Enhanced cold tolerance with increased soil moisture: thermal tolerance variability among alpine *Ranunculus* species and hybrids in Kosciuszko National Park, Australia.

Natasha Erb*[1], Lisa Cary*[1]

* u7127032@anu.edu.au, lisa.cary@anu.edu.au

[1] Research School of Biology, ANU, Canberra, ACT, Australia

Abstract:

Escalating extreme temperature events significantly impact organisms, driving species selection and adaptation. To predict global species distributions in a warmer future, understanding differences in metabolic temperature tolerance and plasticity is crucial. Chlorophyll fluorescence can be used to measure critical temperature of photosynthetic heat tolerance (PHT) in plants. The upper (CT_{max}) and lower (CT_{min}) critical temperature, as well as the difference between these two limits, the thermal tolerance breadth (TTB), were measured for five *Ranunculus* species and four hybrid types, using a high-throughput chlorophyll fluorescence imaging method and apparatus. This study compares PHT between species and hybrids and analyses the correlation of PHT to soil moisture to gain insight into how local microclimatic factors and hybridisation might allow vulnerable species to leverage adaptive traits. Our results show very significant differences between the CT_{max}, CT_{min} and TTB of species, with hybrids often having intermediate values between their parent species. The correlation between soil moisture and CT_{min} was negative and significant, while the correlation with CT_{max} and TTB were both slightly positive, but not significant. Soil moisture marginally increasing thermal tolerance indicates that small pockets with higher soil moisture than the surrounding landscape (such as gullies and intermittent stream beds) may become increasingly important for the persistence of *Ranunculus* populations. This is concerning, given predictions of a drier climate in the future, especially for those species identified as having comparatively lower thermal tolerance. This study demonstrates how plant physiological measurements could aid in the identification of species that are more at risk of climate induced range shifts or local extinction and assist with setting conservation priorities.

Introduction:

Temperature stands out as the primary factor influencing the adaptation and distribution of plant species worldwide (Nievola et al. 2017). Global warming predictions expect heat extremes, and compound heat and drought events, to increase in frequency and severity over the next few decades (Intergovernmental Panel on Climate Change 2023; Cowan et al. 2014; Frank et al. 2015; Intergovernmental Panel on Climate Change 2018). Heatwave maximum temperatures are increasing due to a gradual rise in average temperatures (+0.85°C globally from 1880 to 2012, +1°C for Australia from 1910 to 2016), and this warming trend is projected to escalate to +3°C by 2100 (CSIRO and Bureau of Meteorology 2016; Intergovernmental Panel on Climate Change 2014). By serving as a selection pressure and driving the adaptation of species, increasing temperature extremes impact plants (Gutschick and BassiriRad 2003; Buckley and Huey 2016; Lancaster and Humphreys 2020). Australia's alpine ecosystems will be particularly vulnerable to the effects of global warming, due to their limited extent, fragmented nature, and high level of endemism (Sumner et al. 2022; Steffen 2009). To predict spatial distributions of species in a warmer future, it is crucial to understand the inherent differences in heat tolerance among species and the extent of metabolic heat tolerance plasticity (Reyer et al. 2013). Photosynthesis is widely recognized as one of the most thermally sensitive metabolic processes in plants (Berry and Björkman 1980; Schreiber et al. 1976; Seemann et al. 1984; Zhu et al. 2018), exhibiting defined limits beyond which assimilation is inhibited, irreversible tissue damage and the denaturing of enzymes can occur (Neuner and Pramsohler 2006; Sukhov et al. 2017; Schreiber and Berry 1977). Terminology around the definition of thermal tolerance is debatable, but here we define it as an organism's ability to withstand extreme temperature stress.

Chlorophyll fluorescence is widely used to assess photosynthetic heat tolerance (Schreiber and Bilger 1987). The critical temperature of photosynthesising tissue can be measured by recording and assessing changes in basal chlorophyll-a fluorescence (F_0) during leaf heating or cooling. In a dark-adapted leaf, F_0 can be rapidly measured when exposed to a low intensity modulated measuring light (Yamane et al. 1997). Chlorophyll-a and photosystem II (PSII) complexes embedded in the thylakoid membrane absorb less light at extreme low and high leaf temperatures (Schreiber and Berry 1977; Geange et al. 2021). Consequently, critical temperatures are the temperature thresholds at which stress to PSII begins and a higher amount of light is reflected as fluorescence (Curtis et al. 2016; Krause and Weis 1984; Schreiber and Berry 1977). The temperature dependent change of chlorophyll fluorescence (thermal stability) is measured, and critical temperatures can be determined, by calculating the point where there is a sudden increase in F_0 (Schreiber and Berry 1977; Berry and Björkman 1980). Critical temperature will be used in this paper when referring to both CT_{min} and CT_{max}; and CT_{min} or CT_{max} will be used if referring to only one. The difference between CT_{min} and CT_{max}, referred to as thermal tolerance breadth (TTB), is a measure of the overall range of temperatures the species can tolerate.

