

# Beyond a single temperature threshold: applying a cumulative thermal stress framework to plant heat tolerance

Alicia Cook<sup>1</sup>, Enrico Rezende<sup>2</sup>, Katherina Petrou<sup>1</sup>, and Andrea Leigh<sup>1</sup>

<sup>1</sup>University of Technology Sydney

<sup>2</sup>Pontificia Universidad Catolica de Chile

August 28, 2023

## Abstract

Most plant thermal tolerance studies focus on single critical thresholds, which are arbitrary and phenomenological, limiting the generality of findings across studies. In animals and microbes, thermal tolerance landscapes describe the more realistic, cumulative effects of temperature. We tested this in plants by measuring the decline in leaf photosynthetic efficiency ( $F_V/F_M$ ) of two species following a combination of temperatures and exposure times. As predicted by the thermal tolerance landscape framework, we demonstrate that a general relationship between stressful temperatures and exposure durations can be effectively employed to quantify and compare heat tolerance within and across plant species and over time. We also show how  $F_V/F_M$  curves translate to natural conditions, suggesting that natural environmental temperatures often impair photosynthetic function. Our findings provide more robust descriptors of heat tolerance in plants, and suggest that heat tolerance in disparate groups of organisms can be studied with a single analytical framework.

## Beyond a single temperature threshold: applying a cumulative thermal stress framework to plant heat tolerance

Alicia M. Cook<sup>1\*</sup>, Enrico L. Rezende<sup>2</sup>, Katherina Petrou<sup>1</sup> and Andy Leigh<sup>1\*</sup>

<sup>1</sup> *University of Technology Sydney (UTS), School of Life Sciences, PO Box 123, Broadway, NSW 2007, Australia*

<sup>2</sup> *Departamento de Ecología, Center of Applied Ecology and Sustainability (CAPES), Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago 6513677, Chile.*

\*Corresponding authors: Alicia.Cook@uts.edu.au, Andrea.Leigh@uts.edu.au ph (+61 (02) 9514 1765) other author emails: katherina.petrou@uts.edu.au, erezende@bio.puc.cl

**Running title:** Cumulative thermal stress in plants

**Keywords:** chlorophyll fluorescence, critical thermal limits, heat stress, photosynthetic activity, thermal tolerance landscape, thermal death time, temperature tolerance,  $T_{50}$ threshold.

**Article type:** Letter **Word count:** 148 abstract; 4116 main text; 69 References; 4 figures; 1 table

ORCID numbers: Alicia M. Cook: 0000-0003-3594-3220 Enrico L. Rezende: 0000-0002-6245-9605 Katherina Petrou: 0000-0002-2703-0694 Andy Leigh: 0000-0003-3568-2606

## Authorship

AMC, KP and AL designed the research. AMC collected the data. AMC and ELR analysed the data, and AMC, ELR and AL wrote the first draft. All authors contributed to the final draft.

## Abstract

Most plant thermal tolerance studies focus on single critical thresholds, which are arbitrary and phenomenological, limiting the generality of findings across studies. In animals and microbes, thermal tolerance landscapes describe the more realistic, cumulative effects of temperature. We tested this in plants by measuring the decline in leaf photosynthetic efficiency ( $F_V/F_M$ ) of two species following a combination of temperatures and exposure times. As predicted by the thermal tolerance landscape framework, we demonstrate that a general relationship between stressful temperatures and exposure durations can be effectively employed to quantify and compare heat tolerance within and across plant species and over time. We also show how  $F_V/F_M$  curves translate to natural conditions, suggesting that natural environmental temperatures often impair photosynthetic function. Our findings provide more robust descriptors of heat tolerance in plants, and suggest that heat tolerance in disparate groups of organisms can be studied with a single analytical framework.

## Introduction

The use of critical endpoints or thresholds as thermal tolerance metrics has gained considerable momentum following the seminal work by Deutsch *et al.* (2008). They showed that tropical animals lived at temperatures closer to their physiological limits and were, therefore, more vulnerable to global warming. These authors originally used intrinsic rates of increase to estimate the temperature at which fitness dropped to zero and populations were no longer sustainable. Subsequent analyses have adopted the temperature in which an organism collapses and/or dies during a ramping experiment as a more indirect proxy of tolerance limits (Huey *et al.* 2009; Sunday *et al.* 2010; Clusella-Trullas *et al.* 2011; Sunday *et al.* 2014; Pinsky *et al.* 2019). These so-called ‘critical thermal limits’, which presumably describe the lower and upper temperatures that an organism can tolerate, have been measured in thousands of species (Bennett *et al.* 2018). However, these estimates are not directly comparable to thermal effects on fitness and are highly sensitive to experimental conditions such as initial temperatures and rates of temperature changes (Lutterschmidt & Hutchison 1997; Terblanche *et al.* 2007). These limitations, concomitantly with increasing use of these estimates to predict species’ resilience to warming, have sparked debate on the mechanistic basis of thermal tolerance as a trait and the adequacy of critical endpoints as tolerance proxies (Rezende *et al.* 2011; Santos *et al.* 2011; Terblanche *et al.* 2011; Rezende & Santos 2012; 2014; Huey & Kearney 2020; Ørsted *et al.* 2022).

The fundamental concern is that critical limits, quantified as a single temperature value, ignore the cumulative nature of physiological stress, which includes both the intensity and duration of a thermal challenge. Empirical studies have shown that the logarithm of survival time varies linearly with temperature, a relationship well-established for microorganisms such as bacteria and fungal spores (Bigelow 1921; Watkins & Winslow 1932), mollusks (Ansell *et al.* 1980), insects (Maynard-Smith 1957; Jørgensen *et al.* 2019) and vertebrates (Brett 1956; Somero & DeVries 1967). These thermal-death time (TDT) curves are widely used in the pest control and food processing industry (Stumbo 1973; Tang *et al.* 2007 and references therein). Related approaches, such as thermal tolerance landscapes (*sensu* Rezende *et al.* 2014) that describe TDT effects across different survival probabilities, have been recently employed to predict heat mortality under constant and fluctuating temperature conditions in animals (Jørgensen *et al.* 2019; Rezende *et al.* 2020; Molina *et al.* 2023). There is a growing consensus in animal studies that TDT curves and tolerance landscapes provide a more accurate framework to estimate thermal tolerance in ecological settings (Huey & Kearney 2020; Rezende *et al.* 2020; Jørgensen *et al.* 2022) yet the potential to apply this framework to plants remains virtually unexplored (but see Neuner & Buchner 2023).

