

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

Verónica F. Briceño¹, Alicia M. Cook², Stephanie K. Courtney Jones^{1,3}, Pieter A. Arnold¹, Rachael V. Gallagher⁴, Kris French⁵, León A. Bravo⁶, Adrienne B. Nicotra¹ and Andrea Leigh²

¹Division of Ecology and Evolution, Research School of Biology, Australian National University, Canberra, ACT, Australia.

²School of Life Sciences, Faculty of Science, University of Technology Sydney, Broadway, NSW, Australia.

³Ecosystem and Threatened Species, Northwest Branch, NSW Department of Planning and Environment, Dubbo, NSW, Australia

⁴Hawkesbury Institute for the Environment, Western Sydney University, Sydney, Australia.

⁵Centre for Sustainable Ecosystem Solutions, School of Earth, Atmospheric and Life Sciences, University of Wollongong, Wollongong, Australia.

⁶Departamento de Ciencias Agronómicas y Recursos Naturales, Facultad de Ciencias Agropecuarias y Medioambiente, Universidad de la Frontera, Región de la Araucanía, Temuco, Chile.

Corresponding author: Andy Leigh: andrea.leigh@uts.edu.au ORCID [0000-0003-3568-2606](https://orcid.org/0000-0003-3568-2606)

ORCID details:

Verónica F. Briceño

Alicia M. Cook [0000-0003-3594-3220](https://orcid.org/0000-0003-3594-3220)

Stephanie K. Courtney Jones [0000-0002-0905-0624](https://orcid.org/0000-0002-0905-0624)

Pieter A. Arnold [0000-0002-6158-7752](https://orcid.org/0000-0002-6158-7752)

Rachael V. Gallagher [0000-0002-4680-8115](https://orcid.org/0000-0002-4680-8115)

Kris French [0000-0001-6938-2017](https://orcid.org/0000-0001-6938-2017)

León A. Bravo [0000-0003-4705-4842](https://orcid.org/0000-0003-4705-4842)

Adrienne B. Nicotra [0000-0001-6578-369X](https://orcid.org/0000-0001-6578-369X)

Andrea Leigh [0000-0003-3568-2606](https://orcid.org/0000-0003-3568-2606)

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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 ($T_{\text{crit-cold}}$) and high ($T_{\text{crit-hot}}$) 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 $T_{\text{crit-cold}}$, NT and $T_{\text{crit-hot}}$ 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 $T_{\text{crit-hot}}$ 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

1 **Introduction**

2 Desert and alpine plant species live at the thermal limits of biological processes. Unlike those in
3 temperate regions, plants living in extreme environments are exposed to both very low and very
4 high temperatures and thus to wide thermal range and highly variable thermal regimes, both
5 among days and seasons. Given the important influence of temperature in shaping species
6 distributions (Tattersall *et al.*, 2012; Garcia-Pichel *et al.*, 2013) the innate thermal tolerance
7 limits (lowest and highest temperature that a species can withstand) and thermal tolerance
8 breadths (range of temperature between upper and lower thermal tolerance limits) of species are
9 predicted to correlate with the temperature variation characterising their environment. The
10 climate variability hypothesis (CVH; Janzen, 1967) predicts that species with *wide* thermal
11 tolerance ranges are likely to evolve in environments with *variable* temperatures compared to
12 thermal specialists with *narrow* tolerance ranges that evolved in thermally *stable* environments.
13 This hypothesis is a cornerstone of thermal ecology, but has to date been primarily explored in
14 animals (Addo-Bediako *et al.*, 2000; Compton *et al.*, 2007; Calosi *et al.*, 2010; Baudier *et al.*,
15 2018).