Thermal tolerance exhibits plasticity in response to natural and experimentally controlled temperature changes at both seasonal (Cook et al. 2021; Zhu et al. 2018; Scherrer and Körner 2010) and diurnal (Buchner and Neuner 2003; Neuner et al. 2000) timescales, but less is understood about the influence of water availability on thermal tolerance. Recent research on an Australian alpine grass suggests that water-stressed plants exhibit enhanced thermal tolerance to both heat and cold (Sumner et al. 2022), in line with findings for plants adapted to other environments, such as the desert (Cook et al. 2021). Other research suggests that differences in preferred growing environments determine the response to low water (Curtis et al. 2016; Buchner and Neuner 2003). This underscores the complexity of heat tolerance in plants, highlighting the need for broader studies across multiple biomes and plant functional types to draw firm conclusions on inherent differences in PHT. These findings collectively emphasise the potential role of water availability in heat tolerance and the importance of considering aridity when evaluating variations in critical temperature values between plants (Zhu et al. 2018).

Climate warming projections indicate larger temperature increases in alpine areas compared to lower elevations at the same latitude, making these regions particularly vulnerable (Beniston et al. 1997; Theurillat and Guisan 2001; Shröter et al., 2005; Scherrer and Körner 2010). In alpine environments, small compact growth forms that create a distinct microclimate are common (Korner, 2021; Scherrer and Korner 2010). Leaf temperatures of these plants often decouple from air temperatures and, depending on the conditions, can be warmer or colder than ambient temperatures, which increases the potential of exceeding tolerance ranges (Squeo et al. 1991). Limited studies on heat tolerance in alpine species reveal high CT_{max} (approximately 48–50°C), and a warmer climate in the future increases the risk of surpassing these thermal thresholds (Buchner and Neuner 2003; Dai et al. 2018; Larcher et al. 2010). Notably, recent studies have indicated that microhabitat factors, particularly soil moisture variation, may be more important than macroscale climate or latitude in determining critical damage thresholds (Curtis et al. 2016). Despite the demonstrated ability of plants to acclimate in response to environmental changes, increased pressure from extreme events coupled with changing long term climate patterns threatens to expose plant populations to conditions beyond their tolerance limits, potentially causing distribution shifts or local extinctions (Harris et al. 2018).

To comprehend species' ability to respond to rapid climate change, various evolutionary mechanisms are considered, including genetic adaptation, dispersal, acclimation, and phenotypic plasticity (Hansen et al. 2012). Interspecific introgression through hybridisation, a less explored but potentially valuable mechanism (Brauer et al 2023), allows vulnerable species to leverage adaptive traits from others in response to environmental changes (Aitken and Whitlock, 2013; Becker et al. 2013). Hybrid zones, arising from increased human-mediated species redistribution and climate-driven range shifts, have the potential to contribute to the evolutionary rescue of species facing climate threats (Becker et al. 2013). Unlike standing genetic variation or mutations, hybridization rapidly enhances genetic diversity by combining functional variants from different genomic backgrounds (Ottenburghs 2021) and is predicted to increase due to

climate change, as shifts in species' ranges may increase the geographic overlap of species boundaries (Chunco 2014). However, the novel allele combinations resulting from hybridization may also lead to incompatibilities and reduced fitness (Coyne and Orr, 2004; Kulmuni et al. 2024). Despite some empirical evidence supporting the adaptive potential of hybridization, a systematic evaluation of its value for polygenic traits is lacking (Meier et al. 2017; Kulmuni et al. 2024). There is a need for a comprehensive assessment of when and over what time scale hybridization is expected to facilitate rapid adaptation in evolutionary models (Kulmuni et al. 2024). In Kosciuszko National Park, coexisting *Ranunculus* species hybridise while maintaining distinct species boundaries. Prior research indicates that this is likely influenced by gradients of soil water availability (Hanley, 2023). The Australian Alps offer a unique setting to study plant adaptation to high elevations and variable weather conditions as well as the relationship between thermal tolerance and water availability.

Aims and hypotheses

Understanding the upper and lower limits for photosynthesis in Australian alpine *Ranunculus* will be beneficial for predicting how they might be impacted by changes in temperature extremes and precipitation patterns (Geange et al. 2021). This study sought to (a) determine if there are interspecific differences in extreme temperature tolerance (CT_{min} and CT_{max}) and tolerance breadth (TTB) of *Ranunculus* species and their hybrids under natural growing conditions, and (b) to investigate whether soil moisture content and species, and interactions between these, are predicting factors of thermal tolerance (CT_{min}, CT_{max} and TTB) in *Ranunculus* plants studied. It was hypothesised that (1) that hybrids will outperform their parent species in critical temperature and TTB analyses, due to the potential synergistic effects of combining advantageous traits from both parental sources; and (2) *Ranunculus* plants in locations with lower soil moisture content will have lower CT_{min} values, higher CT_{max} values and consequently a wider TTB, with the hybrids withstanding more extreme temperatures. Furthermore, we hypothesise that the combined effect of hybridization and changing soil moisture conditions will synergistically enhance the thermal tolerance breadth and critical temperatures of *Ranunculus*, contributing to their ability to thrive in a broader range of environmental conditions.

