Drivers of thermal tolerance breadth of plants across contrasting biomes: do mean or seasonality in climate indices matter more?

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Abstract

1. The Climate Variability Hypothesis (CVH) predicts that species from environments with more variable temperatures should have *wide* thermal tolerance breadth. We addressed this question in plants and asked which climate predictors (e.g., temperature, precipitation, variability/seasonality) best predict species thermal limits.
2. Measures of low ($T_{crit\text{-}cold}$) and high ($T_{crit\text{-}hot}$) photosystem II thermal thresholds were used to determine thermal tolerance breadth (TTB), along with ice nucleation temperature (NT) of 69 plant species in alpine, desert and temperate biomes.
3. Tolerance metrics $T_{crit\text{-}cold}$, NT and $T_{crit\text{-}hot}$ all differed among biomes. Notably, desert species had the most cold and heat tolerant leaves with the widest TTB. Alpine and temperate biomes had similar TTB and each biome TTB exceeded their climate thermal ranges. Overall, climate drivers explained the most variation (~50%) in TTB and NT, with species a second strong predictor. The climate variables best explaining tolerance differed for cold and heat: species from more variable (higher temperature seasonality) environments (alpine and desert) were more cold tolerant, whereas mean annual temperature (MAT) was the best predictor of $T_{crit\text{-}hot}$ with species in higher MAT environments having higher heat tolerances. TTB and NT were explained by both seasonality and MAT. Unexplained variation could be due to microclimate-driven plasticity, leaf traits or thermoregulatory mechanisms.
4. Our results provide some support for the CVH. Depending on the thermal limits examined, climate means and seasonality remain important predictors of plant thermal tolerance.

Key words: Climate variability hypothesis, thermal tolerance breadth, heat tolerance, freezing tolerance, climate, ice nucleation temperatures

Introduction

Desert and alpine plant species live at the thermal limits of biological processes. Unlike those in temperate regions, plants living in extreme environments are exposed to both very low and very high temperatures and thus to wide thermal range and highly variable thermal regimes, both among days and seasons. Given the important influence of temperature in shaping species distributions (Tattersall *et al.*, 2012; Garcia-Pichel *et al.*, 2013) the innate thermal tolerance limits (lowest and highest temperature that a species can withstand) and thermal tolerance breadths (range of temperature between upper and lower thermal tolerance limits) of species are predicted to correlate with the temperature variation characterising their environment. The climate variability hypothesis (CVH; Janzen, 1967) predicts that species with *wide* thermal tolerance ranges are likely to evolve in environments with *variable* temperatures compared to thermal specialists with *narrow* tolerance ranges that evolved in thermally *stable* environments. This hypothesis is a cornerstone of thermal ecology, but has to date been primarily explored in animals (Addo-Bediako *et al.*, 2000; Compton *et al.*, 2007; Calosi *et al.*, 2010; Baudier *et al.*, 2018).

Cold and heat tolerance thresholds are rarely studied concomitantly. Cold tolerance studies predominate in alpine environments where plants can withstand very low temperatures and tolerate extracellular ice formation and the resulting cytoplasmic dehydration (Sakai & Larcher, 1987; Larcher, 2003). Heat tolerance studies, historically, are primarily focussed on warm and desert environments (Geange et al., 2021). Mechanisms for tolerating extreme heat have been demonstrated in a range of desert species, including heat avoidance through increased transpiration (Aparecido *et al.*, 2020) with microhabitat conditions setting the scene for heat tolerance (Curtis *et al.*, 2016), whereas in other cases, these differences disappear under common garden conditions (Downton *et al.*, 1984; Knight & Ackerly, 2002; Knight & Ackerly, 2003). In spite of great progress, a recent systematic review revealed that less than 5% of more than 3000 studies examining thermal tolerance of plants considered both heat and cold tolerance thresholds together (Geange *et al.*, 2021). The current focus on either cold or heat tolerance overlooks the fact that plants in extreme biomes can experience both cold and heat stress.

Temperature increases associated with climate change have motivated an increase in studies on plant heat tolerance, particularly in relation to climate variability and the occurrence of aseasonal extreme events (Rummukainen, 2012; Seneviratne *et al.*, 2012). However, *in situ* field sampling

studies rarely encompass multiple biomes or a broad range of thermal environments across landscapes. There remains a critical gap in our understanding of how plants in extreme *vs.* benign environments tolerate both heat and cold stress *in situ*, which limits our capacity to predict where the most thermally sensitive species occur.

Differing patterns of climate among contrasting biomes provide an opportunity to explore the role of climate variability in upper and lower thermal thresholds and on thermal tolerance breadth. Of the few studies that have compared thermal thresholds among biomes, Smillie and Nott (1979) found that heat tolerance was lower in alpine species, followed by temperate and tropical species. In a rare study considering both upper and lower thermal thresholds together, Cunningham and Read (2006) show that, in common garden conditions, temperate origin species have lower heat and higher cold tolerance than tropical species. Meta-analytical studies across a wide range of species and biomes suggest that heat tolerance decreases and cold tolerance increases with latitude; however, these shifts in tolerance do not keep pace with an observed latitudinal temperature gradient (Lancaster & Humphreys, 2020). Further, predictors of thermal tolerance variation with latitude may include mean temperature of the warmest month and mean annual temperature (O'Sullivan *et al.*, 2017; Lancaster & Humphreys, 2020), though threshold-based predictors (e.g. number of days over 35 degrees per year; Maher *et al.*, 2019) are increasingly being explored as drivers of heat tolerance (Mora *et al.*, 2015). Given that water status influences plant heat tolerance (Curtis *et al.*, 2016; Marchin *et al.*, 2022) and freezing tolerance (Sierra-Almeida *et al.*, 2016), precipitation parameters are also likely to affect thermal thresholds. In addition, upper and lower thermal thresholds can vary in relation to the methods used (Lancaster & Humphreys, 2020; Perez *et al.*, 2021), influencing the ability of large-scale meta-analyses to capture such sources of variation and likely limiting their power to reach generalisable conclusions about any association between thermal limits and climate variables. Finally, although we have evidence that climate variables influence heat and cold thermal thresholds independently, the climate predictors of the difference between these thresholds, thermal tolerance breadth, are unknown. If we can reveal general patterns underlying variation and the climate drivers of physiological thermal tolerance thresholds among co-occurring species within and among biomes, we can strengthen predictions of which and where species are likely to be most vulnerable to changing thermal conditions.

Here we assess leaf-level thermal tolerance breadth (TTB) of plant species in extreme and benign environments and the climate drivers of these thresholds to test the CVH. We used chlorophyll fluorescence to determine the low and high photosystem II (PSII) thermal thresholds and thermal tolerance breadth of 22 alpine, 24 desert and 23 temperate rainforest species (temperate hereafter) during the growing season. We also determined ice nucleation temperatures to examine freezing tolerance in all species. To determine relationships between climate variability and physiological thresholds, we extracted long term climate parameters for each sampling locality. First, we hypothesised that the physiological thermal limits will be skewed towards the extremes of the environments; colder temperatures in the alpine; and warmer temperatures in the desert respectively, with temperate species having milder cold and heat tolerance thresholds. Second, we tested if species from more thermally variable biomes have high thermal tolerance to both heat and cold, and thus wider thermal tolerance breadth, in accordance with the CVH. Thirdly, we assessed whether the variation in tolerance thresholds and TTB is explained by climate variability (e.g., seasonality), supporting the CVH, rather than mean climate values.