16 Cold and heat tolerance thresholds are rarely studied concomitantly. Cold tolerance studies
17 predominate in alpine environments where plants can withstand very low temperatures and
18 tolerate extracellular ice formation and the resulting cytoplasmic dehydration (Sakai & Larcher,
19 1987; Larcher, 2003). Heat tolerance studies, historically, are primarily focussed on warm and
20 desert environments (Geange *et al.*, 2021). Mechanisms for tolerating extreme heat have been
21 demonstrated in a range of desert species, including heat avoidance through increased
22 transpiration (Aparecido *et al.*, 2020) with microhabitat conditions setting the scene for heat
23 tolerance (Curtis *et al.*, 2016), whereas in other cases, these differences disappear under common
24 garden conditions (Downton *et al.*, 1984; Knight & Ackerly, 2002; Knight & Ackerly, 2003). In
25 spite of great progress, a recent systematic review revealed that less than 5% of more than 3000
26 studies examining thermal tolerance of plants considered both heat and cold tolerance thresholds
27 together (Geange *et al.*, 2021). The current focus on either cold or heat tolerance overlooks the
28 fact that plants in extreme biomes can experience both cold and heat stress.

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

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

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

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

76 **Materials and Methods**

77 **Study locations**

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

82 ***Species selection***

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

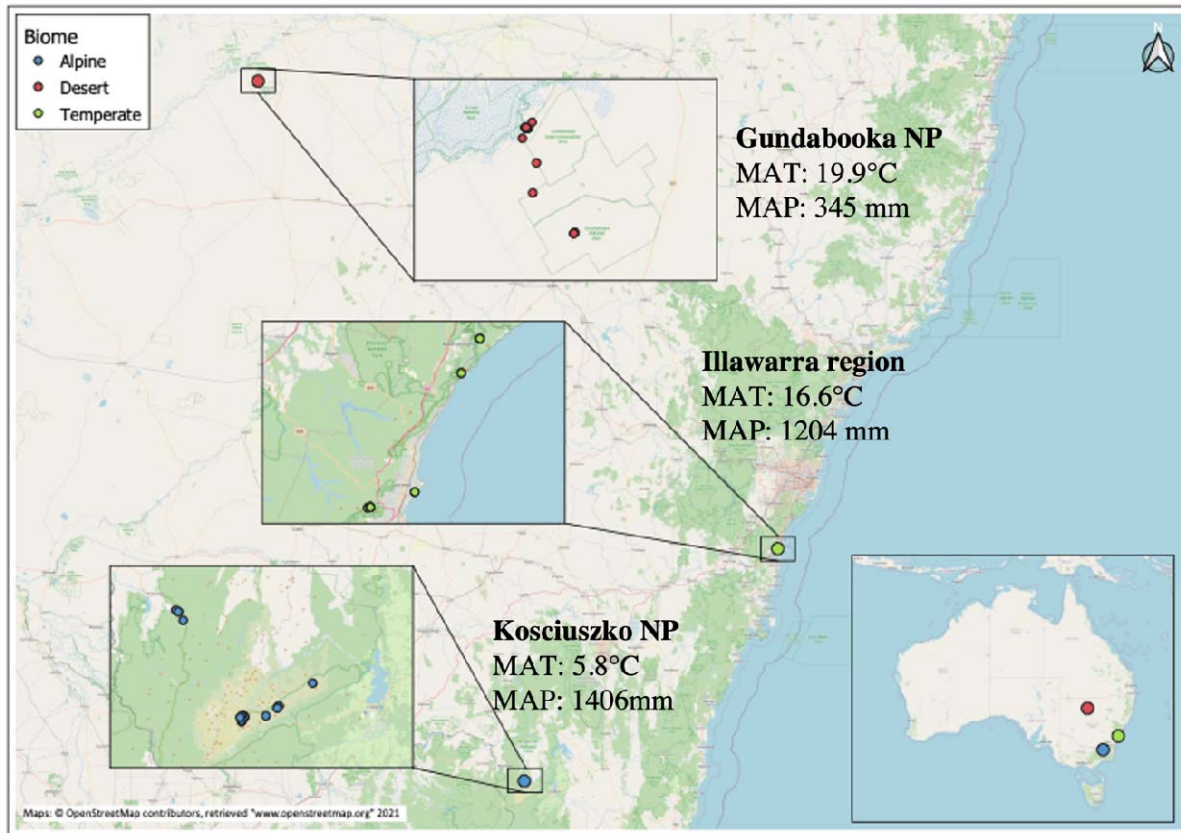


Fig. 1: Locations of study sites across three biomes in NSW, Australia. Desert sites (red) in Gundabooka National Park (GNP), temperate sites (green) in the Illawarra Region, and alpine sites (blue) in Kosciuszko National Park (KNP). GNP sites span open eucalypt woodland, mulga and chenopod shrublands 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.