Thermal tolerance metrics such as the  $T_{50}$  threshold, which describe the temperature required for a 50% drop in physiological function, are now being used to estimate potential vulnerability of a given plant species under a warming climate (Curtis *et al.* 2016; Sastry & Barua 2017; Perez & Feeley 2020; Cook *et al.* 2021; Marchin *et al.* 2022). These metrics usually are based on photosynthetic thermal threshold assays to determine the point of failure based on the physiological response of photosystems to applied thermal stress. All else being equal, such metrics allow the comparison of tolerance among leaves, individual plants, species and environmental effects and have been used to identify different levels of thermal vulnerability (e.g. Knight & Ackerly 2003;

Curtis *et al.* 2014; Curtis *et al.* 2016; French *et al.* 2017; Sastry & Barua 2017; Drake *et al.* 2018; French *et al.* 2019; Slot *et al.* 2019; Feeley *et al.* 2020; Perez & Feeley 2020). However, use of these critical endpoints involves the same conceptual pitfalls discussed in the animal literature, which have sparked debate regarding their adequacy to study plant thermal tolerance (Lancaster & Humphries 2020, 2021; Perez *et al.* 2021). Indeed, several studies have shown that damage to plants caused by heat is not only determined by the stress temperature but also the duration at a given temperature or heat dose (Bilger *et al.* 1984; Colombo & Timmer 1992; Hüve *et al.* 2011). Furthermore, at a constant temperature, increasingly longer periods of the same stress temperature cause more damage to photosystems measured by a range of physiological techniques (Valladares & Pearcy 1997; Dascaluic *et al.* 2007; Hüve *et al.* 2011; Li *et al.* 2014; Marias *et al.* 2016). These results highlight that critical limits in the plant literature likely exhibit limitations similar to those discussed in animal studies. Accordingly, Neuner and Buchner (2023) recently unveiled the typical association between heat intensity and duration described by the TDT curve for leaf damage and photosynthetic dysfunction across five plant species, suggesting that a unified approach to study thermal tolerance across organisms is not only desirable but also realistic.

Here we aim to bridge this gap and explore the potential usefulness of analytical tools developed to study heat tolerance in other systems for application to plant lineages. Addressing this question opens the venue not only to compare thermal tolerance across plant populations and species, but also to employ predictive tools that utilise realistic thermal tolerance landscapes in this group. With this purpose, we investigated the impact of heat stress on plant photosystem function (maximum quantum yield,  $F_V/F_M$ ) in two Australian arid zone species, *Myoporum montanum* R.Br. and *Eucalyptus socialis* F.Muell. ex Miq. First, we examined if the 50% decline in function of Photosystem II (PSII), often employed in plant studies to assess response to heat stress ( $T_{50}$ ), exhibits the expected relationship between temperature intensity and duration (Fig. 1). Second, we analysed how this relationship varied within *M. montanum* longitudinally over multiple trials to estimate intraspecific variability via potential changes in heat tolerance over the course of the summer. Third, we compare our empirical results against recently published estimates by Neuner and Buchner (2023) to illustrate how thermal adaptation may explain differences in heat tolerance across plant species with this framework. And finally, as a proof-of-concept, we applied a dynamic model of thermal damage (Rezende *et al.* 2020) to illustrate how this information can be employed to forecast the effect of heat stress on leaf function under natural conditions.

## Materials and Methods

### Experimental set-up and species

This study was conducted at the Australia Arid Lands Botanic Garden, Port Augusta, South Australia (AALBG, 32°27'45.0"S 137°44'33.7"E), during summer in January and February 2016. The AALBG has a desert climate, with mean annual rainfall of 250 mm and average relative humidity of 35% in summer (AGBoM 2018). We conducted the same set of experiments three times during the summer period, the first set including measurements of *M. montanum* and *E. socialis* (between January 14<sup>th</sup> to 21<sup>st</sup>) and the second and third including only *M. montanum* to obtain a longitudinal record (between January 24<sup>th</sup> to 26<sup>th</sup> and February 15<sup>th</sup> to 19<sup>th</sup>, respectively). *M. montanum* is a desert shrub or small tree to ~8-m tall and *E. socialis* is a slow growing multi-stemmed tree to ~6 to 12-m tall (Royal Botanic Gardens and Domain Trust 2018). To determine how the thermal environment changed across sampling trial, we recorded the mean local air temperatures at 45-min intervals from five shielded temperature sensors (iButton Thermochron®; Ecolutions, Portugal) suspended near the experimental plants. The average, maximum and minimum temperatures reached within a 24-hour period were calculated. Throughout the entire experiment, thermal extremes ranged between 14°C and 47°C (Supplementary Fig. S1).

### Heat stress measurements

For the heat tolerance measurements, we collected leaves from five established individuals of each species grown *in situ* and supplemented with irrigation at the AALBG as part of their living collection. We collected

leaves pre-sunrise and kept them in the dark in plastic humid bags until experimentation. For each batch, 10 randomised leaves were kept on moist paper towel in sealed plastic bags. Bags were submerged in well-mixed, temperature-controlled water baths under sub-saturating light levels ( $337 \pm 63$  SE  $\mu\text{mol m}^{-2}\text{s}^{-1}$  at leaf level underwater). Prior to heat treatment, leaves were light-adapted for 15-min in a  $28^\circ\text{C}$  bath then moved to the stress treatment bath for the allocated stress duration. Leaves were then returned to a recovery bath of  $28^\circ\text{C}$  for 90-min under light conditions, then dark adapted under ambient temperature overnight. This series of baths follow an established  $T_{50}$  threshold assay protocol (Curtis *et al.* 2014). The maximum quantum yield of PSII ( $F_V/F_M$ ) was measured with a Mini-PAM pulse amplitude fluorometer (Heinz Walz, Effeltrich, Germany), prior to stress treatment and overnight post stress (14-16 hours) on dark-adapted leaves. A reduction in  $F_V/F_M$  indicates reduced capacity to effectively handle photons and is a common physiological measurement used to estimate the effects of temperature on the functioning of the PSII (Berry & Bjorkman 1980; Maxwell & Johnson 2000). Declines in overnight  $F_V/F_M$  show continuing photoinhibition and indicate ongoing and/or irreversible damage incurred by the photosystems, thus providing information on damage incurred by high temperature.

To build the temperature-duration curves of heat tolerance, we first estimated how  $F_V/F_M$  decreased following the commonly used 15 min duration in static  $T_{50}$  threshold assays (e.g. Curtis *et al.* 2014; Sastry & Barua 2017) at 44, 46, 48, 50 and  $52^\circ\text{C}$  for *M. montanum*. Temperatures of 48, 50 and  $52^\circ\text{C}$  resulted in a decline in overnight  $F_V/F_M$  of 12, 32 and 73% in *M. montanum* with a 15 min  $T_{50}$  threshold of  $51.1^\circ\text{C}$  (Supplementary Fig. S2). Previous  $T_{50}$  thresholds of *E. socialis* have been similar to *M. montanum*, thus the same test temperatures were used (Cook, unpublished data). Therefore, we selected these three experimental temperatures and estimated the overnight  $F_V/F_M$  following 5, 10, 15, and 30 min exposure at each temperature. As an experimental control, we repeated the procedure at  $28^\circ\text{C}$  treatment. Ten detached whole leaves were used for each temperature intensity and duration combination, totalling 480 leaves tested over three trials for *M. montanum* (10 leaves x 4 durations x 4 temperatures x 3 trials) and 160 leaves for *E. socialis* (10 leaves x 4 durations x 4 temperatures).