Materials and Methods

Study locations

Sites used in this study were designated to one of three biomes in New South Wales, Australia: alpine (Kosciuszko National Park), desert (Gundabooka National Park), and wet temperate (Illawarra Region: Royal National Park, Illawarra State Conservation Area and Bass Point Conservation Area) (**Fig. 1**).

Species selection

A total of 69 species (22 alpine, 24 desert and 23 wet temperate species; **Table S1**) were selected to encompass a representative sample of growth forms and plant families and where possible, included congeneric and/or confamilial species between two or more biomes. We included community dominants and a range of less abundant species to represent a cross section of species occupying each biome. Species replicates were sampled across days within each sampling period to incorporate daily variation in thermal tolerance thresholds.

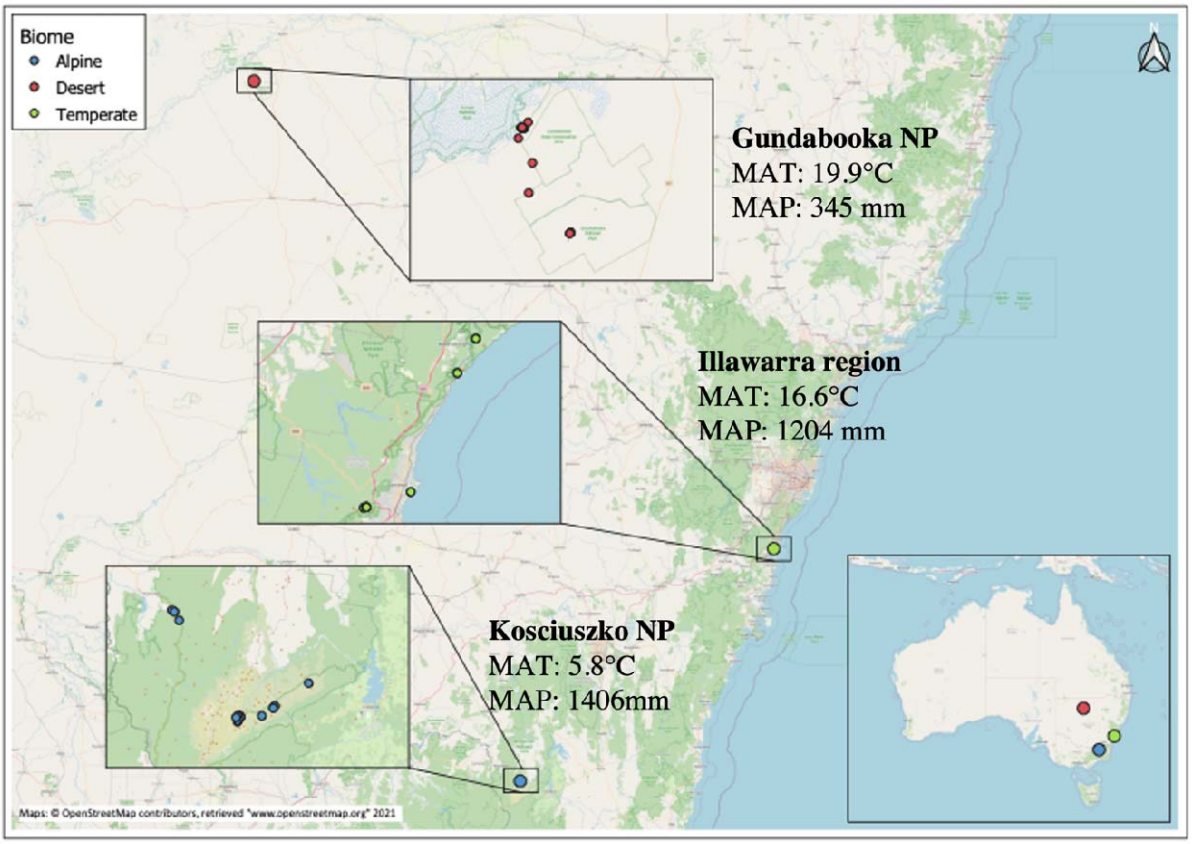

Fig. 1: Locations of study sites across three biomes in NSW, Australia. Desert sites (red) in Gundabooka National Park (GNP), temperate sites (green) in the Illawarra Region, and alpine sites (blue) in Kosciuszko National Park (KNP). GNP sites span open eucalypt woodland, mulga and chenopod shrublands between elevations of 100 to 224 m a.s.l. KNP sites encompassed feldmark, herbfields and sphagnum bog vegetation formations between 1792 and 1855 m a.s.l. In the wet temperate environments, collections were conducted across the Illawarra Subtropical Rainforest including the Tall Open Forest and Rainforest communities between 100 and 400 m a.s.l. MAT = mean annual temperature, MAP = mean annual precipitation.

Leaf sample collection

Mature, fully expanded leaves were harvested from five to seven individuals per species. For shrubs and trees, small branches were collected from the northern facing side of the plant at a height of 1-1.5 m. For smaller growth forms, entire individuals or rosettes were collected. Samples were kept in cool sealed plastic bags with moistened paper towel until they were processed (approx. 1-2 hours after collection). In the field laboratory, leaves (size ranging 0.25 cm^2 to 5 cm^2) were cut (or combined) to fit an approximate area of 1 cm^2 and kept on wet florist foam in humid dark containers until thermal tolerance assays were performed. Measurements occurred during January 2020, March 2020 and December 2020 for the desert, wet temperate and

alpine biomes, respectively. Sampling periods were selected to capture peak growing season in each region.

Thermal tolerance assays

The photosynthetic machinery, specifically photosystem II (PSII), embedded in the thylakoid membrane has long been recognised as a thermally sensitive structure (Schreiber & Berry, 1977; Berry & Bjorkman, 1980; Seemann *et al.*, 1984). The temperature at which the PSII are disrupted causes a rise in minimal chlorophyll a fluorescence (F_0) as a leaf is heated or cooled (Schreiber & Berry, 1977; Neuner & Pramsohler, 2006). This critical temperature indicates a threshold beyond which physiological and photochemical systems become increasingly impaired and damage can occur, if temperatures are sustained. Freezing tolerance is tied to the temperatures at which ice formation occurs (ice nucleation, IN), the measuring of which is required to understand whether temperature dependent damage to photosynthetic machinery occurs in association with ice formation.

Lower and upper thresholds of PSII ($T_{crit\text{-}cold}$ and $T_{crit\text{-}hot}$)

The critical low and high temperature thresholds of PSII ($T_{crit\text{-}cold}$ and $T_{crit\text{-}hot}$) were measured using T- F_0 curves, i.e., the rise in F_0 (Neuner & Pramsohler, 2006; Arnold et al., 2021) with set heating or cooling rates. One leaf per individual (or several small leaves abutting one another) was used for each of the heating and cooling assays with n = 5-7 replicates per species. For each assay run, 45 samples were secured with double-sided tape to paper on a peltier plate (CP-121HT; TE-Technology, Inc., Michigan, USA), used to cool or heat samples at a set rate using LabView-based control software (National Instruments, Texas, USA) and adapted code from TE-Technology, Inc. A vacuum sealed double-glazed glass was placed on top of the leaf samples on the plate to avoid water condensation, particularly at freezing temperatures, and compress samples to ensure maximum leaf contact with the Peltier plate (see Arnold *et al.*, 2021 for more details).

