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Functional Ecology

Water availability influences thermal safety margins for leaves

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Author Contributions

AMC, KVM and AL conceived the ideas, all authors contributed to the design of methodology, NB collected the data, AMC analysed the data, AMC led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Abstract

1. One application of plant physiological heat tolerance measurements is the assessment of vulnerability to increasing environmental temperatures under climatic change. A thermal safety margin, the difference between physiological tolerance and environmental temperature, is a common metric for the assessment of plant thermal vulnerability. However, there are biological and methodological aspects to consider when evaluating thermal vulnerability that have the potential to substantially alter assessments. Two such aspects include the leaf to air temperature relationship and the scale at which air temperature data are collected.
2. We grew plants of a desert species, *Myoporum montanum*, *in situ* under water-stressed and well-watered conditions, measured their leaf temperatures and photosynthetic heat tolerance (T_{50} threshold) every third day over 12 days in summer. Thermal safety margins were calculated based on leaf temperatures and compared to those calculated with local and regional air temperatures.

- Accepted Article
3. We found that heat tolerance and the thermal vulnerability assessment of a plant changed with water status. When water was readily available, plants maintained wide leaf temperature safety margins and displayed partial-homeothermy. When cooling via transpiration was limited, increasing leaf temperature corresponded with occurrences of leaf poikilo- and megathermy, higher heat tolerance, and narrower safety margins.
 4. Our study shows high physiological heat thresholds are not necessarily reflective of wide safety margins, but instead can indicate a greater vulnerability and increased risk of heat stress exposure. Calculating thermal safety margins using air temperatures can also substantially alter margin widths. Where possible, the use of leaf temperatures in assessments of thermal vulnerability will lead to more meaningful vulnerability assessments. We recommend considering the source and temporal pairing of temperature measurements as well as plant water status, when measuring and interpreting plant thermal safety margins.

Keywords: thermal safety margin, heat tolerance, photosynthetic thermal tolerance, thermal tolerance, thermotolerance, thermal vulnerability, climate model predictions

Introduction

To determine an organisms vulnerability to heat stress, a thermal safety margin, the difference between its physiological heat threshold and a temperature it experiences is often estimated (Deutsch *et al.* 2008; Sunday *et al.* 2014; O'Sullivan *et al.* 2017). Thermal safety margins are used to predict which species may be at a greater risk from heat stress now and under climate projections (e.g., Huey *et al.* 2009; O'Sullivan *et al.* 2017; Sastry & Barua 2017; Gallagher, Allen & Wright 2019).

For plants, physiological heat damage to photosystems is frequently measured with chlorophyll *a* fluorescence techniques (Schreiber & Berry 1977). A common heat damage threshold is T_{50} , the 50% decline in maximum quantum yield of Photosystem II (F_V/F_M), which is associated with irreparable damage to the photosystem apparatus (Schreiber & Berry 1977; Downton & Berry 1982). It therefore is logical to know and use leaf tissue temperatures to understand which plants are most likely to experience photosystem damage. However, air temperatures, which are more readily obtained, are typically used instead of leaf temperatures to determine plant safety margins (Curtis *et al.* 2016; O'Sullivan *et al.* 2017; Sastry & Barua 2017). The use of air temperatures assumes that leaf temperatures, and hence the temperatures that leaf photosystems experience, are equal or very close to air temperature.

Work on animals finds thermal safety margins based on ectotherm body temperature to be very different from air temperature-based margins (Bonebrake *et al.* 2014; Sunday *et al.* 2014; Pincebourde & Casas 2015) and behaviour also mediates thermal vulnerability (Kearney, Shine & Porter 2009; Pincebourde & Casas 2019). The scale of climate temperature, whether fine scale microclimate in which insects exist, such as leaf boundary layers (Potter, Davidowitz & Woods 2009), or broader scale climate variables, also influences accuracy of thermal vulnerability assessments (Potter, Davidowitz & Woods 2009; Pincebourde & Casas 2015; Pincebourde & Woods 2020). For plants, great variability exists in reported thermal safety margins. Among the few published leaf-based estimates, margins range from moderate (Perez & Feeley 2020) to wide (Leon-Garcia & Lasso 2019), while air-based margins can be negative to wide (Curtis *et al.* 2016; O'Sullivan *et al.* 2017; Sastry & Barua 2017). Variation in width may be influenced by species and latitude (e.g.

O'Sullivan *et al.* 2017); however, methodological differences may also play a role. The difference between margins using leaf (body) temperature and local air over more macro-environmental air temperatures is yet to be quantified.