89 *Leaf sample collection*

90 Mature, fully expanded leaves were harvested from five to seven individuals per species. For
91 shrubs and trees, small branches were collected from the northern facing side of the plant at a
92 height of 1-1.5 m. For smaller growth forms, entire individuals or rosettes were collected.
93 Samples were kept in cool sealed plastic bags with moistened paper towel until they were
94 processed (approx. 1-2 hours after collection). In the field laboratory, leaves (size ranging 0.25
95 cm² to 5 cm²) were cut (or combined) to fit an approximate area of 1 cm² and kept on wet florist
96 foam in humid dark containers until thermal tolerance assays were performed. Measurements
97 occurred during January 2020, March 2020 and December 2020 for the desert, wet temperate and

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

100 **Thermal tolerance assays**

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

111

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

113 The critical low and high temperature thresholds of PSII ($T_{crit-cold}$ and $T_{crit-hot}$) were measured
114 using T- F_0 curves, i.e., the rise in F_0 (Neuner & Pramsöhler, 2006; Arnold *et al.*, 2021) with set
115 heating or cooling rates. One leaf per individual (or several small leaves abutting one another)
116 was used for each of the heating and cooling assays with $n = 5-7$ replicates per species. For each
117 assay run, 45 samples were secured with double-sided tape to paper on a peltier plate (CP-
118 121HT; TE-Technology, Inc., Michigan, USA), used to cool or heat samples at a set rate using
119 LabView-based control software (National Instruments, Texas, USA) and adapted code from
120 TE-Technology, Inc. A vacuum sealed double-glazed glass was placed on top of the leaf samples
121 on the plate to avoid water condensation, particularly at freezing temperatures, and compress
122 samples to ensure maximum leaf contact with the Peltier plate (see Arnold *et al.*, 2021 for more
123 details).

124

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

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

141
142 $T_{\text{crit-cold}}$ and $T_{\text{crit-hot}}$ were determined as inflection points on the T- F_0 curves, using the *segmented*
143 R package. For further description of T- F_0 curve see Arnold et al., (2021). $T_{\text{crit-cold}}$ and $T_{\text{crit-hot}}$
144 were used to represent the lower and upper bounds of the PSII thermal tolerance breadth, TTB
145 ($\text{TTB} = T_{\text{crit-hot}} - T_{\text{crit-cold}}$).