## Statistical analysis

To assess how heat tolerance varied within and across species, we combined logistic regressions with a model comparison approach (Burnham & Anderson 2002). We included overnight  $F_V/F_M$ , rescaled between 0 and 1 by dividing it by initial  $F_V/F_M$ , as the dependent variable in the following model:

$$F_V/F_M = Ta + time + trial + Sp \text{ (eq. 1)}$$

where  $Ta$  = measurement temperature (28, 48, 50 and  $52^\circ\text{C}$  as a continuous variable),  $time$  = exposure time (5, 10, 15 and 30 min as continuous),  $trial$  = experimental trial (categorical factor with 3 levels) and  $Sp$  = species identity (categorical with 2 levels). We compared the fit of four different models using Akaike Information Criterion (AIC). These models included only main effects versus all interactions on the one hand, and  $time$  in arithmetic versus logarithmic scale and on the other hand. Consequently, the model comparison approach is simultaneously estimating if the slope of the temperature-duration relationship varies across trials and/or species, which should result in a better fit of models including interactions, and the adequate temporal scale that underlies the cumulative impact of heat stress. To estimate the relative support of each model, we employed Akaike weights ( $w_i$ ), which quantify the probability that each model is the best model given the data.

We then calculated the  $T_{50}$  threshold predicted by the logistic model as a standard estimate of heat damage across combinations of temperature and exposure times, which was then employed to fit temperature-duration relationships (Fig. 1). Note that some combinations resulted in predicted  $T_{50}$  threshold temperatures that fell outside the measured temperature ranges and, as such, they represent potential estimates of the thresholds. Consequently, we estimated the uncertainty surrounding  $T_{50}$  estimates employing a bootstrap with replacement (x1000) and included these estimates in the following linear model:

$$T_{50} = T_{50}' - z \log_{10} time \text{ (eq. 2)}$$

where  $T_{50}$  ( $^{\circ}\text{C}$ ) is the intercept, corresponding to the expected  $T_{50}$  following an exposure of 1 min, which describes the elevation of the temperature-duration relationship, and  $z$  ( $^{\circ}\text{C}$ ) its slope, which quantifies thermal sensitivity as the drop in temperature that results in a 10-fold increase in tolerance time (Rezende *et al.* 2014; 2020). With this approach, we were able to quantify both the intercept and slope of the temperature-duration relationship with their respective standard errors. For comparative purposes, these parameters were also calculated for the alpine species *Pinus cembra* L., *Picea abies* (L.) H.Karst, *Larix decidua* Mill., *Kalmia procumbens* (L.) Gift & Kron & P.F.Stevens ex Galasso, Banfi & F.Conti and *Ranunculus glacialis* L., studied by Neuner and Buchner (2023) (also compared with *Picea mariana* (Mill.) Britton and *Phaseolus vulgaris* L. from published heat tolerance and duration data (Yarwood 1961; Colombo & Timmer 1992), see Supplementary Table S3). Parameters and methods employed to estimate heat tolerance (e.g., photosynthetic activity or visual damage) are detailed in Supplementary Table S3.

### Modelling heat stress in the field

To illustrate how differences in temperature-duration relationships obtained in the laboratory might translate into field conditions, we employed the dynamic thermal survival probability model developed by Rezende *et al.* (2020). This model employs differential calculus to predict how thermal damage quantified under constant temperatures translates into variable temperatures. The analysis proceeded as follows. First, we reconstructed hourly air temperatures for Port Augusta between January 2016 and December 2023 from daily minima and maxima data from the Australian Bureau of Meteorology (<http://www.bom.gov.au/climate/data/?ref=fr>), employing the R package ‘chillR’ (vignette ‘hourly temperatures’, both accessed on 17 Feb 2023). Second, we employed simultaneous records of air and leaf temperatures of *M. montanum* to convert weather station temperature data into expected leaf temperatures. The latter was measured with type-t thermocouples and HOBO® dataloggers inserted into the epidermis of leaves. Finally, as explained in Rezende *et al.* (2020, details in their Supplementary Material p. 12), we employed the ad hoc *dynamic.landscape* function to combine leaf temperatures with the  $T_{50}$  temperature-duration relationships obtained for *M. montanum* and predict thermal damage under these thermal conditions. This analytical approach based in differential calculus is applicable to any system that exhibits the typical temperature-duration relationship at constant temperatures (Fig. 1) and has been empirically validated in insects and fish (Rezende *et al.* 2020, unpublished results). For simplicity, we report only predicted daily damage on PSII function (i.e., thermal damage within a 24-hour period), which can be employed to calculate cumulative effects over multiple days with some additional assumptions regarding recovery rates (Rezende *et al.* 2020).

### Results

Estimates of overnight  $F_V/F_M$  for each temperature and time combination are provided in the supplementary information (Supplementary Table S1). The model comparison approach shows that logistic regressions including only main effects and exposure time in logarithmic scale provides the best fit to the data (Table 1). This result suggests that the slopes of the temperature-duration relationships remain relatively constant across trials and/or species, and also provides support to the log-linear relationship predicted by the framework (Fig. 1). To assess how well the best model fits the empirical observations, we performed a regular linear regression between predicted values against observed  $F_V/F_M$  and obtained an  $R^2 = 0.77$  ( $F_{1,638} = 2134$ ,  $P < 2.2 \times 10^{-16}$ ). Thus, the best logistic model explains roughly 77% of the variance in  $F_V/F_M$  observed across leaf samples. Results were qualitatively similar when we repeated the analysis including only *M. montanum* and removing species as a categorical factor (results not shown), and in this instance the model with only main effects and log-transformed exposure time had an even stronger support based on Akaike weights ( $w_i = 0.96$ ). Also, in this analysis the fit of the model improved, based on the stronger regression between predicted and observed  $F_V/F_M$  ( $R^2 = 0.79$ ,  $F_{1,478} = 1848$ ,  $P < 2.2 \times 10^{-16}$ ).

As we hypothesized, empirical values of overnight  $F_V/F_M$  exhibit the response predicted by the temperature-duration framework and a large fraction of the unexplained variance can be attributed to variation in  $F_V/F_M$  within each temperature and exposure time combination (Fig. 2). Thus, the temperature-duration framework appears to describe the overall response of PSII to heat stress remarkably well, with some of the unexplained variation involving differences across leaves and/or individual plants within each sample (Fig. 2,

and Supplementary Figs. S3 and S4). Accordingly, most of the variation around mean estimates was detected at intermediate  $F_V/F_M$  (Supplementary Fig. S5), which is expected as all leaves are likely either unharmed or completely damaged at extremely low and extremely high temperatures, respectively.