Leaf temperature is influenced by a range of environmental conditions: air temperature, solar radiation (Fauset *et al.* 2018; Miller *et al.* 2021), wind speed (Drake, Raschke & Salisbury 1970; Leigh *et al.* 2012), leaf shading (Roden & Pearcy 1993), as well as plant characteristics such as leaf morphological traits (Leigh *et al.* 2017; Fauset *et al.* 2018) and stomatal conductance (Gates, Alderfer & Taylor 1968; Drake, Raschke & Salisbury 1970). When air temperatures are high, leaves can avoid heat stress through stomatal conductance (Gates, Alderfer & Taylor 1968; Drake *et al.* 2018; Aparecido *et al.* 2020). Thus, cooling via transpiration offers plants a degree of control over their temperature, maintaining 'partial' homeothermy (Mahan & Upchurch 1988; Potter, Davidowitz & Woods 2009; Blonder & Michaletz 2018), an analogue to ectotherm behavioural avoidance. Reduced water availability can force a trade-off between the maintenance of leaf temperature and maintenance of hydraulic function by closing stomata, triggered by water stress (Fauset *et al.* 2018). Conditions of water stress therefore can also reduce leaf homeothermy and lead to poikilothermy and megathermy, where leaf temperatures track or exceed air temperature (Blonder & Michaletz 2018). As such, megathermy is predicted to be particularly common in dry, hot environments such as deserts (Blonder & Michaletz 2018). However, there is a growing body of knowledge showing temperatures of leaves exceed high air temperatures across a range of environmental conditions and species (Gates, Alderfer & Taylor 1968; Roden & Pearcy 1993; Ishida, Toma & Marjenah 1999; Buchner & Neuner 2003; Vogel 2009; Krause *et al.* 2010; Schymanski, Or & Zwieniecki 2013; Aparecido *et al.* 2020; Miller *et al.* 2021). The effect of water limiting conditions on thermal safety margins is under explored (Leon-Garcia & Lasso 2019) and may help delineate what plant safety margins could be when under less than optimal environmental conditions.

Not only does the relationship between leaf and air temperature vary but increasing temperature can also stimulate increased leaf physiological heat tolerance thresholds by several degrees (Downton, Berry & Seemann 1984; Seemann, Berry & Downton 1984;

Valladares & Pearcy 1997; Neuner & Buchner 2012; Buchner *et al.* 2017; Drake *et al.* 2018). Increased heat thresholds in response to local environmental (acquired tolerance) triggers can help protect leaves from subsequent high temperatures. Indeed, an upward shift in heat tolerance is potentially an adaptation to extreme leaf temperature (Perez & Feeley 2020). A shift in heat tolerance means that *both* components of a thermal safety margin calculation - the heat tolerance threshold and leaf temperature - can vary with environmental conditions.

In this study, we used chlorophyll fluorometry to determine photosynthetic thermal tolerance thresholds (T_{50}) of Photosystem II (PSII) in leaves of water-stressed and well-watered desert plants. We investigated the extent to which thermal thresholds and subsequent estimates of thermal vulnerability might vary depending on the water status of a plant and how ambient temperature was assessed. We aimed to address three hypotheses: 1) that leaves of water-stressed plants would track (poikilothermy) or exceed ambient temperatures (megathermy) and that this would be associated with higher leaf temperatures and heat tolerance thresholds than well-watered plants. When assessing thermal vulnerability using thermal safety margins, we hypothesised 2) that the water status of plants would alter the estimated safety margins, with well-watered plants maintaining wider margins than water-stressed plants. Finally, we predicted 3) that calculations of thermal safety margins using various ambient sources would differ markedly from margins calculated using leaf temperatures.

Materials and methods

Sampling location and study species plants

This experiment was conducted at the Australian Arid Lands Botanic Garden, Port Augusta, South Australia (32°28'03.5" S, 137°44'36.7" E) during the austral summer (December) of 2017. *Myoporum montanum* (water bush) was selected for study because it has a wide distribution throughout semi-arid and arid Australia (Atlas of Living Australia 2020), with preferred microhabitats along dry water courses with higher water availability than surrounding hard pan areas. *Myoporum montanum* leaves visibly respond to water stress prior to leaf drop, which is ideal for visually monitoring plants throughout a water-stress

experiment. Plants were propagated from cuttings taken in the austral winter (July) and well-watered until the beginning of the water treatments in spring (November). Four weeks after the onset of water treatments, physiological sampling occurred over a 12-day period, with T_{50} measurements made every third day (days three, six, nine and 12; Figure 1). All leaves sampled were mature and fully expanded, having developed prior to the water-stress treatments. Due to the amount of leaf material required for the heat tolerance assay, plants from each water treatment were randomly allocated into three 'sub-populations' of 13 plants and re-sampled on each of the four sampling days, with leaves from the plants within each sub-population randomised for each assay. Sampling from multiple individuals meant there was enough similar age leaf material to select fully expanded mature leaves of similar age across plants and sampling days. To ensure that plants used for measuring T_{50} and water potential remained intact, across the two treatments, an additional subset of plants were reserved for monitoring leaf temperature via embedded thermocouples.