Methods:

Species, study site and sample collection

The *Ranunculus* genus, commonly called buttercups, are small flowering herbs found in alpine and subalpine ecosystems throughout southeastern Australia. There is clear evidence that hybridisation between species is common and produces viable offspring (Briggs 1962; Hammer, 2019; Armstrong, 2003). This study assessed the thermal tolerance of five species from the *Ranunculus* genus (*Ranunculus dissectifolius* F. Muell, *Ranunculus graniticola* Melville, *Ranunculus gunnianus* Hook, *Ranunculus millanii* F. Muell, *Ranunculus muelleri* Benth.) and four hybridisations between these species (*R.*

dissectifolius x *R. graniticola*, *R. dissectifolius* x *R. millanii*, *R. dissectifolius* x *R. muelleri* and *R. graniticola* x *R. muelleri*). *Ranunculus gunnianus* is not reported to hybridise with any of the other species and belongs to a different subgroup of the *Ranunculus* genus to the other species (Briggs 1962; Costin et al. 1979).

Due to the behaviour of the hybridisation complex at the site, characterised by less distinct species boundaries than anticipated, a key (Appendix A) was developed with reference to the descriptions in Costin et al. (1979). Reference photos were also taken prior to sampling and, together with the key, aided identification in the field. The key included the degree of dissection; leaf shape; presence of hairs on the stem or leaf; leaf thickness and texture; flower petal colour, with only *R. millanii* displaying white petals; and finally, soil conditions, where *R. millanii* was specifically found within bogs, sometimes submerged and *R. millanii x R. dissectifolius* grew adjacent to bog communities (Armstrong, 2003). This comprehensive analysis allowed for consistent identification of each species and their hybrids within the diverse ecosystem. Identifications were confirmed by more than one person before being accepted.

Samples were collected from four transects on a slope east of Charlotte Pass, in Kosciuszko National Park, Australia (latitude: -36.430985; longitude: 148.338797; elevation: 1797m). The transects started at Kosciuszko Road (n=3) or Charlotte Pass access road (n=1) and extended downslope to Spencer's creek, the lowest point of each transect. Sampling took place on the 21st and 23rd of November 2023, with temperatures reaching a maximum of 14.8 °C and 16.6°C and a minimum of 8.2°C and 6.3°C, respectively (Australian Government Bureau of Meteorology 2023). The weather was overcast on the 21st, partly cloudy on the 23rd and precipitation fell on both days, with a total of 36.6 mm between the 21st and 23rd and a total of 66.4mm in the month prior to the study (Australian Government Bureau of Meteorology 2023).

Three healthy plants from each *Ranunculus* species and hybrid type were sampled along each transect, where possible. If a transect did not have three plants of one type, missing individuals were supplemented by obtaining additional samples from the next transect to ensure sample sizes were approximately the same across species and hybrids (Appendix B). From each plant, 2-5 fully expanded leaves were picked and placed in a zip lock bag with a moist piece of paper towel. Zip lock bags were labelled with the transect and sample number and placed in an insulated cool bag for the time between collection and processing, before undergoing thermal tolerance testing within 24 hours of collection.

GPS coordinates were recorded for each sample and percentage soil moisture content readings were taken from the ground near the base of each plant using a soil moisture probe (Fieldscout 250 TDR Soil Moisture Meter; Spectrum Technologies, Illinois, USA).

Thermal tolerance testing

Two hot and two cold thermal tolerance assays were conducted for this study, following Arnold et al. (2021), with some modifications to methods. Thermal tolerance was determined using the chlorophyll fluorescence technique, which measures basal chlorophyll reflectance (F_0) under non-photosynthetic light of detached leaves (Arnold

et al. 2021) using a Pulse Amplitude Modulating (PAM) sensor (Maxi-Imaging-PAM; Heinz Walz GmbH, Effeltrich, Germany) (Figure 1A). The temperature of samples was controlled by a bi-polar proportional-integral-derivative controller (TC-36-25) and powered by a fixed voltage supply (PS-24-13). The plate utilised four direct contact thermoelectric modules with a potential thermal range of –20°C to 100°C (Arnold et al. 2021). A steady temperature across the surface using LabVIEW-based control software (National Instruments) was maintained with ± 0.1°C precision and ± 1°C tolerance across the surface, until the specified temperature was reached. Samples were cooled at 15 °C h^{-1} with a final target temperature of -20 °C in cold assays, and heated at 30 °C h^{-1} to 70 °C for hot assays. Using a thermoelectric plate to control temperature and the use of detached leaves allowed concurrent measurement of multiple leaves. Standard protocols for measuring critical temperature use different rates for heat and cold tolerance testing to reflect slower rates of cold transitions compared to heat transitions observed in nature. Factors like wind speed and insolation can rapidly increase leaf temperature on a hot day, while cooling rates on cold frosty nights rarely exceed 5°C h^{-1}, especially below freezing (Vogel 2009; Buchner and Neuner 2009).