We then estimated  $T_{50}$  as the inflection point of the logistic regression (Fig. 2), as well as its standard error, with the bootstrap analysis (Supplementary Table S2). Linear regressions between  $T_{50}$  estimates and log *time* (eq. 2) performed separately for each trial and/or species resulted in a very good fit (Fig. 3). For *M. montanum*, the  $R^2$  ranged between 0.95 and 0.98 across the three trials ( $F_{1,3998}$  [?] 7949 and  $P < 2.2 \times 10^{-16}$  in all cases), with the 1-min exposure time (intercept),  $T_{50}$ , varying between 59.2 and 56.2 degC across trials and the thermal sensitivity (slope),  $z$  remaining virtually constant and corresponding to 6.5 degC (Fig. 3). In contrast, for *E. socialis* we obtained a  $T_{50}$  of 63.1 degC and a  $z$  of 8.2 degC with the linear regression, though with a lower goodness of fit, resulting in an  $R^2 = 0.845$  ( $F_{1,3998} = 2190$ ,  $P < 2.2 \times 10^{-16}$ ). This illustrates how the higher variability in  $F_V/F_M$  across leaf samples and a  $T_{50}$  falling often outside the experimental temperatures for this species results in a lower precision in the estimation of its heat tolerance (Supplementary Fig. S5). Accordingly, the standard errors associated with the temperature-duration parameters were substantially larger for *E. socialis* than for *M. montanum* (Fig. 3), and their overlapping  $z$  support a single slope across species and trials as suggested by the model comparison analysis (Table 1). Parameter estimates for the temperature-duration curves are listed in Supplementary Table S3.

### *Photosynthetic function in the field*

The relationship  $T_{\text{leaf}} = 1.63 + 0.91 T_{\text{air}}$  obtained with a linear regression ( $R^2 = 0.98$ ) was employed to convert air into leaf temperatures in our estimation of heat stress in the field (Fig. 4). Simulations suggest that summer temperatures could often be stressful for *M. montanum*, though there are pronounced differences from year to year. Interestingly, heat tolerance seemed to decrease from trials 1 to 3, resulting in higher vulnerability at relatively lower temperatures. When we analyse the data on 24 h bins, we detect a regular sigmoidal association between daily thermal damage as a function of maximum daily temperatures (Fig. 4), which provides a relatively straightforward rule-of-thumb to diagnose differences in thermal stress based on the temperature-duration curve. For instance, for *M. montanum*, the probably of thermal damage seems to rise in days reaching maximum temperatures of approximately 40, 38.5 and 36 degC for trials 1, 2 and 3, respectively, but nears 100% when temperatures reach 45, 43 and 41 degC (Fig. 4).

## Discussion

Here we show that the temperature-duration TDT framework developed to study thermal tolerance in microorganisms and metazoans can be successfully employed to quantify heat tolerance of photosynthetic tissue in plants. It is not entirely surprising, as multiple studies have previously reported that higher stressful temperatures, on the one hand, or longer exposure durations, on the other hand, increase damage to photosystems (Valladares & Pearcy 1997; Koniger *et al.* 1998; Dascaluc *et al.* 2007; Huve *et al.* 2011; Yan *et al.* 2011; Agrawal & Jajoo 2015; Marias *et al.* 2017). Importantly, this general relationship between temperature and exposure time has also been reported for other plant traits, such as visual cell death analysis in seedlings (Colombo & Timmer 1992), leaf weight loss in green beans (Yarwood 1961) and visual leaf damage and PSII dysfunction in alpine species (Neuner & Buchner 2023). Here we demonstrate how these findings can be merged and analyzed with a single framework for plants for application in climate change ecology.

Our analyses also demonstrate how variation in heat tolerance can be quantified and compared within and across plant species, and subsequently how to employ this knowledge to estimate heat stress in natural populations. For the limited subset employed here, analyses detected differences in heat tolerance corresponding primarily with shifts in the elevation of the temperature-duration curves rather than changes in their respective slopes (Table 1, Fig. 3). Over the course of summer sampling, there was a decline in heat tolerance across trials. While this trend could be interpreted as a decline in tolerance with declining plant condition with the progression of summer, counterintuitively, the environmental conditions became more benign with

the later trials. Air temperatures reduced and rainfall increased for trials 2 and 3 (Supplementary Fig. S1). Photosystem heat tolerance can rapidly change with local environmental conditions and water status affecting leaf temperature (Havaux 1992; Valladares & Pearcy 1997; Buchner & Neuner 2003; Knight & Ackerly 2003; Zhu *et al.* 2018; Cook *et al.* 2021; Sumner *et al.* 2022). As such, progressively lower heat tolerances over the trials in our study most likely reflected the increasingly benign conditions over summer. Interestingly, comparisons between parameters for our temperature-duration curves and those recently reported by Neuner and Buchner (2023) suggest that (i) variation in heat tolerance within a single species, as quantified here for *M. montanum*, tends to be lower than variation across species but is far from negligible and (ii) perhaps not surprisingly, that species inhabiting Australian desert environments seem to exhibit higher tolerance to heat stress than alpine plants (Fig. 3). Additional data are clearly required to properly understand how heat tolerance landscapes vary across plant lineages, contrasting distribution limits, habitats and functional groups such as trees, and herbs. Importantly, while these analyses may not necessarily contradict large-scale trends detected with critical thermal limits across phylogenetically disparate groups across the globe (Lancaster & Humphries 2020), they may dramatically increase the statistical and predictive power of future analyses at smaller spatial scales by controlling for the confounding effects of exposure duration (e.g., see Table 1 in Rezende *et al.* 2014).

Just as with animal studies, many of the limitations of the theoretical framework apply to plants. For example, thermal tolerance studies on leaves from adult plants often neglect the synergistic impact of other stressors, such as dehydration, or how heat tolerance may change during different stages of the lifecycle (Geange *et al.* 2021). Similarly, simulations employing the dynamic model for animals ignore spatial heterogeneity in light and temperature, physiological history before the stress, the existence of microhabitats and, importantly, short- and long-term physiological recovery following a thermal stress (Huey and Kearney 2020). However, one aspect that is unique to plants is that, because they are modular organisms, studies often work with indirect proxies of temperature stress at the leaf level such as  $F_V/F_M$ , and arbitrary thresholds such as  $T_{50}$ , instead of thermal mortality of the whole plant. While this approach is crucial for comparative purposes (Lancaster & Humphries 2020, 2021; Perez *et al.* 2021), it remains to be determined how different thresholds are indicative of heat tolerance in natural plant populations for more reliable predictions. For instance, the temperature-duration curves from Neuner and Buchner (2023) suggest that 50% visual leaf damage is attained at temperatures on average 3.7 degC higher than those required for a similar drop in  $F_V/F_M$ , hence caution is warranted when comparing curves obtained with different methods (Supplementary Table S3). Here, we were able to predict the probability of *M. montanum* leaves of reaching  $T_{50}$  (Fig. 4), but both studies ignored how this threshold translates into leaf death and, subsequently, into plant survival, reproduction and ultimately Darwinian fitness. Future research assessing crown dieback, plant mortality and/or decreased seed production following heat waves in the field (Marchin *et al.* 2020; Breshears *et al.* 2021; Milner *et al.* 2023) may help to calibrate similar predictive models and bridge the gap between physiological proxies of thermal stress and their demographic consequences.