Water treatments

Plants were randomly allocated to each of the watering treatments: well-watered and water stressed. Rainfall was excluded with a rainout shelter made of clear polycarbonate sheeting roof, fine mesh on the sides hanging approximately 40-cm below the roof to reduce incidence of driving rain from the sides (Supplementary Figure S1). Within the rainout shelter, plant blocks were rotated four times during the eight weeks prior to measurement period to minimise position effects on growth. Watering was via a dripper irrigation system which provided 8L/h. Well-watered plants (HW) were watered for five minutes (total 0.7L) every day. For water-stress treatments (LW), water was reduced from the well-watered rate in two steps over four weeks in November. First reduction was to three minutes every two days (total 0.4L), followed by the second reduction to two minutes (total 0.26L) every three days. Watering ceased altogether the week prior measurements. During the 12-day measurement period, 0.06 L was provided by hand to water-stressed treatment plants on days four and nine to prevent plant mortality.

Midday leaf water potentials (Ψ_{leaf}) of each water treatment group were measured daily with a Scholander pressure chamber (PMS Instrument Company, Albany, OR). Once picked,

leaves were kept in dark humid plastic bags and measured within 10 mins (n= 3-6 leaves per watering treatment).

Temperature measurements

Leaf and local air temperatures were recorded every minute for the 12-day measurement period (Figure 1) on eight plants per watering treatment (n=8). Leaf temperatures were measured with t-type thermocouples (Omega Engineering, Singapore; and HOBO; Onset, Bourne, USA) inserted into the centre of the abaxial epidermis layer, away from the midrib. Selected leaves were approximately 10-cm above the soil surface (one leaf per plant). Air temperatures were recorded with eight shielded temperature sensors (iButton®, DS1923, San Jose, CA), suspended 10-cm above the potted soil of plants equally dispersed throughout the rainout shelter. Regional temperature data were downloaded from the Australian Bureau of Meteorology (AGBoM 2017) for the “Port Augusta aeronautical station” (station number 018201), situated 3.17 km from the experimental site.

Thermal tolerance assay (T_{50} threshold)

Photosynthetic heat tolerance was assessed on leaves collected during the hottest part of the day, to represent the point of highest potential risk of temperature stress. The T_{50} threshold was determined with a temperature assay of the maximum quantum yield of PSII (F_V/F_M), following Curtis *et al.* (2014). F_V/F_M is considered a good indicator of the functioning of PSII and is temperature sensitive (Björkman & Demmig 1987; Maxwell & Johnson 2000; DeEll & Toivonen 2003), with declines representing photoinhibition and/or damage to the photosystem (Yamane *et al.* 1997). Chlorophyll fluorescence measurements were taken with a Mini-PAM fluorometer (Heinz Walz, Effeltrich, Germany). Leaves were collected at the hottest time of day, approximately 15:00, and kept in humid dark plastic bags until experimentation. Five 1-cm leaf sections were placed on moist paper towel and sealed in ziplock plastic bags, then dark-adapted for 30-min prior to initial F_V/F_M measurements to check leaf function prior to temperature assay. To begin the assay, leaves were submerged in a 28°C (summer control temperature) water bath under sub-saturating light conditions for 15-min pre-heat treatment to allow photosystems to light adapt. Leaves were then immediately transferred to their assigned treatment water bath for 15-min then

transferred to a second 28°C bath for 90-min. Six temperatures (46, 48, 50, 52, 54°C and a 28°C control) were assayed, totalling 30 leaf disc per replicate sub-population (Supplementary Figure S2). All temperature baths maintained sub-saturating light conditions, were thermo-electrically controlled and monitored with K-type thermocouples (Curtis *et al.* 2014). After the heat treatment and post-heat 90-min control bath, leaves were stored on moist paper towel in ziplock bags at room temperature in the dark. The final F_V/F_M was measured approximately 14 hours after heat treatment.

The T_{50} threshold was defined as the temperature at which F_V/F_M declined by 50% of the control F_V/F_M (28°C), interpolated from a linear equation fit to the two temperatures bracketing the 50% decline temperature (Knight & Ackerly 2003; Curtis *et al.* 2014). The temperature at which there is a 50% decline in F_V/F_M represents the point of irreversible damage to the Photosystem II machinery (Downton and Berry 1982; Schreiber and Berry 1977) and is a widely used metric for assessing thermal limits of PSII (e.g. Bigras 2000; Buchner & Neuner 2003; Knight & Ackerly 2003; Krause *et al.* 2010; Curtis *et al.* 2014; Sastry & Barua 2017; Feeley *et al.* 2020).

