in Australia website (https://www. climatechangeinaustralia.gov.au), because they are representative for Australia and cover a range of scenarios. We focus on climate metrics from the provenance sites and not from across species distributions because we cannot assume that a single location is representative of the species. Instead, we consider differences between locally adapted populations from different species and source climates.

Results

Effects of heatwave on PHT and T_{leaf}

For our selection of native Australian Acacia species, from a broad range of bioclimatic regions (Table S1), we see a consistent increase in thermal tolerance during a controlled 4-day heatwave (Fig. 1a, b). Most of the variation in T_{crit} measurements across the two time points was explained by the fixed effect of the heatwave treatment (LMM: semipartial r = 0.81, $t_{14.8} = 13.22$, P < 0.0001, $mR^2 = 0.67$, Table S2). The variance explained by species was $\sim 10\%$ (ICC = 0.11), so for the entire $T_{\rm crit}$ model cR^2 = 0.78. For $T_{\rm max}$, the heatwave treatment explained slightly less of the variance across the two time points (LMM: semi-partial $r = 0.77, t_{13,3} = 13.97, P < 0.0001, mR^2 = 0.60$). The variance explained by the random factor levels of species was similar to $T_{\rm crit}$ (ICC = 0.10) and for the whole $T_{\rm max}$ model $cR^2 = 0.70$. As expected, T_{leaf} also showed a strong increase during the heatwave (Fig. 1c). Most of the variation in T_{leaf} measurements taken the day before and on Day 3 of the heatwave was explained by the fixed effect of heatwave time points (LMM: semi-partial r = 0.94, $t_{13,5} = 23.52, P < 0.0001, mR^2 = 0.87$) the variance explained by the random factor levels of species was only 8% (ICC = 0.08) and for the whole T_{leaf} model $cR^2 = 0.95$. These three models had a similar model structure with the binary fixed effect of heatwave treatment and random factor levels for species that had an interaction with the treatment effect, so random factor levels had random intercepts and slopes (Table S2). This similar structure makes semi-partial r values comparable across models and we see T_{leaf} changing the most with the heatwave treatment and T_{max} measurements changing the least. We also show current and future projected MTWM as an overlay in Fig. 1, which demonstrates that every Acacia species we measured



Fig. 1. PSII heat tolerance (PHT) acclimation in the response of 15 *Acacia* species to a controlled heatwave. (*a*) The change in species T_{crit} from the day before (preHW) to the morning of the fourth day of the heatwave (D4HW). (*b*) The change in species T_{max} for the same time points. Blue downward pointing triangles indicate current mean temperature of the warmest month (MTWM) at the source location and red upward pointing triangles indicate future MTWM at the source location. (*c*) Mean species T_{leaf} measured on the day before the heatwave and the first 3 days of the heatwave, where error bars show standard deviation.

had both higher pre-heatwave and acclimated PHT than the MTWM, even those projected for years 2061–2080.

Relationships between PHT and MTWM at the source location

Within the pre-heatwave time point, a moderate proportion of variation in T_{crit} and T_{max} was partitioned between species $(T_{\text{crit}} \text{ ICC} = 0.27, T_{\text{max}} \text{ ICC} = 0.24)$, but the fixed effects of MTWM and annual precipitation at the source location as well as mean T_{leaf} pre-heatwave were not significant predictors of PHT in both models (Table S3, $T_{crit} mR^2 = 0.13$ and $T_{\text{max}} mR^2 = 0.08$). The variance partitioned between species was similar at the end of heatwave time point $(T_{\text{crit}} \text{ ICC} = 0.21, T_{\text{max}} \text{ ICC} = 0.25)$ and the fixed effects were also non-significant (Table S3, $T_{\rm crit} mR^2 = 0.03$ and $T_{\rm max}$ $mR^2 = 0.03$). Most of the variation in T_{leaf} before the heatwave was partitioned between species (ICC = 0.56) but during the heatwave there was less differentiation in leaf temperature between species (ICC = 0.43) but the fixed effects of MTWM, annual precipitation, and T_{crit} were also non-significant (Table S3).