To conclude, our analyses show how the theoretical framework currently employed to study heat tolerance in animal research can be successfully employed in plants. Examination of thermal sensitivity across numerous plant species, vegetation systems and environmental growth conditions is required to determine common trends in thermal sensitivity. For example, there may be relationship among species differing in their underlying biological mechanisms for temperature stress response, such as those capable of rapid isoprene production (Siwko *et al.* 2007) and changing membrane lipid compositions (Zheng *et al.* 2011). Modelling thermal sensitivity in the way we present here incorporates an important dimension to estimating plant thermal tolerance in a thermally changing environment. This approach has the potential to highlight not only vulnerable species but also predict their thermally vulnerable periods in greater detail and with greater precision than current approaches, opening the prospect for meaningful predictive comparisons of thermal limits, not only among plants (Lancaster & Humphries 2020), but also across biological kingdoms.

## Acknowledgements

We thank G Neuner and O Buchner for sharing their original  $F_V/F_M$  data for reanalysis. We are grateful for Ryan Hayward and colleagues for tending plants and allowing us to turn their tearoom into a field laboratory. We also gratefully acknowledge the intellectual contribution of James Brown via lengthy and valuable statistical explorations of this dataset in the early analysis phase. AMC and AL were supported by Port Augusta City Council, South Australia, and the Friends of the Australian Arid Lands Botanic Garden (AALBG). AMC was funded by an Australian Government Research Training Program Scholarship. ELR was funded by FONDECYT 1211113 and ANID PIA/BASAL FB0002 grants.

## Data availability statement

All data and R scripts necessary to replicate our analyses and results are archived on the DRYAD repository at [xxlink to be included upon acceptance].

## References

- AGBoM (2018). Climate statistics for Australian Locations: Port Augusta Power Station. Available at: [http://www.bom.gov.au/climate/averages/tables/cw\\_019066.shtml](http://www.bom.gov.au/climate/averages/tables/cw_019066.shtml).
- Agrawal, D. & Jajoo, A. (2015). Investigating primary sites of damage in photosystem II in response to high temperature. *Indian Journal of Plant Physiology* , 20, 304-309.
- Ansell, A.D., Barnett, P.R.O., Bodoy, A. & Masse, H. (1980). Upper temperature tolerances of some European molluscs: *Tellina fabula* and *T. tenuis* . *Marine Biology* , 58, 33-39.
- Bennett, J.M., Calosi, P., Clusella-Trullas, S., Martinez, B., Sunday, J., Algar, A.C. *et al.* (2018). GlobTherm, a global database on thermal tolerances for aquatic and terrestrial organisms. *Scientific Data* , 5, 180022.
- Berry, J.A. & Bjorkman, O. (1980). Photosynthetic response and adaptation to temperature in higher plants. *Annual Review of Plant Physiology* , 31, 491-543.
- Bigelow, W.D. (1921). The Logarithmic Nature of Thermal Death Time Curves. *The Journal of Infectious Diseases* , 29, 528-536.
- Bilger, H.-W., Schreiber, U. & Lange, O.L. (1984). Determination of leaf heat resistance: comparative investigation of chlorophyll fluorescence changes and tissue necrosis methods. *Oecologia* , 63, 256-262.
- Breshears, D.D., Fontaine, J.B., Ruthrof, K.X., Field, J.P., Feng, X., Burger, J.R. *et al.* (2021). Underappreciated plant vulnerabilities to heat waves. *New Phytologist* , 231, 32-39.
- Brett, J.R. (1956). Some Principles in the Thermal Requirements of Fishes. *The Quarterly Review of Biology* , 31, 75-87.
- Buchner, O. & Neuner, G. (2003). Variability of Heat Tolerance in Alpine Plant Species Measured at Different Altitudes. *Arctic, Antarctic, and Alpine Research* , 35, 411-420.
- Burnham, K.P. & Anderson, D.R. (2002). *Model selection and multimodel inference: a practical information-theoretic approach* . Springer, New York.
- Clusella-Trullas, S., Blackburn, T.M. & Chown, S.L. (2011). Climatic predictors of temperature performance curve parameters in ectotherms imply complex responses to climate change. *The American Naturalist* , 177, 738-751.
- Colombo, S.J. & Timmer, V.R. (1992). Limits of tolerance to high-temperatures causing direct and indirect damage to black spruce. *Tree Physiology* , 11, 95-104.



- Cook, A.M., Berry, N., Milner, K.V. & Leigh, A. (2021). Water availability influences thermal safety margins for leaves. *Functional Ecology* , 35, 2179-2189.
- Curtis, E.M., Gollan, J., Murray, B.R. & Leigh, A. (2016). Native microhabitats better predict tolerance to warming than latitudinal macro-climatic variables in arid-zone plants. *Journal of Biogeography* , 43, 1156-1165.
- Curtis, E.M., Knight, C.A., Petrou, K. & Leigh, A. (2014). A comparative analysis of photosynthetic recovery from thermal stress: a desert plant case study. *Oecologia* , 175, 1051-1061.
- Dascaliuc, A., Ralea, T. & Cuza, P. (2007). Influence of heat shock on chlorophyll fluorescence of white oak (*Quercus pubescens* Willd.) leaves. *Photosynthetica* , 45, 469-471.
- Deutsch, C.A., Tewksbury, J.J., Huey, R.B., Sheldon, K.S., Ghalambor, C.K., Haak, D.C. *et al.* (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proceedings of the National Academy of Sciences* , 105, 6668-6672.
- Drake, J.E., Tjoelker, M.G., Varhammar, A., Medlyn, B.E., Reich, P.B., Leigh, A. *et al.* (2018). Trees tolerate an extreme heatwave via sustained transpirational cooling and increased leaf thermal tolerance. *Global Change Biology* , 24, 1-13.
- Feeley, K., Martinez-Villa, J., Perez, T., Silva Duque, A., Trivino Gonzalez, D. & Duque, A. (2020). The thermal tolerances, distributions, and performances of tropical montane tree species. *Frontiers in Forests and Global Change* , 3, 25.
- French, K., Jansens, I.B., Ashcroft, M.B., Ecroyd, H. & Robinson, S.A. (2019). High tolerance of repeated heatwaves in Australian native plants. *Austral Ecology* , 44, 597-608.
- French, K., Robinson, S.A. & Lia, J. (2017). Thermotolerance capacities of native and exotic coastal plants will lead to changes in species composition under increased heat waves. *Conservation physiology* , 5, 1.
- Havaux, M. (1992). Stress tolerance of photosystem ii in vivo: antagonistic effects of water, heat, and photoinhibition stresses. *Plant Physiology* , 100, 424-432.
- Huey, R.B., Deutsch, C.A., Tewksbury, J.J., Vitt, L.J., Hertz, P.E., Alvarez Perez, H.J. *et al.* (2009). Why tropical forest lizards are vulnerable to climate warming. *Proceedings. Biological sciences* , 276, 1939-1948.
- Huey, R.B. & Kearney, M.R. (2020). Dynamics of death by heat. *Science* , 369, 1163-1163.
- Huve, K., Bichele, I., Rasulov, B. & Niinemets, U. (2011). When it is too hot for photosynthesis: heat-induced instability of photosynthesis in relation to respiratory burst, cell permeability changes and H<sub>2</sub>O<sub>2</sub> formation. *Plant, Cell & Environment* , 34, 113-126.
- Jorgensen, L.B., Malte, H. & Overgaard, J. (2019). How to assess *Drosophila* heat tolerance: Unifying static and dynamic tolerance assays to predict heat distribution limits. *Functional Ecology* , 33, 629-642.
- Jorgensen, L.B., Orsted, M., Malte, H., Wang, T. & Overgaard, J. (2022). Extreme escalation of heat failure rates in ectotherms with global warming. *Nature* , 611, 93-98.
- Knight, C.A. & Ackerly, D.D. (2003). Evolution and plasticity of photosynthetic thermal tolerance, specific leaf area and leaf size: congeneric species from desert and coastal environments. *New Phytologist* , 160, 337-347.
- Koniger, M., Harris, G.C. & Percy, R.W. (1998). Interaction between photon flux density and elevated temperatures on photoinhibition in *Alocasia macrorrhiza*. *Planta* , 205, 214-222.
- Lancaster, L.T. & Humphreys, A.M. (2020). Global variation in the thermal tolerances of plants. *Proceedings of the National Academy of Sciences USA* , 117, 13580-13587.