Chlorophyll fluorescence was measured with a Pulse Amplitude Modulated (PAM) chlorophyll fluorescence imaging system (Maxi-Imaging-PAM; HeinzWalz GmbH, Effeltrich, Germany) mounted above the Peltier plate. Leaves were dark-adapted first and the PSII photochemistry maximum quantum efficiency (F_V/F_M) measured to check starting function of leaves before another dark adaption for 15min prior to assay. Circular areas of interest were created in the

middle of leaf samples avoiding edges for F_0 measurements using the Maxi-Imaging-PAM software. During temperature ramping, F_0 was measured every 20 s with a weak blue low frequency (1 Hz) pulse modulated measuring light (0.5 µmol photons m^{-2} s^{-1}). Leaf temperatures were simultaneously measured with 40-gauge type-T thermocouples (OMEGA Engineering, Singapore) attached to undersides of leaves and logged with a multi-channel DT85 Datataker datalogger (Lontek, Australia). For hot T-F_0 measurements, samples were heated to 60°C at a rate of 30°C h^{-1}(0.5°C/min). For cold T-F_0 measurements, the leaves were cooled down to -20°C or -25°C when logistically possible, at a rate of 15°C h^{-1}(0.25°C/min). Temperature rates were selected as moderate temperature changes per Arnold et al., (2021). The starting temperature of each heating/cooling assay depended on average field lab temperature, generally 15°C for alpine and temperate, and 20°C for desert measurements.

$T_{crit\text{-}cold}$ and $T_{crit\text{-}hot}$ were determined as inflection points on the T- F_0 curves, using the *segmented* R package. For further description of T- F_0 curve see Arnold et al., (2021). $T_{crit\text{-}cold}$ and $T_{crit\text{-}hot}$ were used to represent the lower and upper bounds of the PSII thermal tolerance breadth, TTB (TTB = $T_{crit\text{-}hot}$ - $T_{crit\text{-}cold}$).

Ice nucleation temperatures

The ice nucleation temperature was estimated by analysing the freezing exotherms of leaf tissue, using the leaf temperature data collected during $T_{crit\text{-}cold}$ measurements. Temperature data collected for each leaf sample was plotted against time to visualize the freezing exotherms (small peak in temperature). The temperature at which the exotherm began was recorded as the nucleation temperature (Larcher, 2003).

Climate variables for sampling locations

Climate data were obtained from gridded datasets at 1 km resolution based on spatial interpolations of long-term (1981-2010) conditions accessed from the CHELSA v2.1 database (Karger *et al.*, 2017). For each species, a suite of climate variables including means, minimum, maximum and seasonality variables for temperature and precipitation were extracted for each species at leaf collections sites (see **Table S2** for complete list and sampling location coordinates).

Statistical analyses

To test the Climate Variability Hypothesis ability to predict photosystem thermal tolerance, we examined climate by two designations. First, as a-prior categorical variable 'biome' (aims 1 & 2), and secondly, by a comprehensive selection of continuous climate variables (aim 3).

Differences in thermal tolerance metrics among biomes were tested with Linear Mixed Models (LMMs, Bates *et al.*, 2015) from the *lme4* package and post-hoc Tukey's Honest Significant Differences were used to compare biome means. Models included biome (alpine, desert and temperate) as fixed terms and species as a random term. Growth form as a random effect did not significantly improve models thus was excluded. Because the aim of this study was to test the Climate Variability Hypothesis in relation to thermal tolerance limits and TTB, other variables such as local air and leaf temperatures or finer scale environmental characteristics such as aspect were not included in models. Plant evolutionary history was considered in analysis, see below.

LMMs were also used to determine the relative contribution of climate variables to the variation in each thermal tolerance metric. For the contribution of climate variables, LMMs included the climate variables as fixed terms and species as a random term. Several of our climate variables were strongly correlated and to avoid collinearity, we excluded some of those variables. All full models included mean annual temperature (MAT, °C), mean annual precipitation (MAP, mm), temperature seasonality (BIO4, standard deviation of the monthly mean temperatures) and precipitation seasonality (standard deviation of the monthly precipitation estimates expressed as a percentage of the annual mean of those estimates). The best models were selected by backward selection using the function *step* from the *emdi* package (Kreutzmann *et al.*, 2019). The package *lmerTest* (Kuznetsova *et al.*, 2017) was used with *lme4* to calculate LMM degrees of freedom and *P*-values . The *MuMIn* package (Johnson, 2014) was used to calculate the variance explained by fixed effects, (marginal R^2, mR^2) and the variance explained by both fixed and random effects (conditional R^2, cR^2). The Intraclass Correlation Coefficient (ICC) describes the proportion of the total variance in the data that is explained by the random effects alone.

Prior to model fitting we assessed if the shared evolutionary history across our 69 species may also explain differences in thermal tolerance measures. We generated a phylogenetic tree using the R package *V.phylomaker* (Jin & Qian, 2022) and calculated the phylogenetic signal and statistical significance for each $T_{crit\text{-}cold}$, $T_{crit\text{-}hot}$, TTB and nucleation temperature using Pagel's λ

and Blombergs's K (**Table S3**) with the *phylosignal* package (Keck et al., 2016). No evidence of phylogenetic signal was present and thus phylogenetic non-independence was not detected therefore, we did not include the phylogenetic structure in any of our models. All analyses and visualizations were carried out in R version 3.6.1 (R Core Team, 2018).

Results

Biome differences in thermal tolerance

There were significant differences among biomes in all the individual thermal tolerance metrics measured on plant species (**Fig. 2a-d**, **Table 1**). Unexpectedly, desert species exhibited the most extreme cold thresholds (-13.3 ± 0.5°C), followed by alpine (-11.0 ± 0.5°C) and then temperate species (-8.8 ± 0.5°C), all of which differed significantly from one another (**Fig. 2a**, **Table 1**). As predicted, desert species had significantly higher heat tolerance (49.3 ± 0.6°C), followed by temperate (46.6 ± 0.6°C), then alpine (43.1 ± 0.6°C) species (**Fig. 2b**, **Table 1**). Similar to $T_{crit-cold}$ values, nucleation temperatures were coldest in desert species (-16.0 ± 0.6°C), followed by alpine (-10.8 ± 0.6°C) and warmest in temperate species (-7.5 ± 0.6°C); again, these differed significantly from one another (**Fig. 2c**, **Table 1**). Interestingly, $T_{crit-cold}$ values for alpine and temperate species were very close to their nucleation temperature, whereas in desert species, ice formation occurred at markedly lower temperatures than $T_{crit-cold}$ (**Fig. 2a, c**).