Thermal safety margin (TSM)

The thermal safety margin (TSM) is the difference between the temperature a plant can tolerate biologically (measured here as the T_{50} threshold) and the maximum temperature to which it is exposed in its habitat. For comparison of TSM based on different maximum exposure temperatures, we calculated the safety margins for the maximum recorded leaf temperature, maximum local air temperature adjacent to experimental plants and regional maximum temperature each day of experimental measurement. Maximum leaf and local air temperatures used in TSM calculations were calculated from a 15-min moving average to match the T_{50} assay duration of 15-min. Unlike the leaf and local air temperature values, regional maximum temperatures were single values, so leaf and local air temperatures were averaged across plants and sensors and TSM replication was at the T_{50} level (n=3). For qualitative comparison of the effects of unpaired temporal physiological tolerance and air temperature on the TSM, the regional maximum air temperature during the sampling month (December), the sampling year regional maximum air temperature and the regional long term mean maximum summer temperature were downloaded from AGBoM (2019).

Statistical analysis

Differences between water treatments for each measured parameter were assessed with linear or linear mixed effects models when the random effect of either day or plant improved the model. Watering treatment and day were fixed categorical factors. Thermal safety margin models also included “measurement type” (leaf, local or regional temperature) as a fixed factor. Models were fitted in R (R Core Team 2018) with the package “nlme” (Pinheiro J *et al.* 2018). To control for potential temporal autocorrelation, three defined correlation structures were fitted to the models: 1) no correlation structure, 2) compound symmetry structure and 3) a first-order autoregressive structure. Variances were allowed to vary for each factor level of day or watering treatment, depending on the model. Models were compared with AIC and log-likelihood values and those with the lowest AIC value and best fit assumptions were selected. Assumptions of normality, homoscedasticity and degree of autocorrelation were evaluated through graphical assessment of residual-fitted scatter plots, histograms, factor level boxplots and autocorrelation figures. Where applicable, pairwise comparisons were analysed with Tukey HSD post-hoc tests in the “emmeans” R package (Lenth 2019).

Results

Leaf water potential

Withholding water from plants led to significantly lower and more variable leaf water potentials compared to well-watered plants (Table 1). The Ψ_{leaf} of well-watered plants ranged from -0.1 to -0.5 MPa, whereas water-stressed plants reached a mean minimum of -2.2 MPa on day nine (Figure 2). On days four and nine, leaves of water-stressed plants were visibly wilted, with mean leaf water potentials of -1.7 and -2.2 MPa, respectively. On each occasion, 0.06 L of water was applied to the water-stressed plants to avoid leaf death. Leaf water potentials subsequently increased (days five, six, ten and eleven) before declining again (Figure 2).

Relationships between leaf and air temperature

Maximum local air temperature was generally significantly different from maximum leaf temperatures of both well-watered and water-stressed plants (on eight of the 12 days, Table 1; Table S1). Only on day eight was maximum air temperature similar to the maximum leaf temperatures of both water treatments (Figure 3). The temperature of water-stressed leaves peaked at 3 to 5°C above the daily maximum air temperature, except for days one, two, seven and eight, when leaf and air maximums were similar to one another. By contrast, leaves of well-watered plants maintained maximum temperatures 3 to 7°C lower than maximum air temperatures (Figure 3, Table S1). Consequently, the leaves of water-stressed plants generally reached significantly higher maximum temperatures (up to 57°C) than those of well-watered plants (up to 46°C, Figure 3; Table 1).

Water availability altered the relationship between leaf and air temperatures. Leaves of well-watered plants generally maintained partial homeothermy (slope less than one) ($T_{\text{Leaf}} \sim 3.93^{\circ}\text{C} (\pm 0.01) + 0.76^{\circ}\text{C} (\pm 0.0004) * T_{\text{Air}}$, $R^2 = 0.9589$, $p < 2.2^{-16}$, Figure 4A). Leaves of water-stressed plants on average followed a poikilothermic response (leaf temperatures tracking ambient air temperatures) with the mean slope close to one ($T_{\text{Leaf}} \sim -1.26^{\circ}\text{C} (\pm 0.02) + 1.04^{\circ}\text{C} (\pm 0.0006) * T_{\text{Air}}$, $R^2 = 0.9615$, $p < 2.2^{-16}$, Figure 4B). However, there were also cases of megathermy (leaf temperatures above air temperatures at high temperatures) and partial

homeothermy (leaf temperatures below ambient at high air temperature), depending on the plant and day (Figure 4B, Supplementary Figure S3).

T₅₀ threshold temperatures

The T_{50} threshold temperatures varied between 48 and 53°C across days and water treatments. Water-stressed plants consistently had significantly higher T_{50} thresholds than well-watered plants (Table 1). Thresholds also significantly increased over the nine-day period over which they were measured (Table 1), tracking a general increase in air and leaf temperatures (Figure 3). Interestingly, on day nine, when leaf temperatures and air temperatures were notably cooler than the several preceding and subsequent days, T_{50} thresholds remained similarly high across all measurement days (ΔT_{50} of +0.4 and -0.7°C in well-watered and water-stressed plants, respectively).