- Lancaster, L.T. & Humphreys A.M. (2021). Reply to Perez et al.: Experimental duration unlikely to bias global variation in plant thermal tolerances. *Proceedings of the National Academy of Sciences USA* , 118, e2102037118.
- Li, L., Li, X.-Y., Xu, X.-W., Lin, L.-S. & Zeng, F.-J. (2014). Effects of high temperature on the chlorophyll a fluorescence of *Alhagi sparsifolia* at the southern Taklamakan Desert. *Acta Physiologiae Plantarum* , 36, 243-249.
- Lutterschmidt, W.I. & Hutchison, V.H.J.C.J.o.Z. (1997). The critical thermal maximum: data to support the onset of spasms as the definitive end point. *Canadian Journal of Zoology* , 75, 1553-1560.
- Marchin, R.M., Backes, D., Ossola, A., Leishman, M.R., Tjoelker, M.G. & Ellsworth, D.S. (2022). Extreme heat increases stomatal conductance and drought-induced mortality risk in vulnerable plant species. *Global Change Biology* , 28, 1133-1146.
- Marchin, R.M., Ossola, A., Leishman, M.R. & Ellsworth, D.S. (2020). A simple method for simulating drought effects on plants. *Frontiers in Plant Science* , 10, 1715.
- Marias, D.E., Meinzer, F.C. & Still, C. (2016). Leaf age and methodology impact assessments of thermotolerance of *Coffea arabica* . *Trees* , 31, 1091-1099.
- Marias, D.E., Meinzer, F.C. & Still, C. (2017). Impacts of leaf age and heat stress duration on photosynthetic gas exchange and foliar nonstructural carbohydrates in *Coffea arabica* . *Ecology and Evolution* , 7, 1297-1310.
- Maxwell, K. & Johnson, G.N. (2000). Chlorophyll fluorescence—a practical guide. *Journal of Experimental Botany* , 51, 659-668.
- Maynard-Smith, J. (1957). Temperature tolerance and acclimatization in *Drosophila subobscura* . *Journal of Experimental Biology* , 34, 85-96.
- Milner, K.V., French, K., Krix, D.W., Valenzuela, S.M. & Leigh, A. (2023). The effects of spring versus summer heat events on two arid zone plant species under field conditions. *Functional Plant Biology* , 50, 455-469.
- Molina, A.N., Pulgar, J.M., Rezende, E.L. & Carter, M.J. (2023). Heat tolerance of marine ectotherms in a warming Antarctica. *Global Change Biology* , 29, 179-188.
- Neuner, G. & Buchner, O. (2023). The dose makes the poison: the longer the heat lasts, the lower the temperature for functional impairment and damage. *Environmental and Experimental Botany* , 212, 105395.
- Orsted, M., Jorgensen, L.B. & Overgaard, J. (2022). Finding the right thermal limit: a framework to reconcile ecological, physiological and methodological aspects of CTmax in ectotherms. *The Journal of Experimental Biology* , 225, jeb244514.
- Perez, T.M. & Feeley, K.J. (2020). Photosynthetic heat tolerances and extreme leaf temperatures. *Functional Ecology* , 34, 2236-2245.
- Perez, T.M., Feeley, K. J., Michaletz, S. T. & Slot, M. (2021) Methods matter for assessing global variation in plant thermal tolerance. *Proceedings of the National Academy of Sciences USA* , 118, 30, e2024636118.
- Pinsky, M.L., Eikeset, A.M., McCauley, D.J., Payne, J.L. & Sunday, J.M. (2019). Greater vulnerability to warming of marine versus terrestrial ectotherms. *Nature* , 569, 108-111.
- Rezende, E.L., Bozinovic, F., Szilagyi, A. & Santos, M. (2020). Predicting temperature mortality and selection in natural *Drosophila* populations. *Science* , 369, 1242-1245.
- Rezende, E.L., Castaneda, L.E. & Santos, M. (2014). Tolerance landscapes in thermal ecology. *Functional Ecology* , 28, 799-809.