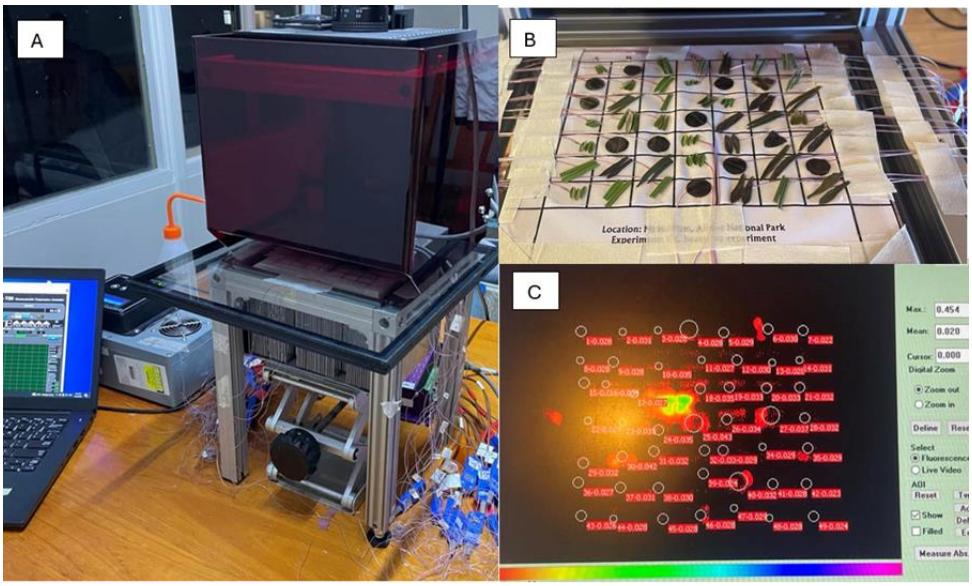


Figure 1: Apparatus used to conduct critical temperature assays. (A) Peltier plate and Maxi-Imaging-PAM system setup, (B) thermocouple and sample placement on the grid prior to critical temperature testing and (C) software used to draw circular areas of interest on the maximal fluorescence image, taken after dark adaptation. Images supplied by Thomas Hanley.

To prepare the leaves for thermal tolerance testing, portions of approximately 1cm x 1cm were cut from larger leaves, or where this was not possible due to their small size, multiple portions were laid next to each other to cumulatively equal the area of one larger section. Cut leaf portions were placed in small pill boxes with a piece of moist sponge, to prevent desiccation while the rest of the samples were prepared.

A paper grid (7 x 8, labelled A1-G8) was affixed to a Peltier plate (CP-121HT; TE-Technology, Inc., Michigan, USA; 152 × 152 mm) positioned 185 mm below the Maxi-Imaging PAM (Arnold et al. 2021). Each run could test 56 samples, randomly assigned to grid squares and adhered with double-sided tape over a thermocouple (Figure 1B), and

a piece of double-glazed glass was placed over the samples to promote even contact with the Peltier plate. After preparation, samples underwent a 20-minute dark adaptation to oxidise all PSII acceptors (Arnold et al. 2021; Huang et al. 2016). A red Perspex hood surrounding the Peltier plate and PAM (Figure 1A) and black fabric covering the whole assembly were used to ensure external light did not interfere with measurements.

A single saturating pulse (10,000µmol photons m^{-2} s^{-1}; 720ms duration) was used to measure maximal fluorescence (F_m) when photosystem reaction centres were closed (Arnold et al., 2021). After the dark adaptation period, a fluorescence image was taken to obtain F_0 values and select a circular Area of Interest on each sample to indicate where F_0 should be measured from (Figure 1C). These areas were drawn using Maxi-Imaging-PAM software and maximised to fit the widest part of each sample whilst staying within the boundary of the leaf tissue. Leaf temperature, measured by the thermocouples on the underside of the leaves, and F_0 were collected by data loggers at set intervals with specifics varying depending on duration of the assay, reflecting memory capacity limits of the Maxi-Imaging-PAM. For each assay, the heating or cooling program was started simultaneously with the recording of F_0 values.