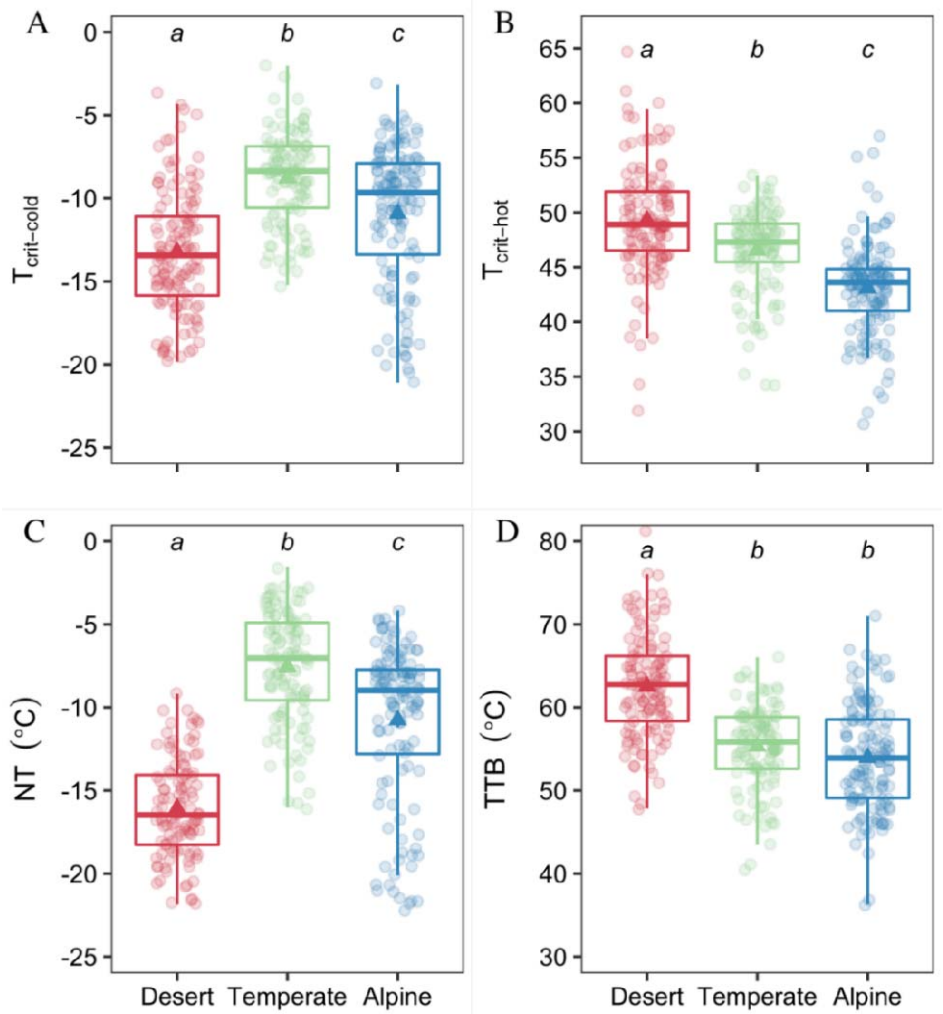

Fig. 2: Heat ($T_{crit-hot}$) and cold ($T_{crit-cold}$) thresholds, ice nucleation temperatures (NT) and thermal tolerance breadth (TTB) for desert (n = 24 spp), temperate (n = 23 spp) and alpine (n = 22 spp) biomes. Circles represent individual replicates, triangles represent biome means across species; letters denote significant differences between biomes tested using linear mixed models and post-hoc Tukey's Honest Significant Differences test for comparing means with significance at 0.05 alpha level.

At a species level, temperate species *Oplismenus hirtellus* was the least cold tolerant (-4.0ºC, $T_{crit-cold}$), whereas two alpine species *Epacris paludosa* and *Hovea montana* were the most cold tolerant (-18.5ºC $T_{crit-cold}$) and the desert species *Atriplex stipitata* had the most extreme nucleation temperature (-20.5°C). The alpine species *Pimelea ligustrina* was the least heat tolerant (38.1ºC, $T_{crit-hot}$) and the desert species *Rhagodia spinescens* was the most heat tolerant ($T_{crit-hot}$ = 54.9ºC, **Fig. 3**, **Table S1**).

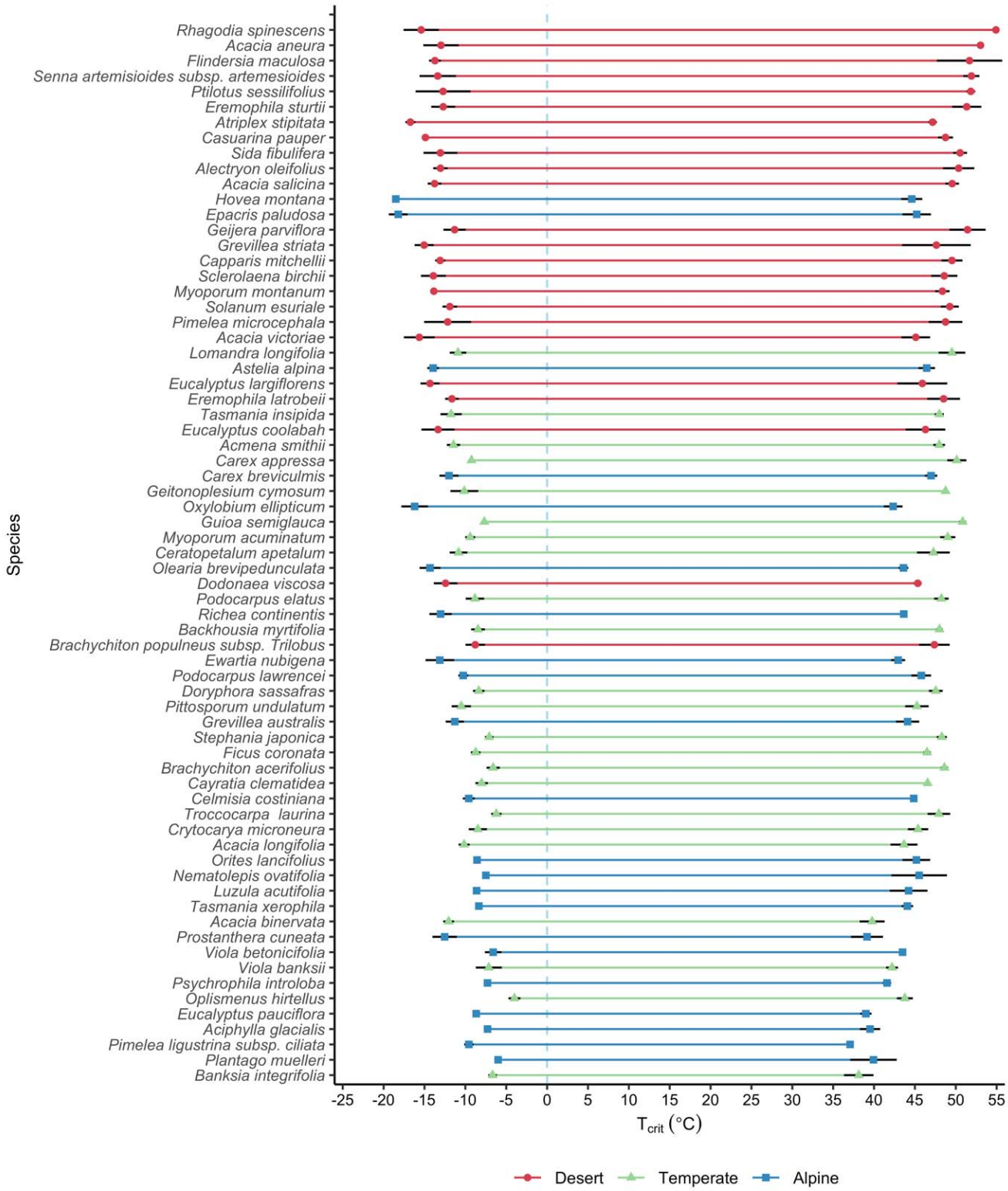

Fig. 3: Thermal tolerance breadth (TTB = $T_{crit\text{-}hot}$-$T_{crit\text{-}cold}$) for all the studied species. Species are sorted top to bottom from widest to narrowest TTB. Red circles and lines represent desert species (n = 24 spp), green triangles and lines represent wet temperate species (n = 23 spp) and blue squares and lines represent alpine species (n = 22 spp). Black horizontal lines represent the standard error. The vertical dashed line represents 0°C. Each TTB, $T_{crit\text{-}hot}$ and $T_{crit\text{-}cold}$ value is from n = 5-6 replicates per species.