146

147 *Ice nucleation temperatures*

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

153 **Climate variables for sampling locations**

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

160

161 **Statistical analyses**

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

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

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

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

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

195 **Results**

196 **Biome differences in thermal tolerance**

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

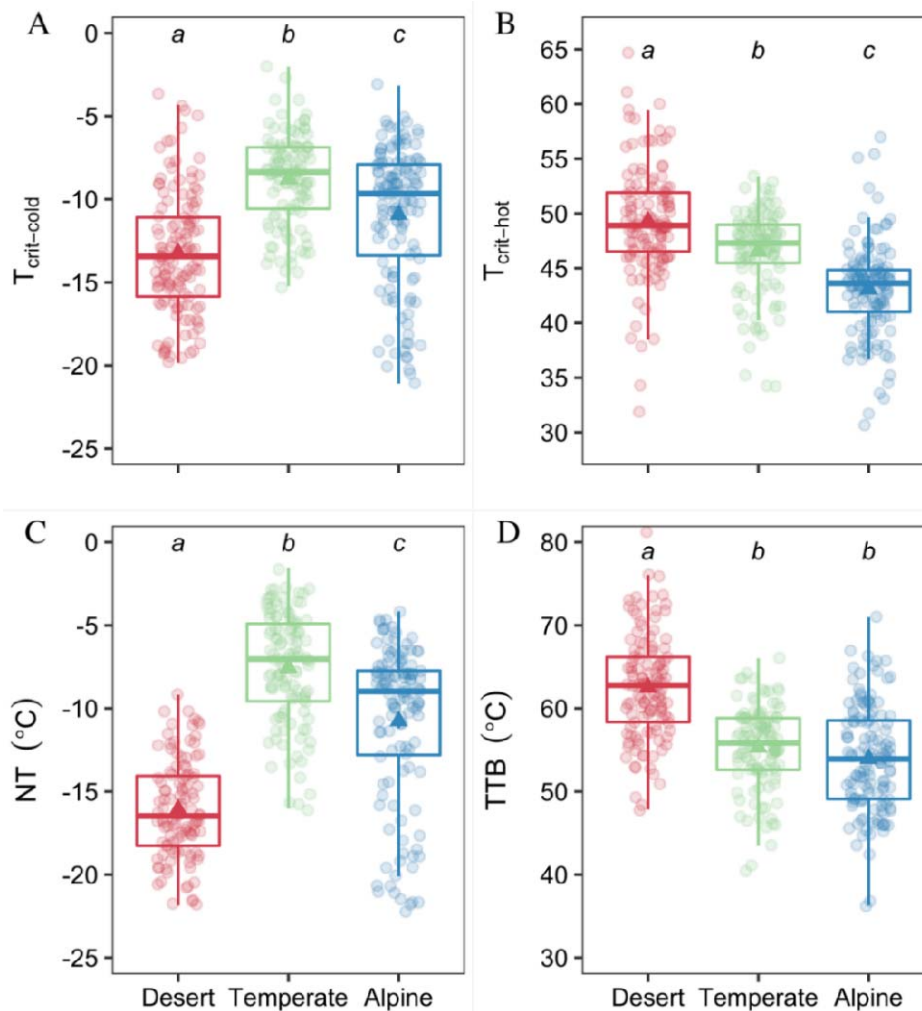


Fig. 2: Heat ($T_{\text{crit-hot}}$) and cold ($T_{\text{crit-cold}}$) thresholds, ice nucleation temperatures (NT) and thermal tolerance breadth (TTB) for desert ($n = 24$ spp), temperate ($n = 23$ spp) and alpine ($n = 22$ spp) biomes. Circles represent individual replicates, triangles represent biome 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.

208 At a species level, temperate species *Oplismenus hirtellus* was the least cold tolerant (-4.0°C,
209 $T_{\text{crit-cold}}$), whereas two alpine species *Epacris paludosa* and *Hovea montana* were the most cold
210 tolerant (-18.5°C $T_{\text{crit-cold}}$) and the desert species *Atriplex stipitata* had the most extreme
211 nucleation temperature (-20.5°C). The alpine species *Pimelea ligustrina* was the least heat
212 tolerant (38.1°C, $T_{\text{crit-hot}}$) and the desert species *Rhagodia spinescens* was the most heat tolerant
213 ($T_{\text{crit-hot}} = 54.9^\circ\text{C}$, **Fig. 3, Table S1**).