We expected the magnitude of PHT acclimation to be correlated with MTWM at the source location (Fig. 2a) where seed was collected. Because different individuals were sampled for the pre-heatwave and Day 4 time points, plastic change in PHT could only be calculated using species averages. Species mean T_{max} changes were significantly lower than changes in T_{crit} (Fig. 2b, ANOVA: F-value = 16.04, P < 0.001). Species from localities with lower MTWM also showed higher acclimation response across the heatwave (Fig. 2b). However, these negative trends with MTWM were non-significant (linear models T_{crit} : $R^2 = 0.12$, $t_{13} = -1.30$, P = 0.22 and T_{max} : $R^2 = 0.18$, $t_{13} = -1.67$, P = 0.12). Heat tolerance acclimation was also not related to the change in T_{leaf} between pre-heatwave and Day 4 (Fig. 2c; linear models T_{crit} : $R^2 < 0.01$, $t_{13} = -0.06$, P = 0.95 and T_{max} : $R^2 = 0.05, t_{13} = 0.78, P = 0.45$).

Heatwave effect on leaf temperature and TSM

TSM, which is the differences between species mean PHT and T_{leaf} , were all positive when calculated with T_{crit} (Fig. 3*a*;



Fig. 2. PSII heat tolerance (PHT) acclimation responses in relation to biogeography and climate. (a) Map of the mean maximum temperature of the warmest month (MTWM) across Australia with points indicating seed collection sites. (b) Mean species PHT acclimation values are plotted on the y-axis with T_{crit} differences between pre-heatwave and Day 4 of the heatwave plotted in black and T_{max} differences in red. The species differences between time points were calculated by subtracting pre-heatwave PHT values from the Day 4 heatwave measurements (°C; ΔT_{crit} and ΔT_{max}). These differences in species means are plotted against the MTWM at seed collection sites for each study species. The ΔT_{max} (red) were smaller than ΔT_{crit} (black), indicating that T_{max} showed less acclimation due to the heatwave. Negative trends for both T_{crit} and T_{max} indicating the species sourced from hotter locations had a smaller response to the controlled heat stress exposure. (c) PHT acclimation response against the change in leaf temperature during the heatwave.



Fig. 3. Leaf Thermal Safety Margins (TSM) in relation to the mean maximum temperature of the warmest month (MTWM). The TSMs were calculated as the difference between mean species PHT and mean species T_{leaf} . The preheatwave margins are plotted in black and during heatwave in red. (a) T_{crit} TSMs and (b) T_{max} TSMs. For both PHT traits, the TSMs declined during the heatwave. The slope also became less positive during the heatwave indicating that species from a range of environments maintained more similar TSMs during the heatwave while before the heatwave species from hotter climates (higher MTWM) generally had larger TSMs.

pre-heatwave mean = 18.72° C, range = $10.18-25.74^{\circ}$ C, during heatwave mean = 15.97° C, range = 11.30-18.46°C). $T_{\rm crit}$ TSM pre-heatwave were just significantly larger than during the heatwave (linear model: $R^2 = 0.13$, $t_{28} = -2.03$, P = 0.05). The T_{max} TSM were also smaller during the heatwave, but this difference was more significant (linear model: $R^2 = 0.59$, $t_{28} = -6.40$, P < 0.0001; pre-heatwave mean = 29.64° C, range = $25.65-35.39^{\circ}$ C during mean = 23.08° C, range = $17.50-26.77^{\circ}$ C). There were no significant relationships between TSMs and MTWM at the source locations (Fig. 3), but there was a positive trend that was nearing significance for $T_{\rm crit}$ TSM pre-heatwave (linear model: $R^2 = 0.14$, $t_{13} = 1.48$, P = 0.16). Changes in T_{leaf} were greater than the changes in T_{crit} (ANOVA: *F*-value = 7.26, P = 0.01) but generally these increases were consistent (Fig. 1). Therefore, we might expect TSM pre-heatwave to be able to predict TSM during the heatwave but there was no strong relationship ($T_{\rm crit}$ TSM during vs pre-linear model: $R^2 = 0.25$, $t_{13} = 2.09$, P = 0.056, and T_{max} TSM during vs pre-linear model: $R^2 = 0.23$, $t_{13} = 2.00$, P < 0.067). T_{crit} and T_{max} measurements were taken for each individual and were significantly correlated for the two time points with moderate R^2 values (Fig. S2: pre-heatwave $R^2 = 0.37$ and during heatwave $R^2 = 0.35$).

Discussion

Our study assessed how the heat tolerance of PSII changed in response to a controlled heatwave across 15 congeneric *Acacia* species from a wide range of bioclimatic regions (mean annual temperature ranging from 9.9 to 27.6°C, Table S1). We found that these related species display a surprisingly large and consistent PHT acclimation response ($\Delta T_{\rm crit}$ mean = 12.1°C, range = 7.7–19.1°C, $\Delta T_{\rm max}$ mean = 8.2°C, range = 4.4–12.1°C). To the best of our knowledge, these are some of the largest and most consistent plastic responses of PHT across species during a controlled heatwave experiment, reported in the literature. Although $T_{\rm crit}$ showed greater acclimation than $T_{\rm max}$, neither trait matched the change seen in $T_{\rm leaf}$ ($\Delta T_{\rm leaf}$ mean = 14.8°C, range = 10.2–20.9°C).