Table 1: Linear mixed model output for all thermal limits considering biome as a fixed effect and species as random effect. Model biome mean estimates were significant with P values of <0.001.

Biome	**$T_{crit\text{-}hot}$**		**$T_{crit\text{-}cold}$**		**NT**		**TTB**	
	Estimate	**Std.Error**	**Estimate**	**Std.Error**	**Estimate**	**Std.Error**	**Estimate**	**Std.Error**
Alpine	43.1	0.6	-11.0	0.5	-10.8	0.6	54.1	0.9
Desert	49.3	0.6	-13.3	0.5	-16.0	0.6	62.6	0.8
Temperate	46.6	0.6	-8.8	0.5	-7.5	0.6	55.4	0.9
	Variance		**Variance**		**Variance**		**Variance**	
Species	5.0		5.2		7.4		12.3	
Residual	14.9		7.7		7.1		23.0	

The thermal tolerance breadth (TTB) was significantly wider in desert species (62.6 ± 0.8°C) than temperate (55.4 ± 0.9°C) or alpine species (54.1 ± 0.6°C) (**Fig. 2d**, **Table 1**). However, there was also considerable variation among species within biomes (**Fig. 3**). Within the desert biome TTB was more similar across species (lower variability), (56.2 to 70.3°C TTB, Δ14.1°C), compared to the alpine biome which had the greatest variation among species (45.9 to 63.1°C TTB, Δ17.2°C) followed by the wet temperate species (44.8 and 60.4°C TTB, Δ15.6°C). For species from all three biomes, TTB was much wider than the extreme temperature range calculated for the sampling sites, thus most thermal thresholds lay well beyond the minimum and maximum temperatures expected for each species in their local environment. The only notable exception was for the alpine biome species, where the cold threshold was closer to the minimum temperatures representing our alpine sites (**Fig. 4**).

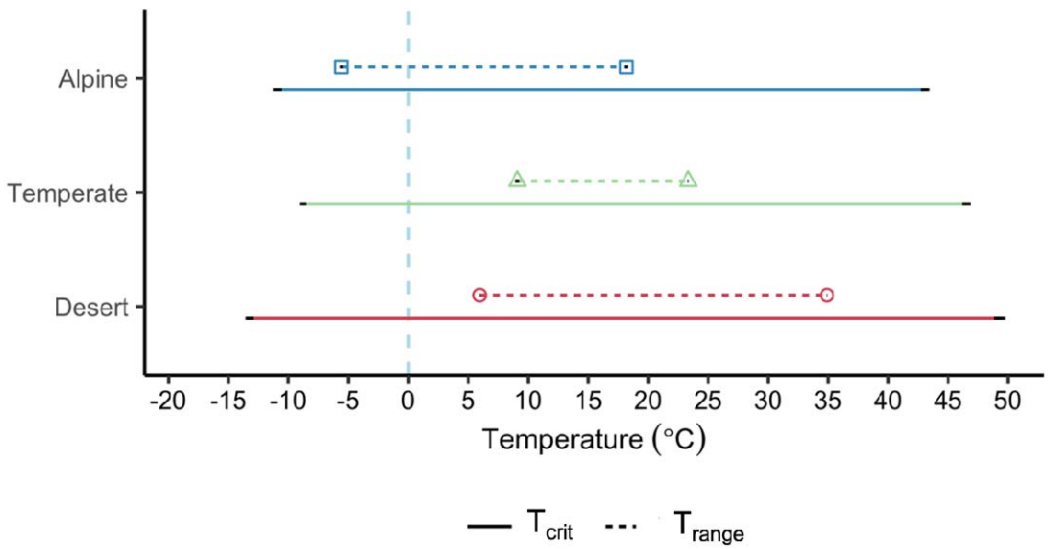

Fig. 4: Mean thermal tolerance breadth (TTB, solid lines) for alpine, temperate and desert species and their respective mean thermal range (T_{range} = mean MaxT- mean MinT based on the locations of the sample sites, see **Table S2**). Black lines represent standard errors.

Contribution of climate variables to the variation on thermal tolerance metrics

We investigated the relationship between climate variables at the site of plant sampling and each of the thermal tolerance traits using backward model selection to identify the most parsimonious linear mixed models. None of the precipitation variables included in our models explained considerable variation in thermal tolerance thresholds. Whereas the best models included either mean annual temperature or temperature seasonality or both.

$T_{crit\text{-}cold}$ was best explained by temperature seasonality (19% of the variation in $T_{crit\text{-}cold}$), although a much larger proportion of the variation in $T_{crit\text{-}cold}$ was explained by species (32%, **Table 2**). There was a negative relationship between $T_{crit\text{-}cold}$ and temperature seasonality, indicating that species from environments with greater seasonality, our alpine and desert sites, had a more extreme $T_{crit\text{-}cold}$ than species from the less seasonal wet temperate rainforest (**Fig. 5a**). By contrast, rather than seasonality, $T_{crit\text{-}hot}$ was best explained by mean annual temperature (MAT, 23%), with a much smaller proportion of the variation in this variable explained by species (19%, **Table 2**). There was a positive relationship between $T_{crit\text{-}hot}$ and site MAT (**Fig. 5b**), such that species from, on average, warmer environments, also had higher $T_{crit\text{-}hot}$.