Thermal safety margins

The thermal safety margins calculated with leaf temperatures (difference between T_{50} threshold and *in situ* leaf temperature) for plants with ample water ranged from 11.6 to 21.4°C, whereas the margins of water-stress plants ranged from 4.6 to 18.7°C. The leaf safety margins of well-watered plants were consistently wider (by 3 to 7°C) than those of water-stressed plants, although there was considerable variation among days (Figure 5, Table 2). Changes in safety margins each day depended on increases in T_{50} threshold and changes in leaf temperature, but the latter had a greater influence.

We also investigated how the source of maximum exposure temperature, whether leaf, local or regional air temperature, altered the calculation of thermal safety margins. In well-watered plants, the use of local air temperature measurements resulted in significantly narrower thermal safety margins (< 8°C) than margins based on leaf temperatures (Figure 5). Using regional air temperature data significantly overestimated the margins around half the time (by > 3°C on days three and nine) compared to those calculated with leaf temperatures (Figure 5, Table 2). For plants with low water availability, a more consistent trend occurred. Both local and regional air temperatures always overestimated the breadth of the safety margins relative to leaf temperature-based predictions, although the extent of overestimation varied from 1 to 9°C (Figure 5, Table 2). The local and regional air

temperatures differed up to 8°C. Part of this difference may have been an effect of the rainout shelter and proximity of the sensors to the ground. Nonetheless, this discrepancy between our local and regional air temperatures was similar to the difference between daily air temperatures logged at the AALBG and recorded regional temperatures (data not shown).

Discussion

We tested three hypotheses about how water availability and sources of temperature measurement may influence assessments of thermal vulnerability for plants. In support of our first hypothesis, leaf temperatures were markedly influenced by plant water availability and varied throughout the 12-day experimental period. Under maximum local ambient air temperatures, which ranged from 30 to 47°C, plants with adequate soil water were able to maintain maximum leaf temperatures up to 7°C below air temperature and up to 11°C lower than maximum leaf temperatures of water-stressed plants (Figure 3). This finding of partial-homeothermy supports recent studies suggesting that plants with adequate water will limit high leaf temperatures during hot conditions by active cooling through transpiration (Urban *et al.* 2017; Drake *et al.* 2018; French *et al.* 2019; Marchin *et al.* 2020). It was also our expectation that water-stressed plants would have higher photosynthetic heat tolerance thresholds than well-watered plants with occurrences of poikilo- and megathermy, which we also found to be the case. Increased heat thresholds can be triggered by temperature increases (Schreiber & Berry 1977; Havaux 1992; Buchner & Neuner 2003; Knight & Ackerly 2003; Zhu *et al.* 2018) and in response to water stress (Havaux 1992; Valladares & Pearcy 1997; Ladjal, Epron & Ducrey 2000). Which mechanism or the degree to which both may be contributing to the higher heat tolerance thresholds requires further work.

Irrespective of water treatment, we found that T_{50} thresholds did not always follow maximum leaf temperatures. Thresholds tracked the increase in ambient temperature between days three and six, but then stayed high, even after air and leaf temperatures declined from day eight (Figure 3). Of the few studies exploring fine-scale temporal changes in heat threshold with temperature, mismatches in threshold relaxation and leaf temperature have been observed in alpine species (Buchner & Neuner 2003; Neuner & Buchner 2012) and delayed relaxation post exposure to a heatwave in a temperate

Eucalyptus tree (Drake *et al.* 2018). In other words, the relationship between photosystem thermal tolerance and leaf temperature is not linear (Buchner & Neuner 2003). It is likely that following and/or alongside the initial acclimatory response, oxidative damage caused by accompanying stress-related processes may also alter photosynthetic heat tolerance (Seemann, Downton & Berry 1986; Havaux 1992; Epron 1997; Valladares & Pearcy 1997; Ladjal, Epron & Ducrey 2000; Ghouil *et al.* 2003). Either way, the non-linear relationship between leaf temperature and tolerance thresholds has implications for interpreting plant responses to repeated heat events. An increase (or decrease) in air temperature on any given day may not always result in the same degree of change in thermal threshold as another day of the same temperature.

Rates of heat tolerance threshold acclimation with increases in temperature are usually reported to be below unity (from 0 to $\sim 0.3^{\circ}\text{C}$ per $^{\circ}\text{C}$ of warming, Braun, Buchner & Neuner 2002; Buchner & Neuner 2003; Sastry & Barua 2017; Zhu *et al.* 2018). It is for this reason that thermal safety margins are generally predicted to narrow with future warming, with species having lower heat thresholds expected to be at greater risk (Curtis *et al.* 2016; Sastry & Barua 2017; Sastry, Guha & Barua 2018; Zhu *et al.* 2018). In the current study, the widest safety margins were associated with the *lowest* heat thresholds, due to accompanying lower leaf temperatures (Figures 3 and 5), supporting findings in tropical species (Perez & Feeley 2020). This apparent reversal of risk was the case when leaf, rather than air temperatures were considered in safety margins. Vulnerability to future heat stress events is dependent on a plants ability to regulate leaf temperature as a first line of defence; something that may be more challenging with increasing dry conditions and humidity (De Boeck *et al.* 2016; Perez & Feeley 2018). A subsequent line of defence for plants is their ability to increase physiological heat tolerance. Further work pairing leaf temperatures with tolerance measurements under present and warming conditions will advance our ability to estimate more realistic risk of heat stress in plants.