In total, leaves from 109 individual plants underwent both a cold and hot assay, producing 218 sets of temperature and fluorescence data. For each assay and leaf section, a graph of the temperature-dependent change in fluorescence (T-F_0 curve), was produced in R. Initially, F_0 rises slowly until the critical temperature of photosynthesis is reached, after which F_0 increases rapidly (Knight and Ackerly 2003; Neuner and Pramsohler 2006). On the T-F_0 graphs, separate linear trend lines were drawn for the slow and fast fluorescence rise phases, with their intersection marking the critical temperature. This point was marked numerically on the T-F_0 graphs, accompanied by an error value provided for the model (Fig. 2). Samples with a model error greater than or equal to 0.2 were removed (n=48) and the remaining graphs were manually reviewed to remove those with incorrect shapes or critical temperature values not between the slow and fast rise phases (n=20), leaving 150 valid critical temperature values (54 CT_{min} and 96 CT_{max}). Due to the removal of some critical temperature values during data cleaning, sample sizes differ slightly between species.

In samples where the cold and hot assay both produced a critical temperature value (n=50), it was possible to calculate the range of temperatures that the plant could tolerate by subtracting the CT_{min} from the CT_{max}, giving the thermal tolerance breadth (TTB) in degrees Celsius, as shown in equation (1).

Equation (1): $$TTB = CT_{max} - CT_{min}$$

Statistical analysis

Statistical analysis of the data was undertaken using R programming software (v 4.3.2; R Core Team 2023) in the RStudio environment (Posit team 2023).

Data were checked for normality before performing statistical analyses. The assumption of normality was violated for the CT_{min} and soil moisture data. CT_{min} values were normally distributed after being power transformed using the '*car*' package in R (Fox

and Weisberg 2019) and statistical analyses were done using the transformed data. Due to the non-parametric distribution of CT_{min}; critical temperature and TTB data are presented graphically using the median, quartiles, and range. Soil moisture data could not be made normal using a variety of transformations. As a result, analyses involving soil moisture were done using non-parametric statistical tests.

Differences in CT_{min}, CT_{max} and TTB between *Ranunculus* species and hybrids were determined using single factor ANOVA tests. In cases where differences were significant, post hoc testing was undertaken using Tukey's Honestly Significant Difference (Tukey's HSD) tests. Assessing the correlation of soil moisture with CT_{min}, CT_{max} and TTB was done using Spearman's rank correlation.

Results:

High temperature tolerance (CT_{max})

The mean upper critical temperature for all species was 43.90 °C, with a range of 3.29 °C between species means. *R. muelleri* had the highest mean CT_{max} of the *Ranunculus* types studied, followed by *R. graniticola* x *R. muelleri* and *R. gunnianus*. The species with the lowest mean CT_{max} was *R. millanii* (Table 1). Single factor ANOVA of CT_{max} values showed that there was a significant difference between species ($F_{8,87}$ = 4.353; p <0.001) (Table 2). Post-hoc testing revealed four significant differences (Appendix C). *Ranunculus muelleri* was significantly higher than three species (*R. dissectifolius* x *R. graniticola*, *R. graniticola* and *R. millanii*) and in addition to *R. muelleri*, *R. millanii* was also significantly lower than *R. gunnianus* (Figure 2A).

Table 1: Sample size (n), mean and standard error (SE) of the lower critical temperature (CT_{min}) and upper critical temperature (CT_{max}) of each *Ranunculus* species and hybrid type, measured in degrees Celsius (°C). The minimum and maximum values for each measure are displayed in blue and red text, respectively.

		CTmin			CTmax	
Species	n	Mean (°C)	SE	n	Mean (°C)	SE
Ranunculus dissectifolius	5	-10.91	0.90	13	44.62	0.51
R. dissectifolius x *R. graniticola*	4	-8.67	0.81	10	42.68	0.44
R. dissectifolius x *R. millanii*	6	-9.01	0.58	9	43.60	0.86
R. dissectifolius x *R. muelleri*	9	-9.21	0.74	11	43.60	0.58
R. graniticola	4	-4.49	1.15	11	42.86	0.53
R. graniticola x *R. muelleri*	5	-9.59	0.96	6	45.19	0.55
R. gunnianus	7	-10.42	0.71	15	44.99	0.47
R. millanii	5	-4.11	2.10	9	42.13	0.50
R. muelleri	9	-9.84	0.43	12	45.42	0.58
Average	**6**	**-8.47**	**0.93**	**10.67**	**43.90**	**0.56**