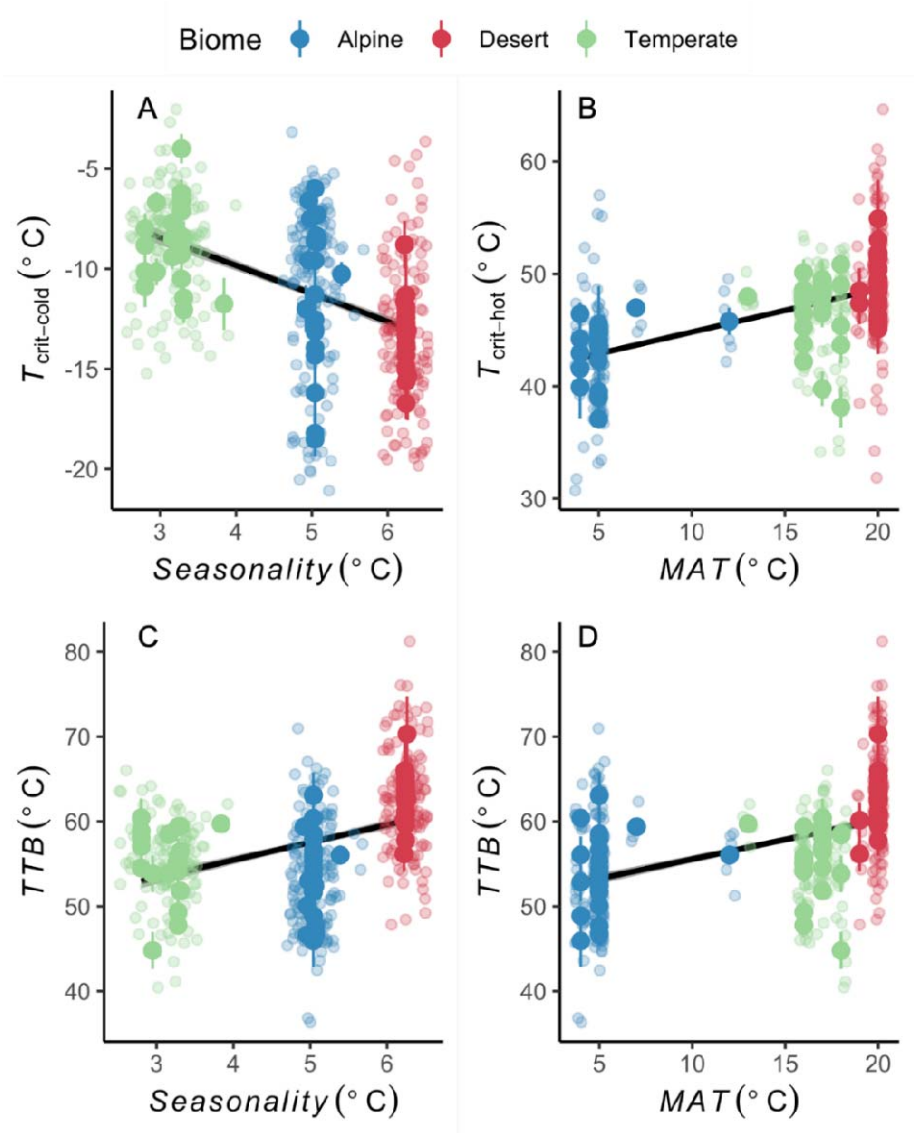

Fig. 5: Climatic variables that best predicted the variation in thermal thresholds across all biomes. Environmental variables are temperature seasonality (°C) and mean annual temperature (MAT, °C). Best models included: for $T_{crit-cold}$, seasonality; for $T_{crit-hot}$, MAT; for TTB both seasonality and MAT. See **Table 2** for statistics. Solid circles are species means, open circles are individual replicates which are jittered around mean x axis points.

TTB was best predicted by seasonality and mean annual temperature (**Table 2**), although a similar proportion of the variance was explained by the species effect compared to climate variables (29% and 24% respectively). There was a positive relationship between TTB and seasonality as well as with mean annual temperature (**Fig. 5c,d**). Notably, TTB was wider in species from desert biomes, which experience considerably greater seasonality and MAT than those in alpine and temperate environments.

Table 2: Best models predicting climatic drivers of physiological thermal limits across all species. Linear mixed model fit using a restricted maximum likelihood approach and t-tests using Satterthwaite's method. Climatic conditions at the sites where leaf samples were collected were included as fixed effects and species as a random effect. These models were selected using a backward model selection using the function *step* from the *emdi* package in R. Along with species, mean annual temperature (MAT) and temperature seasonality were significant predictors in our models.

$T_{crit\text{-}cold}$ ~ Seasonality + (1|Species)								
	Estimate	**SE**	**t-value**	**Df**	**P-value**	**mR^2**	**ICC**	**cR^2**
Intercept	-4.20	1.21	-3.48	70	<0.001			
Seasonality	-1.41	0.24	-5.82	70	<0.001			
	Variance		**N**			0.19	0.32	0.52
Species	5.25		69					
Residual	7.66	2.76						
$T_{crit\text{-}hot}$ ~ MAT + (1|Species)								
	Estimate	**SE**	**t-value**	**Df**	**P-value**	**mR^2**	**ICC**	**cR^2**
Intercept	41.00	0.81	50.24	61	<0.001			
MAT	0.38	0.05	7.22	63	<0.001			
	Variance		**N**			0.23	0.19	0.43
Species	5.22		69					
Residual	14.87							
TTB ~ Seasonality + MAT + (1|Species)								
	Estimate	**SE**	**t-value**	**DF**	**P-value**	**mR^2**	ICC	**cR^2**
Intercept	42.39	2.11	20.06	67	<0.001			
Seasonality	1.88	0.07	4.91	68	<0.001			
MAT	0.42	0.07	5.51	64	<0.001			
	Variance		**N**			0.29	0.24	0.53
Species	13.14		69					
Residual	22.94							
NT ~ MAT + Seasonality + (1|Species)								
	Estimate	**SE**	**t-value**	**DF**	**P-value**	**mR^2**	ICC	**cR^2**
Intercept	3.15	1.52	2.06	65	0.043			
MAT	-0.14	0.05	-2.68	66	0.009			
Seasonality	-2.60	0.27	-9.38	65	<0.001			
	Variance		**N**			0.45	0.27	0.72
Species	7.22		69					
Residual	7.12							

Unlike the cold PSII thermal threshold, both mean annual temperature (MAT) and seasonality explained a considerable proportion of variation (45%) among ice nucleation (NT) temperatures while species had comparatively less of an effect (27%) (**Table 2**). There was a negative relationship between nucleation temperature and temperature seasonality, indicating that species

from environments with higher seasonality froze at lower temperatures than species in environments where temperature is more stable (**Fig. 6a**). There was also a negative relationship between nucleation temperature and MAT, where desert species froze at more extreme temperatures than most species in colder climates (**Fig. 6b**).

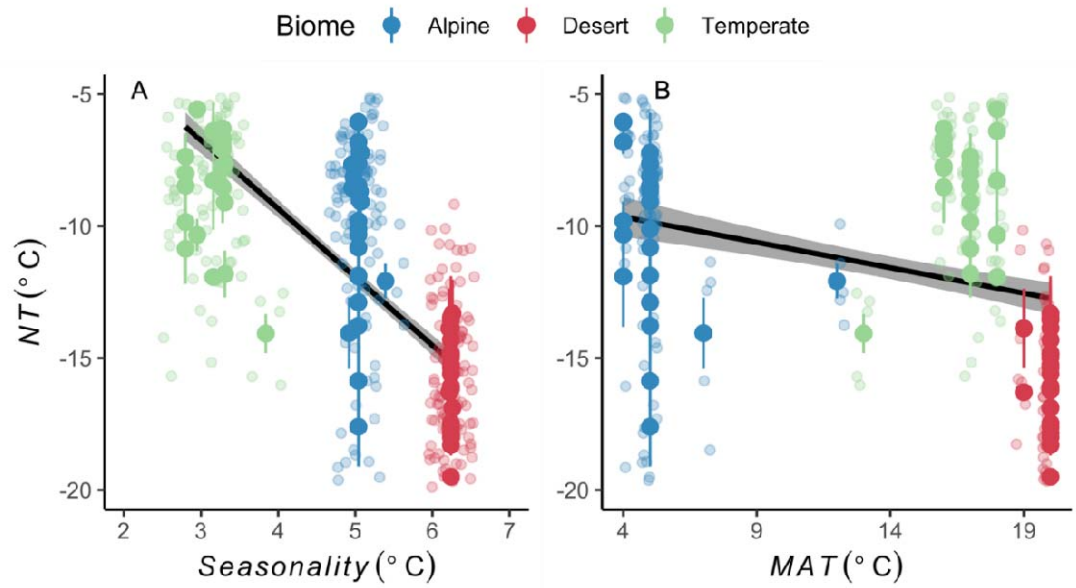

Fig. 6: Climatic variables (temperature seasonality and mean annual temperature (MAT)) that best explained the variation in ice nucleation temperature (NT), see **Table 2** for statistics.