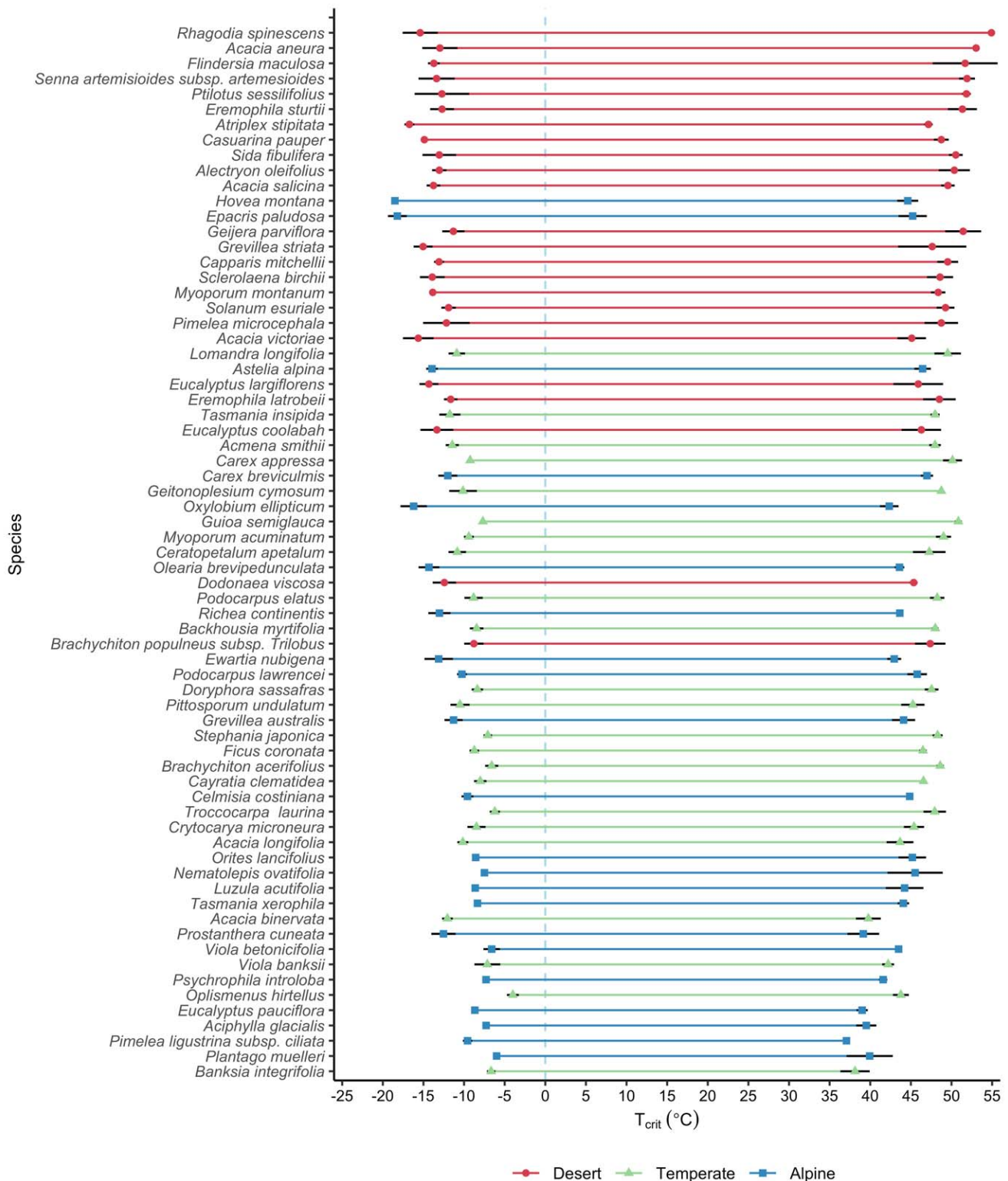


Fig. 3: Thermal tolerance breadth (TTB = $T_{crit-hot} - T_{crit-cold}$) for all the studied species. Species are sorted top to bottom from widest to narrowest TTB. Red circles and lines represent desert species (n = 24 spp), green triangles and lines represent wet temperate species (n = 23 spp) and 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, $T_{crit-hot}$ and $T_{crit-cold}$ value is from n = 5-6 replicates per species.

215

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	$T_{crit-hot}$		$T_{crit-cold}$		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	

216

217 The thermal tolerance breadth (TTB) was significantly wider in desert species ($62.6 \pm 0.8^\circ\text{C}$)
 218 than temperate ($55.4 \pm 0.9^\circ\text{C}$) or alpine species ($54.1 \pm 0.6^\circ\text{C}$) (**Fig. 2d, Table 1**). However,
 219 there was also considerable variation among species within biomes (**Fig. 3**). Within the desert
 220 biome TTB was more similar across species (lower variability), (56.2 to 70.3°C TTB, $\Delta 14.1^\circ\text{C}$),
 221 compared to the alpine biome which had the greatest variation among species (45.9 to 63.1°C
 222 TTB, $\Delta 17.2^\circ\text{C}$) followed by the wet temperate species (44.8 and 60.4°C TTB, $\Delta 15.6^\circ\text{C}$). For
 223 species from all three biomes, TTB was much wider than the extreme temperature range
 224 calculated for the sampling sites, thus most thermal thresholds lay well beyond the minimum and
 225 maximum temperatures expected for each species in their local environment. The only notable
 226 exception was for the alpine biome species, where the cold threshold was closer to the minimum
 227 temperatures representing our alpine sites (**Fig. 4**).