Our second and third hypotheses were that the plant water status and the source of temperature (leaf, local air or average regional air) used to calculate thermal safety margins would alter those margins. Both predictions were supported. When comparing thermal safety margins calculated with leaf, local and regional air temperature, the three estimates were rarely similar, and the availability of water altered the relationship further through

influencing leaf temperatures (Figure 5). Calculating safety margins using local air temperatures compared to leaf temperatures reduced the mean safety margin by $> 5.5^{\circ}\text{C}$ (32% narrower) for well-watered plants and, conversely, increased it by 1.5°C (12% wider) for water-stressed plants. Regional air temperature overestimated margins compared with those calculated with leaf temperature by $>1^{\circ}\text{C}$ (8%) for well-watered plants and, by $>8^{\circ}\text{C}$ (70%) when plants were water stressed.

We note that our study was based on only one desert plant species, but the thermal safety margin values we report here are within the range that can be derived from studies on alpine, tropical and US desert species (Knight & Ackerly 2003; Leon-Garcia & Lasso 2019; Perez & Feeley 2020). Our safety margins using regional yearly air temperature values were also similar to those of dry climate tropical species (Sastry & Barua 2017). Applying the question about under- or overestimation of regional *versus* leaf temperature to Andean highland species suggests that safety margins would be overestimated by up to 83%, depending on species (back-calculated from Leon-Garcia & Lasso 2019). In the absence of leaf temperatures, Sastry and Barua (2017) considered three scenarios of potential leaf temperature under future warming, but only considered cases where leaf temperature equalled or exceeded air temperature (an appropriately conservative approach, given a lack of leaf temperature data). However, in light of the extensive partial-homeothermy observed in our well-watered plants, not accounting for water status may mask a lower risk for plants in some conditions. Leaf-to-air-temperature relationships are variable (Blonder *et al.* 2020). For example, some wet tropical plant species are partially-homeothermic by avoiding high leaf temperatures during peak air temperature (Dong *et al.* 2017), whereas megathermy can be the case for outer canopy sunlit leaves (Miller *et al.* 2021). Nevertheless, the influence of partial-homeothermy, poikilothermy and megathermy on safety margins, as demonstrated in our study, are expected to be similar across environmental contexts.

Safety margins calculated on long term climate variables will be, by their nature, spatially and temporally asynchronous with leaf physiological measurements. At a finer scale, our paired safety margins using leaf temperature varied by up to 14°C in nine days. This variation was largely driven by changes in leaf temperature, which was an order of magnitude larger ($>16^{\circ}\text{C}$) than thresholds ($>4^{\circ}\text{C}$). Using a regional monthly maximum, annual maximum or seasonal mean maximum temperature created less variable safety

margins (see paired vs unpaired error and range bars, Figure 5b). However, we suggest that this reduced variation hides the potential risk of heat stress occurring on a daily scale and, importantly, does not account for plant acclimation or acquired tolerance. Ultimately, leaf temperature provides the essential context for interpreting heat thresholds and making thermal vulnerability assessments.

Recommendations

A thermal safety margin can be a valuable heuristic indicator of potential vulnerability to increased high temperature extremes (Deutsch et al, 2008), but there are some important aspects to consider for improving and interpreting the estimate for plants. The use of maximum leaf temperatures is a more biologically relevant determination of the risk of leaf heat stress than air temperature. We recognise that the measurement of leaf temperatures in the field, especially for large numbers of species or over time, can be impracticable. We therefore outline the following considerations in determining plant thermal vulnerability with thermal safety margins.

In the absence of leaf temperature data, an air temperature measured more proximal to the plants of interest than a regional weather station is more likely to be representative of the environment leaves may experience; this includes positioning sensors at an appropriate height relative to the ground and considering canopy orientations when collecting leaf samples (Curtis, Knight & Leigh 2019). If measuring safety margins based on air temperature, consider that they may over- or underestimate the risk, depending on plant water status influence on leaf temperature. If using regional air measurements, ideally one should calibrate with a series of spot leaf temperatures. If this is infeasible, we suggest calibration using the difference in local air temperature to regional station data repeatedly for key points in time, such as the hottest time of day for a given period. The most vital consideration is the pairing of the maximum temperature to the day and time of collection for tolerance testing to capture the biologically relevant daily scale to which thermal safety margins apply. In temporally unpaired scenarios, sampling physiological tolerance over several days, although increasing variation, will also capture potential plastic responses and increase the range of tolerance responses sampled. Accounting for leaf to air relationships is

clearly fundamental to improving estimates of current and future predicted plant thermal safety margins.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section.