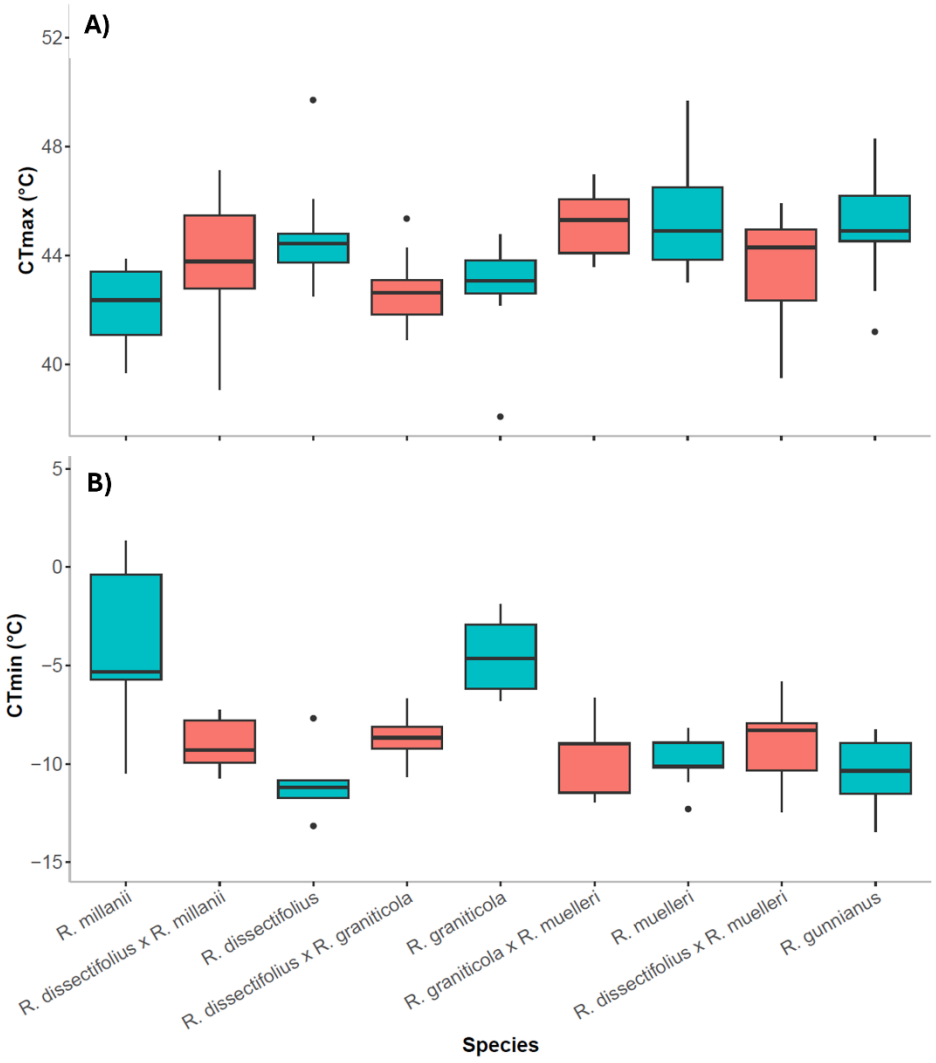


Figure 2: Boxplots showing (A) upper critical temperature (CT_{max}) and (B) lower critical temperature (CT_{min}) of photosynthesis in Ranunculus plants by species. Plot made from minimum, maximum and quartile values, with outliers represented by black dots. Teal colour represents species and orange represents hybrids, noting that all hybrids are placed between their two parent species except *Ranunculus dissectifolius* x *R. muelleri*.

Low temperature tolerance (CT_{min})

Mean CT_{min} had a range of 6.80 °C between the highest and lowest species, with an average of -8.47 °C. The lowest mean CT_{min} value was for *Ranunculus dissectifolius*, followed by *R. gunnianus* and *R. muelleri* and the highest was *R. millanii* (Table 1). Single factor ANOVA found a significant difference in CT_{min} between species ($F_{8,45}$ = 4.196; p<0.001) (Table 2). Post-hoc testing revealed 5 significant differences between species (Appendix D). *Ranunculus millanii* and *R. graniticola* were significantly higher than three and two other species, respectively and were not significantly different to each other (Figure 2B).

Thermal tolerance breadth (TTB)

Thermal tolerance breadth of all species studied had a mean value of 52.27 ^{o}C, with a difference of 9.22 ^{o}C between the highest (*R. muelleri)* and lowest (*R. millanii)* means (Table 3). Tolerance breadths differed significantly between species ($F_{8,41}$ = 5.514; p<0.001) (Table 2), with post-hoc testing identifying 9 significant differences between *Ranunculus* types (Appendix E). *Ranunculus graniticola* had a significantly lower TTB than three species (*R. dissectifolius*, *R. gunnianus* and *R. muelleri*) and one hybrid (*R. graniticola* x *R. muelleri*). *Ranunculus millanii* was significantly lower than all four types different to *R. graniticola*, as well as *R. dissectifolius* x *R. Muelleri*. *Ranunculus graniticola* and *R. millanii* were not significantly different and there were no significant differences between the other 7 types (Figure 3).