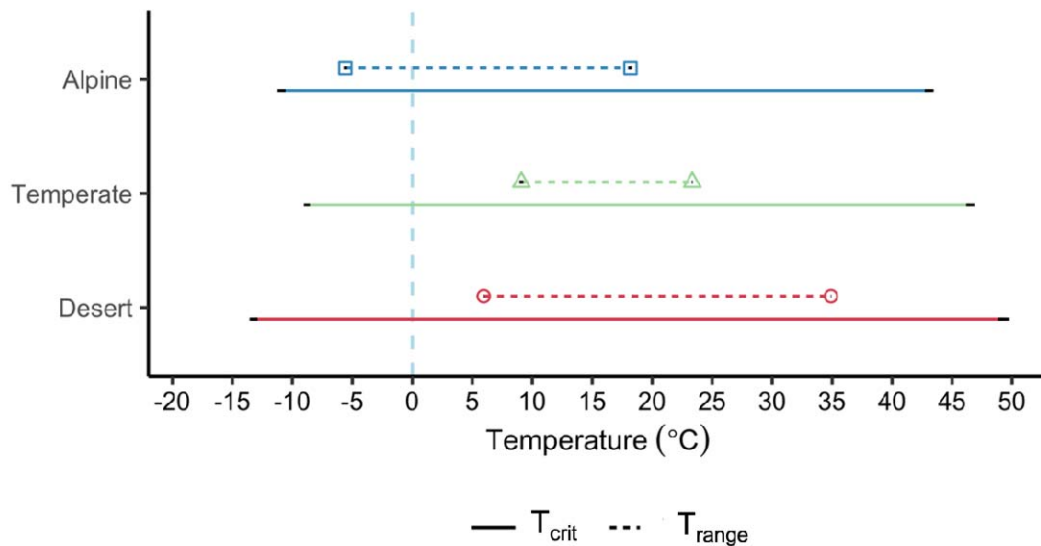


Fig. 4: Mean thermal tolerance breadth (TTB, solid lines) for alpine, temperate and desert species and their respective mean thermal range ($T_{\text{range}} = \text{mean MaxT} - \text{mean MinT}$ based on the locations of the sample sites, see **Table S2**). Black lines represent standard errors.

228 Contribution of climate variables to the variation on thermal tolerance metrics

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

234 $T_{\text{crit-cold}}$ was best explained by temperature seasonality (19% of the variation in $T_{\text{crit-cold}}$), although
235 a much larger proportion of the variation in $T_{\text{crit-cold}}$ was explained by species (32%, **Table 2**).
236 There was a negative relationship between $T_{\text{crit-cold}}$ and temperature seasonality, indicating that
237 species from environments with greater seasonality, our alpine and desert sites, had a more
238 extreme $T_{\text{crit-cold}}$ than species from the less seasonal wet temperate rainforest (**Fig. 5a**). By
239 contrast, rather than seasonality, $T_{\text{crit-hot}}$ was best explained by mean annual temperature (MAT,
240 23%), with a much smaller proportion of the variation in this variable explained by species
241 (19%, **Table 2**). There was a positive relationship between $T_{\text{crit-hot}}$ and site MAT (**Fig. 5b**), such
242 that species from, on average, warmer environments, also had higher $T_{\text{crit-hot}}$.