Extreme weather events have the potential to kill plants and reshape ecosystems as these events increase in severity and frequency (Tabassum *et al.* 2021; Marchin *et al.* 2022). The surprisingly high and uniform acclimation response of *Acacia* species to a heatwave could partly explain why species from the genus are successful across such a broad range of biomes. The high temperature and fire tolerance of the taxa (Renner *et al.* 2020) could mean the genus will be a climate change winner and thrive in more extreme heat and fire prone future conditions across Australia. Below, we contrast our findings to previous studies and explore their implications under predicted future climate scenarios.

PSII heat tolerance in Acacia species

Our study included *Acacia* species sourced from a range of bioclimatic regions (latitude ranging from 12.32°S to 42.97°S). Mean PHT pre-heatwave ranged from 39.9°C in *Acacia crassicarpa* A.Cunn ex Benth. to 50.4°C in *Acacia pruinocarpa* Tindale for T_{crit} , and from 52°C in *Acacia melanoxylon* R.Br. to 60°C in *A. pruinocarpa* for T_{max} . The range of PHT and TSM values that we report here pre- and during heatwave are at the upper end of TSM values reported in a global study spanning seven biomes (O'Sullivan *et al.* 2017), implying that the *Acacia* species we measured here have relatively high heat tolerance compared to a range of other plant species.

The MTWM at source locations currently and under future climate conditions was often well below these T_{crit} and T_{max} values (Fig. 1a, b). This result indicates that only the most extreme summer days are likely to limit PSII activity or induce leaf damage in these species, particularly if the plants have access to water and thus have capacity to cool their leaves via transpiration (Drake et al. 2018). However, an important caveat is that macroclimate metrics included here like MTWM do not include the effects of microclimate, which mean that individual plants can be exposed to temperatures well above or below location averages. Therefore, leaf temperature rather than macroclimatic variables can be a better predictor of PHT (Perez and Feeley 2020). Leaf temperature clearly increases under heat stress but leaf cooling capacity decreases under drought and heat stress, particularly under elevated CO₂, which are all important facets of climatic stress (Li et al. 2019). However, we note that the $T-F_0$ method to measure PSII heat tolerance captures only one aspect of plant sensitivity to heat stress and should be considered as a partial representation of overall heat tolerance, which is a multifaceted trait that is difficult to quantify.

Even though these *Acacia* seedlings were grown in a common environment, there was still large intra- and interspecific variation in PHT (Fig. 1, Table S3) that was not explained by the source climate predictors tested here. Other aspects of the species' biology likely explain some of this variation, for instance the variation in plant size, morphology, and leaf biochemical and physiological traits among our species might contribute to the variation in PHT as seen in other species and cultivars (Sharma *et al.* 2012; Brestic *et al.* 2018; Slot *et al.* 2021). Due to the variability of seedling leaf morphology and the logistical limitations of measuring multiple traits across species, in our study we captured these species differences through use of a random factor in our models.

The pre-heatwave relationships between PHT and source climate did show the expected slopes (positive for MTWM and negative for annual precipitation, Table S3), but these relationships were not significant, likely due to our relatively small number of data points. Similar positive relationships between PHT and MTWM were found in a global study, indicating that species from warmer climates have larger PHT than species from cooler environments (O'Sullivan *et al.* 2017). Another comparative study (Zhu *et al.* 2018) also found that PHT was inherently higher in species from warmer habitats. However, other recent studies on diverse species that found climate is a weak predictor of PHT (Perez and Feeley 2021; Slot *et al.* 2021), similar to our results in these *Acacia* species.

Large increases in PHT during heatwaves

Heatwaves can have a range of sub-lethal, negative effects on function, growth and reproduction (Breshears et al. 2021). Here, our controlled heatwave treatment of 38°C during the day may not be that different to the MTWM at some species' source locations (Table S1, Fig. 2a), and may not have had a serious negative impact on plants in the glasshouse. However, it was enough to stimulate a strong response from these seedlings that had grown in temperatures that were more benign. The species mean T_{max} values showed less change during the heatwave treatment than species mean $T_{\rm crit}$ values (Fig. 2), which suggests that the plastic response of the plants is increasing the point at which damage to PSII begins to accumulate rapidly, more than the temperature at which PSII function is impaired. This could be because the seedlings are reaching a limit to how far they can increase $T_{\rm max}$ or perhaps only a hotter and longer heatwave would stimulate individuals to increase T_{max} further.