Discussion

The climate variability hypothesis (CVH) predicts that species will evolve wider thermal tolerance breadths in environments with more variable temperatures and thermal specialisation (narrow TTB) in thermally stable environments, a concept largely supported in animals (i.e Compton *et al.*, 2007; Sunday *et al.*, 2011). Of the few studies testing this hypothesis on plants, investigations have included phenotypic plasticity increases with latitude (Molina-Montenegro & Naya, 2012), species distribution ranges with seasonality across elevation (Mumladze *et al.*, 2017) and the breadth of photosynthetic thermal response with environmental temperature variability (Perez *et al.*, 2023). Here we explored the CVH by sampling for thermal tolerance metrics including TTB on species growing in three contrasting biomes, two extreme and more variable, one benign and more thermally stable.

Using the CVH framework, we hypothesised that the thermal tolerance limits of alpine and desert species would be skewed to their respective habitat extremes (aim 1). These more skewed

tolerances in the thermally extreme environments would also drive wider TTB than the more benign temperate thermal environment (aim 2). We found that variation in tolerance breadth in the more seasonal desert and alpine environments was indeed wider than temperate species, with desert TTB values skewed towards higher temperatures, alpine TTB values skewed towards colder temperatures and wet temperate TTB sitting in between. However, the mean tolerance breadths of alpine and temperate species were not statistically different, while desert species had both greater heat and cold thresholds. When looking at the climate drivers of tolerance breadth, we found that not only thermal seasonality, but also mean annual temperature and predicted TTB, providing some, but not conclusive support for the CVH (aim 3). When we explored the climate drivers of thermal thresholds, we found that temperature seasonality, our proxy for climate variability, and MAT both contributed to explaining the variation in TTB: seasonality best explained the variation in $T_{crit\text{-}cold}$, whereas MAT was the strongest predictor of $T_{crit\text{-}hot}$. Below we explore our findings in terms of physiology and the ecological implications of these results for species persistence in a changing climate.

TTB exceeds thermal ranges in each biome and varies within biomes

Thermal tolerance breadth values reported here are notably wide; wider than the thermal ranges in each environment and larger than TTB in other organisms. For instance, across a range of marine and terrestrial ectotherm taxa, the widest recorded TTB was 60°C in terrestrial arthropods (Sunday *et al.*, 2011), while the widest TTB in our dataset was 70°C in the desert species *Rhagodia spinescens*, with several other desert species also exceeding 60°C. Within each biome, we found species with a range of wide and narrow TTB (**Fig. 3**). For example, several alpine species across growth forms had relatively narrow TTB, such as the snow bed species *Psychrophila introloba* (45°C TTB) and the treeline species *Eucalyptus pauciflora* subsp. *niphophila* (47°C TTB). The narrow TTB of some alpine species is mainly driven by their low heat tolerance ($T_{crit\text{-}hot}$ = around 40°C), while other co-occurring species (*Hovea montana* and *Epacris paludosa*) had TTB similar to desert species (around 63°C) driven by extreme cold tolerance (around -18°C). This within-biome variation in TTB might be due to variation in microclimate 'preferences' of species within biomes, which vary depending on species growth forms, topography and different transpirational cooling limitations (Curtis *et al.*, 2016; Curtis *et al.*, 2019; Aparecido *et al.*, 2020).

Although thermal seasonality was greater in alpine than temperate regions sampled here, the average TTB for these two biomes is remarkably similar (**Fig. 2d**). Temperate species can tolerate colder temperatures than expected, and it is possible that their wide thermal tolerance is a legacy of exposure to climate extremes through geological time (Byrne *et al.*, 2008). The climate legacy effect on thermal tolerance seems to be stronger for cold than heat tolerances (Bennett *et al.*, 2021) and might explain why some temperate and desert species have greater freezing tolerance than expected by their current climate. Although metrics based on air temperatures for our alpine biome (e.g., MAT, temperature seasonality) confirm that thermal range and seasonality are high in this biome, mountain plants spend 4-5 months under the insulating cover of snow, which stabilizes temperatures (Briceño *et al.*, 2014). This dampening of climate extremes might contribute to the similarity between alpine and temperate species TTB.

Heat and cold thermal limits vary among biomes

Plants exposed to drought and/or freezing share a similar physiological response: cell dehydration (Anisko & Lindstrom, 1996; Blake & Hill, 1996; Lintunen *et al.*, 2013). The physiological response to drought conditions may partially explain our somewhat counterintuitive result that desert species were more freezing tolerant (more extreme $T_{crit\text{-}cold}$ and nucleation temperature) than either alpine or temperate species. Plants can use osmotic adjustments to offset water loss from cells along the water potential gradient caused by both drying and freezing (Siminovitch & Cloutier, 1983; Larcher, 2003). Thus, the ability of desert species to cope with dehydration induced by long periods of drought might also confer the ability to cope with freezing, despite the fact that these desert species are unlikely to ever be exposed to such extreme freezing temperatures. In addition, leaf adaptations to aridity such as smaller leaves, thick cuticles, smaller, more densely packed cells, thicker cell walls with small capillary structures and small xylem vessel diameter (Dörken *et al.*, 2020) also can prevent ice formation, which increases supercooling capacity and freezing tolerance (Lintunen *et al.*, 2013). This may reflect that species living in environments where freezing temperatures are rare use supercooling as a mechanism to avoid freezing damage, while in alpine environments species tolerate ice formation. Indeed, the desert species studied here generally had nucleation temperatures much lower than their $T_{crit\text{-}cold}$, from which we can infer a high supercooling capacity that effectively serves to delay freezing. Indeed, Andean alpine species' low ice nucleation temperatures at the end of the growing season are attributed to low soil moisture, thus potential water stress, rather than low temperatures.(Sierra-Almeida *et al.*, 2016). Likewise, drought exposure can influence

heat tolerance through raising intracellular sugar contents which stabilize thylakoid membranes, increasing heat tolerance (Huve *et al.*, 2006; Brestic *et al.*, 2012). The extreme freezing tolerance and high heat tolerances found in our desert species might reflect a secondary outcome of plant adaptation to aridity.