Figure Legends

Figure 1. Average local air and leaf temperatures throughout the 12-day summer measurement period, recorded every minute. Different coloured lines indicate leaf temperatures for well-watered plants (HW, pale blue), water-stressed plants (LW, red), local air temperature (solid black) and regional maximum air temperature at 3pm (Air, dashed); vertical grey dashed lines indicate when T_{50} thresholds were sampled.

Figure 2. Mean (\pm SE) midday leaf water potentials of well-watered (HW) and water stressed (LW) plants over the 12 measurement days. Blue vertical shaded bar indicates the starting leaf water potentials before the application of the water treatment, eight days prior to the first experimental measurements. Grey dashed lines indicate days on which T_{50} thresholds were measured and stars indicate a significant difference between HW (blue circles) and LW (red squares) leaf water potentials, based on Tukey post-hoc tests, where $p < 0.05$.

Figure 3. The mean maximum (\pm SE) leaf (T_{Leaf}) and local air (T_{Air}) temperatures measured every day and mean (\pm SE) T_{50} thresholds every three days during the 12-day measurement period. Temperatures were measured every minute. Blue circles indicate well-watered leaves (HW); red squares and diamonds indicate water-stressed leaves (LW) and black triangles, air temperature. Open symbols are leaf and air temperatures; filled symbols are T_{50} threshold temperatures. Vertical dashed lines indicate days on which T_{50} thresholds were measured.

Figure 4. Relationship between leaf and local air temperatures for well-watered (a) and water-stressed plants (b). Values on the 1:1 line at high air temperatures indicate that leaves are tracking air temperature (poikilothermy); values below the line show leaf temperatures cooler than air (partial homeothermy) and above the line are leaves warmer than air temperature (megathermy). Linear relationship fit to all $T_{\text{air}}:T_{\text{leaf}}$ measurements, individual short lines were fits per plant per day.

Figure 5. a) Daily changes in thermal safety margins (TSM, means (\pm SE)) for *M. montanum* determined with three temperature sources: maximum leaf temperature (circles), mean maximum local air temperature (triangles) and maximum regional temperature (squares). Each margin was calculated for well-watered (blue) and water-stressed plants (red) on the

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day of T_{50} threshold (T_{50}). To match the T_{50} assay duration of 15-min, maximum leaf and air temperatures were calculated from a 15-min moving average rather than the sampled 1-min data of Figure 2. Letters indicate significant differences within water treatment comparisons ($p < 0.001$), with HW comparisons in lower case and LW in upper case. b) Mean TSM (\pm SE and range) calculated on temporally paired and temporally unpaired measurements of temperature and physiological heat threshold. Paired measurements (black closed symbols), i.e., those measured on the same day as T_{50} , include daily maximum leaf temperature, maximum local air temperature and maximum regional air temperature averaged across all measurement days. Unpaired measurements (open symbols) are TSMs calculated with the regional monthly maximum air temperature of the experimental month (December, 45.9°C), regional yearly maximum air temperature (MxAT, 47.2°C) and the regional long term mean maximum summer temperature (MMxAT, 33.3°C). Grey vertical bars through points indicate the range in mean TSM across all experimental days and water treatments.

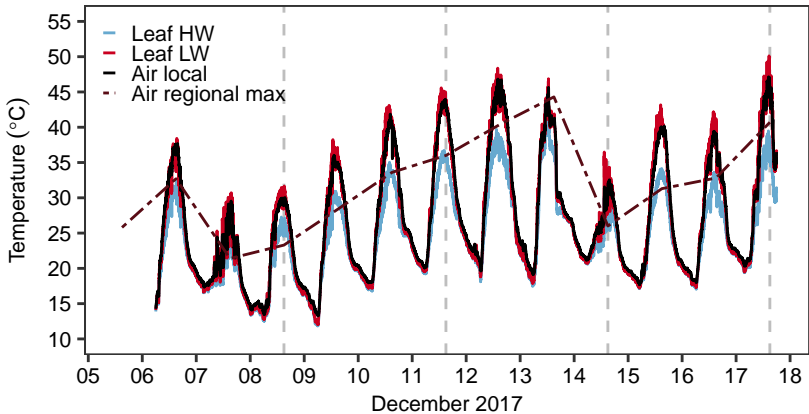
Tables

Table 1. Linear and linear mixed model results for measured parameters: leaf water potential (Ψ_{Leaf}), maximum temperature (T_{max}) and heat tolerance threshold (T_{50}). For variables Ψ_{Leaf} and T_{50} , treatment levels were well-watered (HW) and water stressed (LW), while for T_{max} , treatment levels also included air temperature.