Our study reinforces that the upper thermal limits of leaf function is not a fixed trait, and that Acacia species are capable of adjusting their PHT rapidly in response to heatwaves. However, it is not known to what extent the large acclimation response seen here translates into photosynthetic acclimation. There is evidence that PHT derived from chlorophyll fluorescence methods correlate with the breadth of temperatures over which carbon assimilation is possible as well as its optimum temperature (Perez et al. 2021b). Relationships between PHTs and fitness components such as growth, biomass, and reproduction are not yet known. These associations are worth exploring in the future to develop a better understanding of how PHT relates to whole-plant and ecosystem level impacts on carbon uptake and even species composition. Previous studies have shown that moderate heat stress can reduce carbon uptake through increases in mitochondrial respiration and photorespiration, and reduction in stomatal conductance and photosynthesis that are transitory and reversible (Bilger et al. 1984; Hüve et al. 2006). While photosynthesis can still occur at positive rates even at high temperatures up to around 45°C in some species, carbon assimilation rates decline well before plants reach their heat tolerance limits, such as T_{crit} or T_{50} (Hüve *et al.* 2011; Drake *et al.* 2018; Perez *et al.* 2021*b*).

The responses of PHT to heatwaves vary depending on species and context, for which there are several examples. Eucalyptus parramattensis E.C.Hall trees can increase PHT by \sim 3°C (up to 51°C) during a 4-day 43°C heatwave and maintain leaf temperature below critical temperatures by latent cooling (Drake et al. 2018), which is a more limited response compared to those reported in the present study. A test of the effects of heatwave severity in Corymbia calophylla (Lindl.) K.D.Hill & L.A.S.Johnson showed a 1.1°C increase during a 5-day heatwave where temperatures ranged from 40°C and 46°C, with the 40°C heatwave treatment maintaining heat tolerance at 1.5°C above the 29°C control (Ahrens et al. 2021). Six species of small Australian native forbs, shrubs, and grasses showed remarkably little adjustment of their thermal physiology in response to 3-day 39°C heatwaves, even when repeated up to three times, but their basal capacity to tolerate heat (~42-50°C) exceeded the temperatures of the heatwave (French et al. 2019). In an Australian alpine herb species Wahlenbergia ceracea Lothian, although a warming treatment increased heat tolerance by 0.7°C on average (up to 46-52°C), later exposure to a 4-day 34°C heatwave lead to very little change or even a small reduction in the heat tolerance of leaves (Notarnicola et al. 2021). Interestingly, a study on PHT in several alpine species found that certain species had a much greater heat hardening capacity (i.e. increase in T_{crit} after 5–8-day heatwaves) than others (Buchner et al. 2017).

Therefore, while rapid adjustments of PHT in response to heatwaves can be highly variable among species, responses are possibly more consistent in closely related species like in our study (despite high morphological differentiation between our seedlings). It will be valuable to explore the mechanisms behind variation in PHT acclimation in response to heatwaves in further detail. Such interspecific variation could be explained by a range of mechanisms that include a suite of biochemical and molecular responses to avoid or tolerate heat stress (Wahid *et al.* 2007; Mathur, Agrawal and Jajoo 2014; Nievola *et al.* 2017), the net result of which can be measured by the changes in chlorophyll fluorescence that we have used here (Schreiber and Berry 1977; Neuner and Pramsohler 2006).

Heat stress often occurs in conjunction with high irradiance, and the combination of these two factors has been shown to improve heat tolerance (Weis 1982; Havaux *et al.* 1991; Yin *et al.* 2010; Buchner *et al.* 2015). In addition, performing chlorophyll fluorescence measurements under light also seems to increase PHT (Buchner *et al.* 2015; Krause *et al.* 2015). Here, we measured PHT in the darkness, and perhaps even higher values of PHT could be obtained if these measurements were taken under light. It is also possible that more contrasting interspecific differences could be found if the species were locally adapted to different light conditions, though in our experiment all were grown under the same conditions.