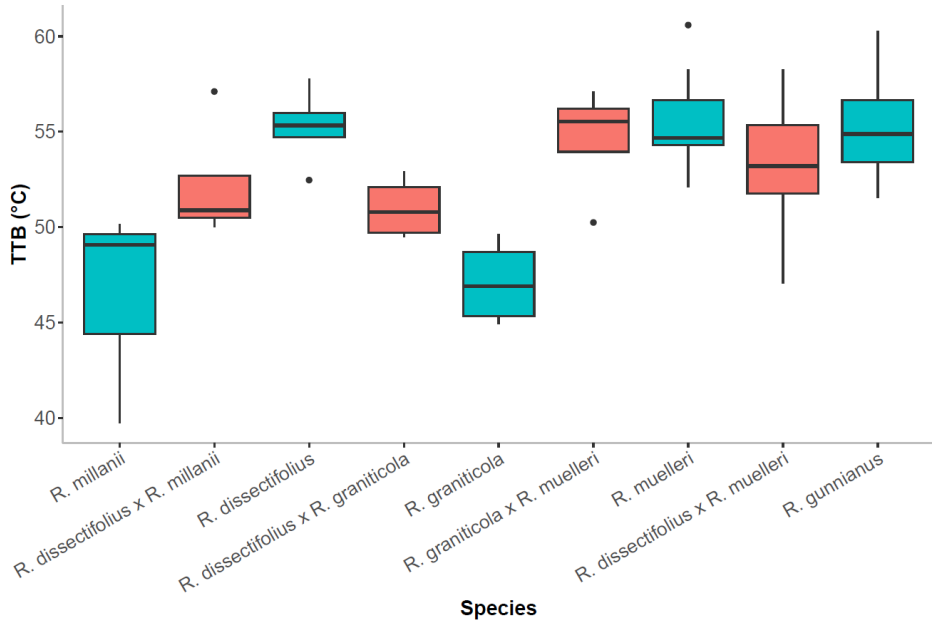


Figure 3: Boxplots showing the thermal tolerance breadth (TTB) of photosynthesis in Ranunculus plants by species. Plot made from minimum, maximum and quartile values, with outliers represented by black dots. Teal colour represents species and orange represents hybrids, noting that all hybrids are placed between their two parent species except *Ranunculus dissectifolius* x *R. muelleri*.

Table 2: Single-factor ANOVA results of lower critical temperatures (CT_{min}), upper critical temperatures (CT_{max}) and thermal tolerance breadth (TTB) in different Ranunculus species and hybrids from Kosciuszko National Park.

ANOVA	Source of variation	df	SS	MS	F value	p
CT_{min}	Species	8	1.240	0.15500	4.196	<0.001
	Residuals	45	1.662	0.03694		
CT_{max}	Species	8	118.7	14.835	4.353	<0.001
	Residuals	87	296.5	3.408		
TTB	Species	8	421.6	52.70	5.514	<0.001
	Residuals	41	391.9	9.56		

Table 3: Sample size (n), mean and standard error (SE) of thermal tolerance breadth (TTB) of each Ranunculus species and hybrid type sampled. The minimum and maximum values are displayed in blue and red text, respectively and means are measured in degrees Celsius (°C).

Species	n	Mean	SE
R. dissectifolius	5	55.26	0.87
R. dissectifolius x R. graniticola	4	51.00	0.83
R. dissectifolius x R. millanii	5	52.24	1.30
R. dissectifolius x R. muelleri	9	53.11	1.29
R. graniticola	4	47.11	1.14
R. graniticola x R. muelleri	4	54.61	1.51
R. gunnianus	7	55.26	1.09
R. millanii	3	46.33	3.31
R. muelleri	9	55.55	0.89
Average	5.56	52.27	1.36

Effect of soil moisture on CT_{min}, CT_{max} and TTB

Soil moisture had a significant, but weak, negative correlation with CT_{min} ($n = 48$, $r_s = -0.34$, p-value = 0.017) indicating slightly higher tolerance of extreme cold temperatures at higher soil moisture content (Appendix F). The relationship between soil moisture and CT_{max} was positive ($n = 84$, $r_s = 0.12$, $p = 0.258$) (Appendix G) and the effect of soil moisture on thermal tolerance breadth was positive ($n = 44$, $r_s = 0.27$, $p = 0.075$) (Appendix H), however, both were not significant.

Discussion:

This study assessed thermal tolerance in five species and four hybrid combinations of alpine buttercups in Kosciuszko National Park. Two hypotheses were tested with the aim of determining species and hybrid differences in thermal tolerance, and to investigate whether soil moisture affects thermal tolerance. Our research could not determine if there was a combined effect of soil moisture and hybridisation on critical temperature values due to data limitations, and hence was unable to test for interactions between soil moisture and species. It did, however, reveal significant independent effects of species and soil moisture on critical temperature, contributing to our understanding of *Ranunculus* thermal tolerance.