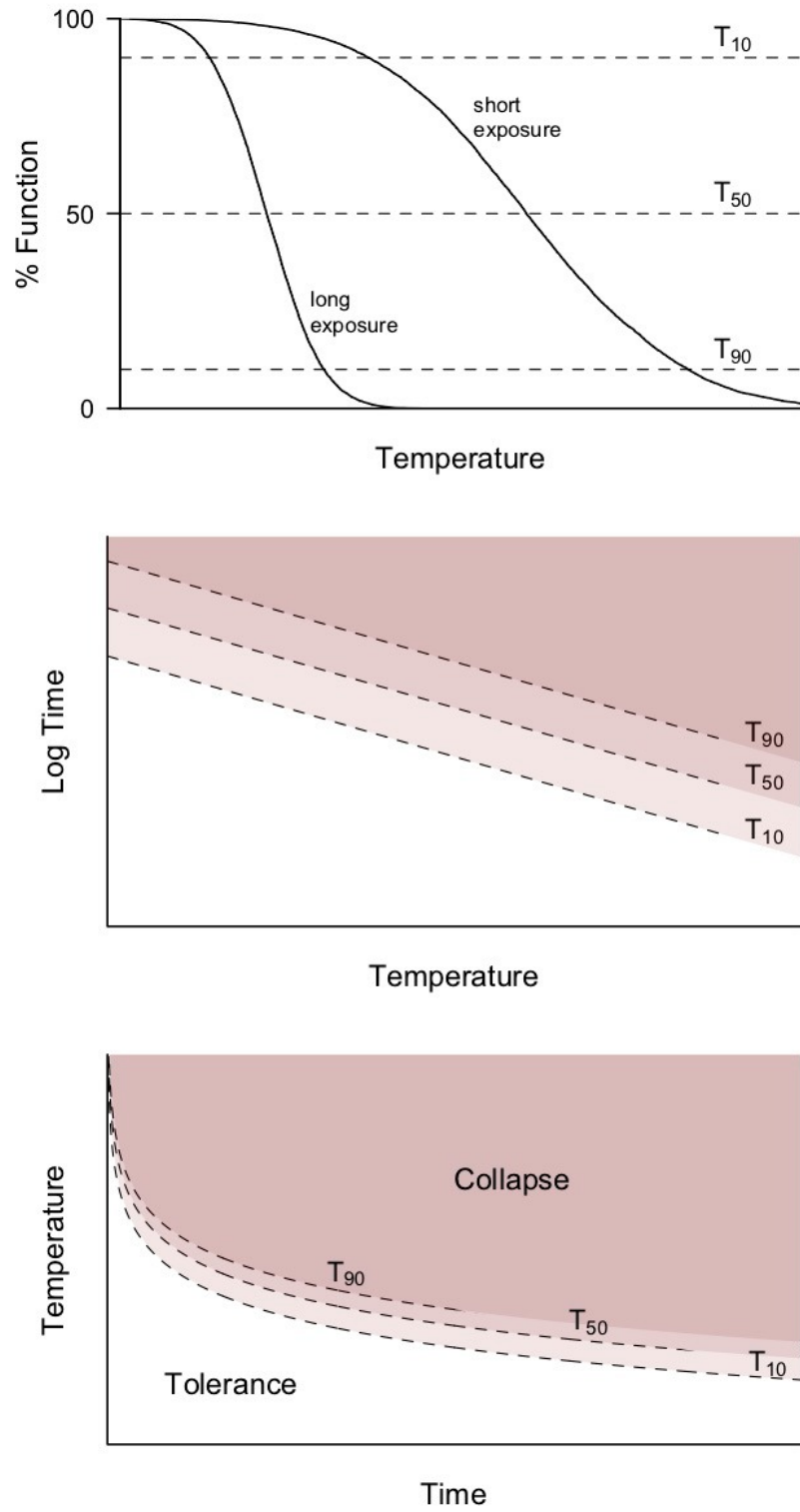
- Rezende, E.L. & Santos, M. (2012). Comment on 'Ecologically relevant measures of tolerance to potentially lethal temperatures'. *The Journal of Experimental Biology* , 215, 702-703.
- Rezende, E.L., Tejedo, M. & Santos, M. (2011). Estimating the adaptive potential of critical thermal limits: methodological problems and evolutionary implications. *Functional Ecology* , 25, 111-121.
- Santos, M., Castaneda, L.E. & Rezende, E.L. (2011). Making sense of heat tolerance estimates in ectotherms: lessons from *Drosophila* .*Functional Ecology* , 25, 1169-1180.
- Sastry, A. & Barua, D. (2017). Leaf thermotolerance in tropical trees from a seasonally dry climate varies along the slow-fast resource acquisition spectrum. *Scientific Reports* , 7, 11246.
- Siwko, M.E., Marrink, S.J., de Vries, A.H., Kozubek, A., Schoot Uiterkamp, A.J.M. & Mark, A.E. (2007). Does isoprene protect plant membranes from thermal shock? A molecular dynamics study.*Biochimica et Biophysica Acta - Biomembranes* , 1768, 198-206.
- Slot, M., Krause, G.H., Krause, B., Hernandez, G.G. & Winter, K. (2019). Photosynthetic heat tolerance of shade and sun leaves of three tropical tree species. *Photosynthesis research* , 141, 119-130.
- Somero, G.N. & DeVries, A.L. (1967). Temperature tolerance of some Antarctic fishes. *Science* , 156, 257-258.
- Stumbo, C.R. (1973). *Thermobacteriology in Food Processing* . 2 edn. Academic Press.
- Sumner, E.E., Williamson, V.G., Gleadow, R.M., Wevill, T. & Venn, S.E. (2022). Acclimation to water stress improves tolerance to heat and freezing in a common alpine grass. *Oecologia* , 199, 831-843.
- Sunday, J.M., Bates, A.E. & Dulvy, N.K. (2010). Global analysis of thermal tolerance and latitude in ectotherms. *Proceedings of the Royal Society B, Biological Sciences* , 278, 1823-1830.
- Sunday, J.M., Bates, A.E., Kearney, M.R., Colwell, R.K., Dulvy, N.K., Longino, J.T. *et al.* (2014). Thermal-safety margins and the necessity of thermoregulatory behavior across latitude and elevation.*Proceedings of the National Academy of Sciences* , 111, 5610-5615.
- Tang, J., Mitcham, E.J., Wang, S. & Lurie, S. (2007). Heat treatments for postharvest pest control: theory and practice. CABI.
- Terblanche, J.S., Deere, J.A., Clusella-Trullas, S., Janion, C. & Chown, S.L. (2007). Critical thermal limits depend on methodological context. *Proceedings of the Royal Society B: Biological Sciences* , 274, 2935-2942.
- Terblanche, J.S., Hoffmann, A.A., Mitchell, K.A., Rako, L., le Roux, P.C. & Chown, S.L. (2011). Ecologically relevant measures of tolerance to potentially lethal temperatures. *Journal of Experimental Biology* , 214, 3713-3725.
- Valladares, F. & Pearcy, R.W. (1997). Interactions between water stress, sun-shade acclimation, heat tolerance and photoinhibition in the sclerophyll *Heteromeles arbutifolia*. *Plant, Cell & Environment* , 20, 25-36.
- Watkins, J.H. & Winslow, C.E. (1932). Factors determining the rate of mortality of bacteria exposed to alkalinity and heat. *Journal of Bacteriology* , 24, 243-265.
- Yan, K., Chen, P., Shao, H., Zhang, L. & Xu, G. (2011). Effects of short-term high temperature on photosynthesis and photosystem ii performance in sorghum. *Journal of Agronomy and Crop Science* , 197, 400-408.
- Yarwood, C.E. (1961). Acquired tolerance of leaves to heat.*Science* , 134, 941-942.
- Zheng, G., Tian, B.O., Zhang, F., Tao, F. & Li, W. (2011). Plant adaptation to frequent alterations between high and low temperatures: remodelling of membrane lipids and maintenance of unsaturation levels.*Plant, Cell & Environment* , 34, 1431-1442.