TSMs in Acacia species

While leaf temperature increased during the heatwave, PHT thresholds were always well above T_{leaf} . The range of values for species leaf TSMs did reduce during the heatwave for T_{crit} , but the minimum and mean values did not change much (Fig. 3). This suggests that T_{crit} increased in parallel with the change in T_{leaf} to maintain T_{crit} at least 10°C above T_{leaf} (Fig. 3a). For T_{max} , TSMs showed a significant reduction during the heatwave coupled with a reduction in the range of values (Fig. 3b). This indicates that while T_{max} showed less acclimation than T_{crit} , species were still maintaining a minimum buffer between T_{max} and leaf temperature of about 17°C. The temperature at which these buffers start to significantly reduce could indicate the upper limits to acclimation in these PHT traits. For example, a moderate heatwave allows trees to maintain a much larger TSM than does an extreme heatwave (Ahrens et al. 2021). The PHT of plants could also improve over multiple heatwaves (Sgrò et al. 2010; Ahrens et al. 2021) and be adapted to the climatic environment where species originated (Ahrens et al. 2021). TSMs of the magnitude we report are not necessarily unusual for the leaf tissue of many species (e.g. Leon-Garcia and Lasso 2019). However, to the best of our knowledge, large TSMs in response to a heatwave have not been reported previously, and the generality of this response needs further consideration.

The PHT traits and TSMs demonstrate that there is a remarkable and consistent buffer for PSII in this range of Acacia species even in severe heatwaves predicted for the future. However, we recognise that heatwaves are frequently associated with periods of drought (Breshears et al. 2021). Our study provided water for the plants, which may have reduced their stress state and increased their capacity for PHT to respond to the heatwave and for transpirational cooling (Drake et al. 2018). Further investigations are needed to establish the degree to which PHT and TSM responses to heatwaves are also affected by compounding stressors of prolonged and acute drought-stress (Breshears et al. 2021) and the concomitant effects on photosynthetic performance under stress. In addition, our metric of heat tolerance on detached leaves reflects the stability of PSII under high temperatures and thus it might not translate into what a whole plant might be able to cope with, but rather it represents the thermal stability of a key component of the photosynthetic machinery.

Conclusions and future directions

Taken together, our results suggest that heat tolerance is highly plastic in 15 Australian *Acacia* species and shows an

increase that mirrors increased environmental and leaf temperatures, where $T_{\rm crit}$ is a more malleable heat tolerance trait compared to T_{max} . These results could contribute to explaining why Acacia species are successful across such a broad range of climates. The extent to which PHT relates to growth, survival, and reproductive capacity of plants are important questions that remain to be fully explored for a range of PHT methods and metrics. Exploring these heat tolerance traits further remains a valuable and relevant area of research given the impacts of climate change and extreme weather events on plants. Because most species maintained similar TSM between PHT and T_{leaf} measurements during the heatwave it is difficult to determine the species with the highest PHT capacity or tolerance niche. An experiment that ramps up temperature across multiple days with daily measurements could help define limits to species plasticity (i.e. the point at which leaf TSMs start to shrink) to differentiate the adaptive capacity of species. Measurements of PHT thresholds at neutral temperatures could also prove informative since species sourced from hotter climates generally maintain higher TSMs at neutral temperatures for our study. Therefore, the size of TSMs at benign temperatures could prove to be an effective and efficient surrogate measure of plants overall PHT. The impacts of climate change are already being felt. A rapid method for assessing species vulnerability to climate change, such as PHT and its plasticity in response to heat stress events, could help finding vulnerable species and predict future changes to ecosystems.

Supplementary material

Supplementary material is available online.

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Data availability. All data and R code for replicating our results are published on figshare link: https://doi.org/10.6084/m9.figshare.21085108.v1.

Conflicts of interest. The authors have no conflicts of interest to disclose.

Declaration of funding. This work was supported by the CSIRO's Environomics Future Science Platform, the Australian Research Council (grant number DP170101681, LP180100942, DE180100046, and DP200101382).

Acknowledgements. The authors would like to acknowledge the Australian Tree Seed Centre and the Australian National Botanical Gardens for providing seeds for the experiment. We acknowledge the National Collaborative Research Infrastructure Strategy of the Australian Government for supporting The Australian National University with the phenotyping equipment from the Australian Plant Phenomics Facility. The authors would also like to thank Adrienne Nicotra, Karel Mokany, Timothy Perez, and Nicole Bison for their useful reviews and comments on an earlier draft of this manuscript.

Author contributions. SCA and AKS designed and ran the experiment. PAA and VFB performed heat tolerance measurements. SCA and PAA carried out statistical analyses with support from co-authors. All authors contributed to writing and reviewing the manuscript and gave approval for publication.

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