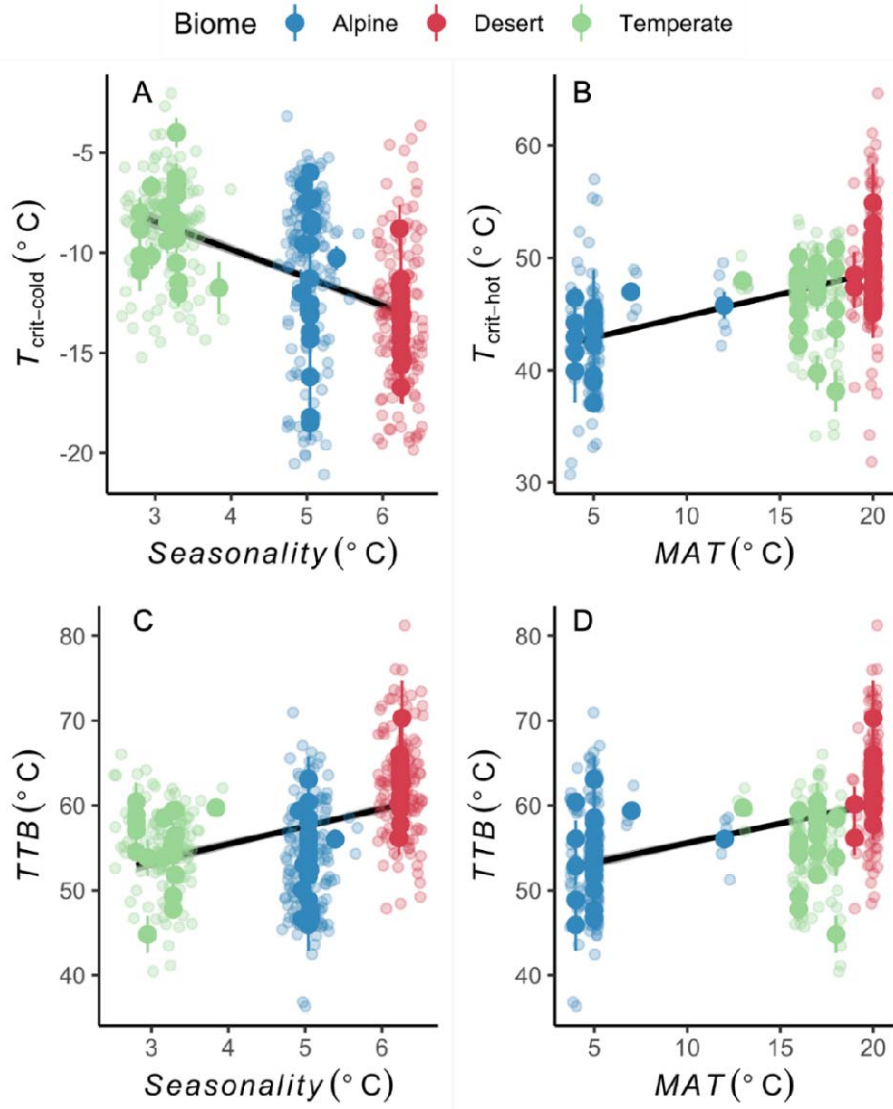


Fig. 5: Climatic variables that best predicted the variation in thermal thresholds across all biomes. Environmental variables are temperature seasonality (°C) and mean annual temperature (MAT, °C). Best models included: for $T_{crit-cold}$, seasonality; for $T_{crit-hot}$, MAT; for TTB both seasonality 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.

243 TTB was best predicted by seasonality and mean annual temperature (**Table 2**), although a
244 similar proportion of the variance was explained by the species effect compared to climate
245 variables (29% and 24% respectively). There was a positive relationship between TTB and
246 seasonality as well as with mean annual temperature (**Fig. 5c,d**). Notably, TTB was wider in
247 species from desert biomes, which experience considerably greater seasonality and MAT than
248 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-cold} ~ Seasonality + (1 Species)								
	Estimate	SE	t-value	Df	P-value	mR²	ICC	cR²
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
	e							
Species	5.25		69					
Residual	7.66	2.76						
T_{crit-hot} ~ MAT + (1 Species)								
	Estimate	SE	t-value	Df	P-value	mR²	ICC	cR²
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
	e							
Species	5.22		69					
Residual	14.87							
TTB ~ Seasonality + MAT + (1 Species)								
	Estimate	SE	t-value	DF	P-value	mR²	ICC	cR²
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
	e							
Species	13.14		69					
Residual	22.94							
NT ~ MAT + Seasonality + (1 Species)								
	Estimate	SE	t-value	DF	P-value	mR²	ICC	cR²
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
	e							
Species	7.22		69					
Residual	7.12							