Zhu, L., Bloomfield, K.J., Hocart, C.H., Egerton, J.J.G., O’Sullivan, O.S., Penillard, A. *et al.* (2018). Plasticity of photosynthetic heat tolerance in plants adapted to thermally contrasting biomes. *Plant, Cell & Environment* , 41, 1-12.

Table 1. Comparing different logistic regression models.

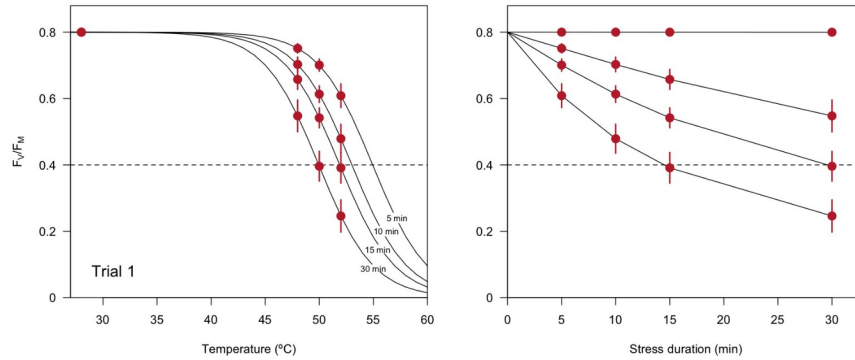
Model	$K$	$AIC_c$	$\Delta AIC_c$	$w_i$	$LL$
$F_V/F_M \sim Ta + time + trial + Sp$	6	427.99	3.42	0.15	-207.93
$F_V/F_M \sim Ta * time * trial * Sp$	16	438.2	13.63	0	-202.66
$F_V/F_M \sim Ta + \log_{10} time + trial + Sp$	6	424.57	0	0.84	-206.22
$F_V/F_M \sim Ta * \log_{10} time * trial * Sp$	16	434.73	10.16	0.01	-200.93

$K$  = number of parameters,  $AIC_c$  and  $\Delta AIC_c$  = Akaike Information Criterion,  $w_i$  = Akaike’s weights,  $LL$  = log-likelihood of each model.

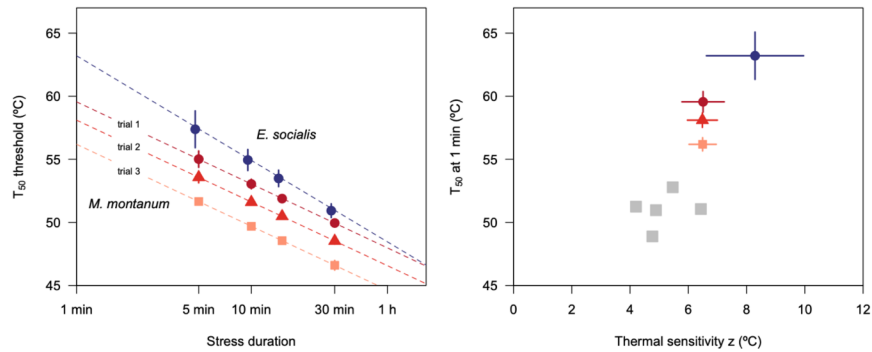


**Figure 1 .** Cumulative heat stress and temperature-duration effects. Thermal damage has been measured

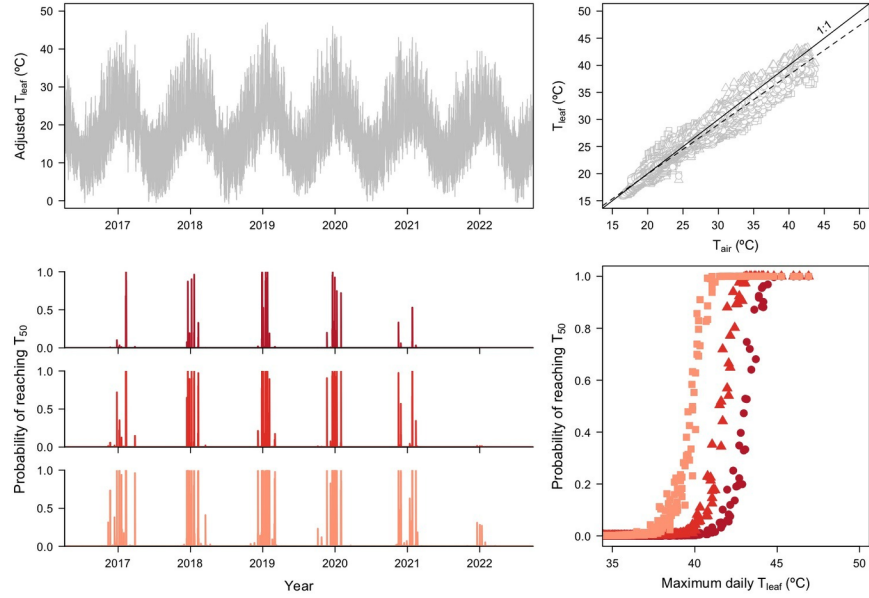
at different temperatures while controlling for duration time. Here we show that this approach can be generalized for multiple temperatures and exposure times, in what is known as thermal-death time curves or thermal tolerance landscapes in the animal literature (Rezende et al. 2014).



**Figure 2** . Variation in overnight  $F_V/F_M$  as a function of measurement temperature and exposure time for *M. montanum* . The fitted logistic models rescaled between 0 and 0.8 are shown in the left panels, with the dotted line indicating a 50% decline of  $F_V/F_M$  employed to estimate  $T_{50}$  in subsequent analyses. For simplicity, only trial 1 is shown (for the whole dataset, see Supplementary Figs. S3 and S4). Values are shown as means  $\pm$  SD.



**Figure 3** . Log-linear relationship between  $T_{50}$  threshold for 50% collapse in  $F_V/F_M$  and exposure time, for *M. montanum* and *E. socialis* . These linear functions can be described with two parameters, namely their intercept  $T_{50}$  at 1 min because  $\log_{10} \text{time} = 0$  and their slope  $z$ . We also include comparable estimates for alpine species *Pinus cembra* , *Picea abies* , *Larix decidua* , *Kalmia procumbens* and *Ranunculus glacialis* based on published data (see Supplementary Table S3), which seem to exhibit lower intercepts and slopes than our estimates. Values are shown as means  $\pm$  SD obtained from the bootstrap analysis.



**Figure 4.** Predicted heat damage under field conditions for *M. montanum* measured in different trials. We employed a linear transform to convert air temperature data from Port Augusta into predicted leaf temperature, and then the dynamic approach by Rezende et al. (2020) to quantify the expected heat damage based on these temperatures and the temperature-duration curves obtained in the laboratory. Note that heat tolerance decreases from trials 1 to 3. Colors as in Fig. 3.