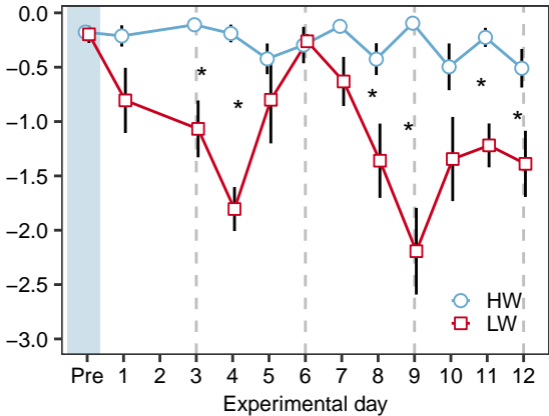
	Ψ_{Leaf}			T_{max}			T_{50}		
	df	F-value	p-value	df	F-value	p-value	df	F-value	p-value
<i>Fixed effects</i>									
Intercept	1, 95	207.1882	<0.0001	1, 217	8117.097	<0.0001	1, 19	131538.6	<0.0001
Treatment	1, 95	69.61736	<0.0001	2, 21	26.41	<0.0001	1, 19	47.8	<0.0001
Day	11, 95	5.74812	<0.0001	11, 217	475.475	<0.0001	3, 19	14.1	<0.0001
Treatment* Day	11, 95	7.94377	<0.0001	22, 217	10.202	<0.0001		ns	ns
<i>Random effects</i>		<i>Variance (%)</i>							
Individual plant		na	na		3.457	(86.4)		na	na
Residual		na	na		0.544	(13.6)		na	na
Total variance	119, 95	0.014			4.0017		24, 19	0.686	

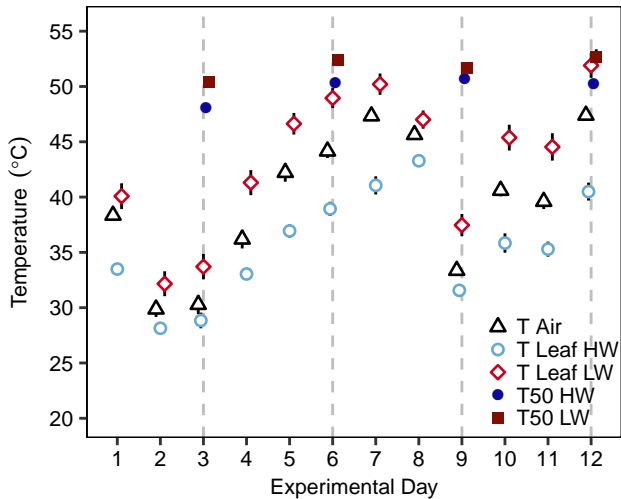
Table 2. Linear model results for thermal safety margin (TSM) comparisons investigating the influence and interaction of plant water availability (Treatment; HW or LW), variability between days (Day) and source of temperature (Measurement; T_{leaf} , T_{air} , T_{regional}) on the TSM estimation.

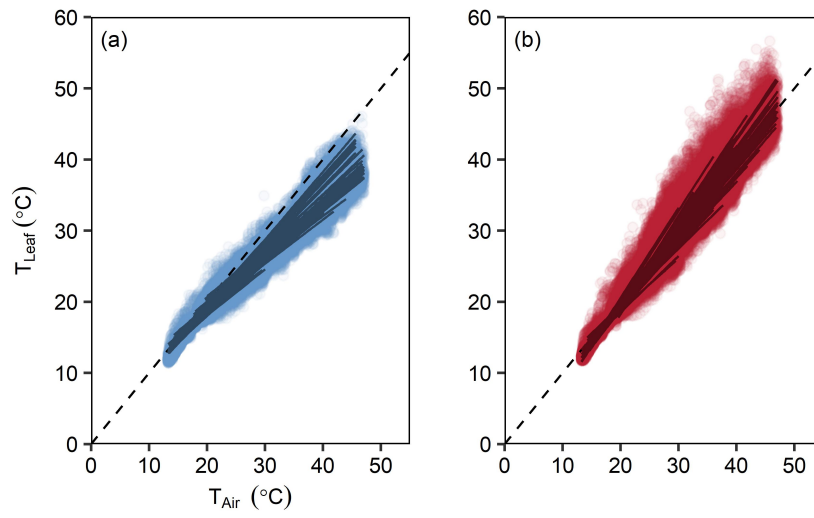
Factors	TSM		
	df	F-value	p-value
<i>Fixed effects</i>			
Intercept	1, 48	120861.42	<0.0001
Treatment LW	1, 48	10.39	0.0023
Day	3, 48	6109.8	<0.0001
Treatment* Day	3, 48	12.08	<0.0001
Measurement (T_{leaf} , T_{air} , T_{regional})	2, 48	1991.75	<0.0001
Treatment* Measurement	2, 48	544.49	<0.0001
Day*Measurement	6, 48	31.1	<0.0001
Treatment*Day*Measurement	6, 48	15.86	<0.0001
Residual variance	0.597		



Leaf water potential (MPa)







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