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

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

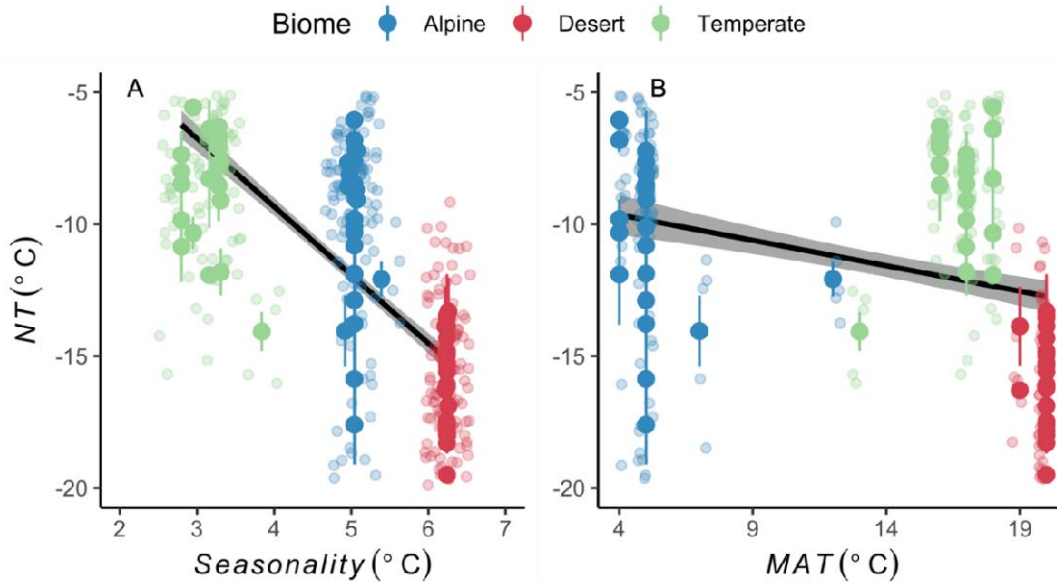


Fig. 6: Climatic variables (temperature seasonality and mean annual temperature (MAT)) that best explained the variation in ice nucleation temperature (NT), see **Table 2** for statistics.

257 Discussion

258 The climate variability hypothesis (CVH) predicts that species will evolve wider thermal
259 tolerance breadths in environments with more variable temperatures and thermal specialisation
260 (narrow TTB) in thermally stable environments, a concept largely supported in animals (i.e
261 Compton *et al.*, 2007; Sunday *et al.*, 2011). Of the few studies testing this hypothesis on plants,
262 investigations have included phenotypic plasticity increases with latitude (Molina-Montenegro &
263 Naya, 2012), species distribution ranges with seasonality across elevation (Mumladze *et al.*,
264 2017) and the breadth of photosynthetic thermal response with environmental temperature
265 variability (Perez *et al.*, 2023). Here we explored the CVH by sampling for thermal tolerance
266 metrics including TTB on species growing in three contrasting biomes, two extreme and more
267 variable, one benign and more thermally stable.

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

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

284 **TTB exceeds thermal ranges in each biome and varies within biomes**

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

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

311 **Heat and cold thermal limits vary among biomes**

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

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

336 The relatively high heat tolerance of some of our alpine species is at first counterintuitive, as
337 alpine areas are conventionally seen as primarily cold environments. Yet, alpine plants can be
338 exposed to very high temperatures, especially those with prostrate growth forms (Körner, 2003).
339 Studies on leaf temperature in alpine species in Europe have shown that leaf temperature can
340 reach up to 50°C in summer, especially on calm sunny days, with leaf heat tolerance higher than
341 50°C (Buchner & Neuner, 2003). Maximum leaf temperature has been measured at 38°C in early
342 summer in one Australian alpine species (Danzey *et al.*, 2021 unpublished data) and may be
343 higher in suitable hot and still conditions in late summer. Therefore, thermal thresholds could be
344 breached in alpine species if the combination of heat with other environmental stress, such as
345 high light, decreased heat tolerances, as found for some European alpine species (Buchner &
346 Neuner, 2003; Buchner *et al.*, 2015).

347 **Heat and cold thermal limits relate to different macroclimatic temperature variables**

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

362 2023). On the other hand, mean annual temperature explained 23% of the variance in $T_{\text{crit-hot}}$,
363 which contrasts a common garden study of 123 taxonomically diverse species, for which climate
364 variables generally had very weak predictive power (<5%) for heat tolerance (Perez & Feeley,
365 2021).

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

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

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

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

412 **Conclusion**

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

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

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444 **Author Contribution**

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

448 **Conflict of interest**

449 The authors have no conflicts of interest to disclose.

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453 **Ethics information**

454 This study was conducted under approval from the NSW Department of Planning, Industry and
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456

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