The relatively high heat tolerance of some of our alpine species is at first counterintuitive, as alpine areas are conventionally seen as primarily cold environments. Yet, alpine plants can be exposed to very high temperatures, especially those with prostrate growth forms (Körner, 2003). Studies on leaf temperature in alpine species in Europe have shown that leaf temperature can reach up to 50°C in summer, especially on calm sunny days, with leaf heat tolerance higher than 50°C (Buchner & Neuner, 2003). Maximum leaf temperature has been measured at 38°C in early summer in one Australian alpine species (Danzey *et al*., 2021 unpublished data) and may be higher in suitable hot and still conditions in late summer. Therefore, thermal thresholds could be breached in alpine species if the combination of heat with other environmental stress, such as high light, decreased heat tolerances, as found for some European alpine species (Buchner & Neuner, 2003; Buchner *et al.*, 2015).

Heat and cold thermal limits relate to different macroclimatic temperature variables

Predicting organismal responses to changing temperature regimes is a significant global challenge and it requires a mechanistic understanding of how temperature affects species physiology and distributions. Although the physiological mechanisms underpinning freezing and cold tolerance (see Guy, 2003 for a review) and heat tolerance (Wahid *et al.*, 2007) have been established in model plants, responses in species exposed to natural conditions remain understudied. While the thermal limits of plants have recently been shown to broadly vary with latitude (O'Sullivan *et al.*, 2017; Lancaster & Humphreys, 2020), comparisons employing standard techniques across multiple biomes remain rare, particularly for both heat and cold tolerance concomitantly (Geange *et al.*, 2021). Importantly, our capacity to predict thermal tolerance from key bioclimate parameters such as MAT and MAP remains complex. In our cross-biome dataset, we found that neither mean annual temperature or precipitation explained variation in cold tolerance ($T_{crit\text{-}cold}$), which was instead strongly predicted by thermal seasonality. For heat tolerance, again climate precipitation was not a significant driver, aligning with the findings for Australian *Acacia* species in an experimental heatwave (Andrew *et al.*,

2023). On the other hand, mean annual temperature explained 23% of the variance in $T_{crit\text{-}hot}$, which contrasts a common garden study of 123 taxonomically diverse species, for which climate variables generally had very weak predictive power (<5%) for heat tolerance (Perez & Feeley, 2021).

Despite some clear macroclimatic patterns in our study, a large proportion of the variation in cold and, to a lesser extent, heat tolerance was explained by species (32% and 19%, respectively). The contribution of species could reflect micro-climate adaptation among species or differences in species' leaf traits that contribute to within-species variation in leaf temperature, the signal of which may be amplified when sampled *in situ*. Mahan and Upchurch (1988) proposed that the lower limit of temperature for a plant is controlled by its environment, while the upper limit can be controlled by the plant, suggesting that plants are "limited homeotherms". Recent studies have shown that what drives variation in heat tolerance is leaf temperature, rather than large scale measures of air temperature (Perez & Feeley, 2020; Cook *et al.*, 2021). Leaves can avoid heat stress through different mechanisms that decouple leaf from air temperature such as anatomical traits (Leigh *et al.*, 2012; Leigh *et al.*, 2017; Tserej & Feeley, 2021), patterns of display such as leaf inclination (Ball *et al.*, 1988) and transpirational cooling through stomatal conductance and can vary among co-existing species (Urban *et al.*, 2017; Drake *et al.*, 2018; Deva *et al.*, 2020; Marchin *et al.*, 2022). The thermoregulatory patterns and physical properties of leaves can differ greatly among species, such that different species exhibit different leaf temperatures even when they co-exist in close proximity. Leaf to air temperature decoupling (Blonder *et al.*, 2020) might also explain why relationships between heat tolerance and latitude are negligible or smaller than expected for the temperature span considered (Curtis *et al.*, 2016; O'Sullivan *et al.*, 2017; Lancaster & Humphreys, 2020).

Species' TTB is a more holistic measure of species thermal limits given that it encompasses both lower and upper limits. Unlike for animals, global patterns of variation in the TTB of plants have received little attention. Despite the existence of large thermal tolerance datasets, the lack of paired, concurrently measured low and high thermal thresholds measurements for a given species constrains our ability to adequately assess TTB globally. We found that TTB was best predicted by the combination of seasonality and mean annual temperature: species exposed to the high seasonality and mean annual temperatures in our desert biome had wider thermal tolerance breadth. However, species played an equal role in explaining variation in TTB and the capacity

for species to respond to increasingly extreme high temperatures and greater climate variability by broadening their TTB is unknown. Previous research generally suggests that plant niche thermal evolution is constrained by heat tolerance (Aruajo et al 2013) as evident through reduced variation in heat tolerance across landscapes (Lancaster and Humphreys, 2020). However, we found the reverse, with heat tolerance having greater variability (~30°C) than cold tolerance (~20°C) across our diverse biomes. Future studies could examine whether wider TTB or more extreme thermal thresholds in fact equate to greater plasticity to warming and future climate extremes.

Narrow thermal physiological limits have been identified as an aspect of species vulnerability to climate change (Williams *et al.*, 2008; Pacifici *et al.*, 2015). The use of PSII thermal tolerance metrics determined at the leaf-level, however, requires careful interpretation. When plants reach their critical thermal limits, damage to PSII can occur, leading to decreased electron transport rate and photosynthetic failure, thus compromising leaf survival. If this risks whole plant survival, however is less clear. In 21 species, heat tolerance thresholds have been correlated with carbon assimilation limits (Perez *et al*, 2021) which influence plant productivity. To further unpick these patterns, we need to better understand the relationship between physiological thermal limits with plant survival and fitness and how plastic these metrics are over space, time and across the plant life cycle – for example, thermal tolerance of seedlings may be more critical than that of adults.

Conclusion

Thermal tolerance breadth (TTB), a parameter broadly used in animal thermal biology literature, has not previously been measured using a consistent method in such a diverse range of plant species and environments. Here we concomitantly measured the lower ($T_{crit\text{-}cold}$ and NT) and upper ($T_{crit\text{-}hot}$) thermal thresholds of PSII and quantified the TTB of 69 plant species across three biomes: two climatically extreme (alpine and desert) and one more stable (wet temperate). Our results show partial support for the climate variability hypothesis. Looking at climate from a discreet biome category perspective, TTB only partially supported the CVH with the climatically variable desert biome having the widest but only significantly different TTB. Using coarse climate variables at sampling locations, we found support for CVH with temperature seasonality predicting cold tolerance, TTB and NT but was lacking in heat tolerance (MAT not seasonality

was the significant predictor) with species explaining substantial portions of variation. Interestingly we found that leaf-level TTB was extremely wide, much wider than the air temperature extremes to which plants are exposed in these biomes. It is important to remember, however, that these measured thermal limits represent a point beyond optimal photosynthetic function and consistent temperatures inside these limits could have far-reaching effects, depending on species life history, morphological traits and plasticity, as well as microclimatic and temporal heterogeneity. Incorporating species traits, leaf temperature and microhabitat information over space and time in future work is important next step to improving predictive power of thermal tolerance metrics for understanding vulnerability of different vegetation communities to climate extremes.

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Author Contribution

VFB, AMC, SKCJ, ABN, LABR and AL designed the study, PAA, RVG, VGB and AMC collated and analysed the data, all authors contributed to data collection, interpretation of results and writing.

Conflict of interest

The authors have no conflicts of interest to disclose.

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Ethics information

This study was conducted under approval from the NSW Department of Planning, Industry and Environment (Scientific Licence SL102330).

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