Whilst populations differed significantly in CT_{max}, CT_{min} and TTB, the hybrids did not outperform their parent species, and hence hypothesis 1 was not supported. Of the species studied, *R. millanii* and *R. graniticola* were least thermally tolerant and *R. muelleri*, *R. dissectifolius* and *R. gunnianus* were most tolerant, with hybrids predominantly falling between these two groups. *R. millanii* was the least cold and heat tolerant and had the narrowest thermal tolerance breadth. *R. graniticola* was also in the three least tolerant species for all measures. Unlike for the least tolerant species, there was no one species that was most tolerant in all three thermal tolerance measures. *R.*

muelleri, *R. dissectifolius* and *R. gunnianus* were the three most tolerant species to low temperatures, had the three highest thermal tolerance breadths and had the top four CTmax means. These findings are supported by existing research on plants of other growth forms, and in other ecosystems, with thermal tolerance differing inherently in species (Buchner and Neuner 2003; O'Sullivan et al. 2017) and that species tolerant of high temperatures often also have wide thermal tolerance breadths (Perez et al. 2020). Additionally, we observed that the Ranunculus types with high $CT_{max,}$ also exhibit lower CT_{min} and also had high TTBs, emphasising the interrelation of these factors, similar to results of a study on the CT_{min}, CT_{max} and TTB of Australian alpine plants (Danzey et al. 2024).

The relationship between thermal tolerance and soil moisture align with previous observations in Australian desert plants highlighting the importance of water availability in influencing thermal damage thresholds (Curtis et al. 2016), however, they contradict the suggestion that water-stress increases thermal tolerance (Sumner et al. 2022; Cook et al. 2021), and hence hypothesis 2 was not supported. Whilst we anticipated that plants from drier areas would exhibit greater tolerance to extreme temperatures, our results indicated a slight positive correlation between CT_{max} and TTB values with soil moisture and a weak, but significant, negative correlation between CT_{min} and soil moisture. These findings suggest that *Ranunculus* plants growing in moist soil had higher extreme temperature tolerance, potentially enabling these plants to photosynthesise over a wider temperature range before inhibition occurs. Species that grow in less arid conditions, such as herbaceous and shrubby plants, have not been found to exhibit increased heat tolerance in response to decreased water availability unlike their desert counterparts (Curtis et al. 2016; Buchner and Neuner 2003), as it decreases the ability of plants to cool their leaves through transpiration (Curtis et al. 2016).

Our findings indicate that soil moisture and species alone do influence thermal tolerance, however they do not account for all the variation observed. Plants with higher tolerance to heat also exhibited increased tolerance to cold temperatures, which suggests that alpine plants may employ similar physiological adaptations to counter both heat and cold extremes. The observed increase in CT_{max} with increasing soil moisture, coupled with lower CT_{min}, could be influenced by other factors not studied. Certain alpine plants possess leaves with specific adaptations, such as succulence or dense trichomes, providing protection against temperature extremes by reducing water loss, which is beneficial in hot and cold environments alike (Pérez-López et al. 2023) and as such, assessing leaf variables and whether they correlate with soil aridity and critical temperature would indicate whether this is an important factor to consider in future studies. Adequate soil moisture might enhance heat dissipation, preventing overheating (Korner 2021), while increased moisture levels could reduce oxygen availability, affecting root metabolism and potentially impacting cold tolerance negatively (Dickopp et al. 2018). Furthermore, determining if plant species respond differently to variations in soil moisture would be beneficial to know which species may be more vulnerable to future climate changes.

The present study is subject to several limitations that warrant consideration. One notable limitation pertains to the sample size for soil moisture readings, which was hindered by complications with the soil moisture probe, and exacerbated by recent rain, where a large proportion of the site's precipitation for the entire month fell during the three days of sampling, which impacted the comprehensiveness of the analysis. Experimentally limiting water to plants with the intention of creating water stress, rather than sampling a naturally growing population in conditions where water has not been limited, could provide better data to test whether soil moisture influences thermal tolerance. Additionally, sampling bias was introduced due to the availability of species at each site, with *R. gunnianus* being readily accessible and consequently overrepresented in comparison to other species like *R. graniticola x R. muelleri.* Furthermore, the time constraint on the study reduced the number of variables assessed, preventing a more comprehensive leaf phenotypic trait examination. This limits the depth of our understanding of the relationship between these traits and thermal tolerance. Finally, studies using T-F_0 methods with different heating and cooling rates may not be directly comparable, even for the same species, as plant species may exhibit different responses to variations in methodology (Arnold et al. 2021). These limitations underscore the need for caution in generalising our findings and highlight avenues for future research to address these constraints for a more robust investigation.

Acclimation of thermal tolerance is common in response to temperature changes. Over the course of a single day acclimation of up to 9.5 °C has been reported (Buchner and Neuner 2003). The plasticity of this physiological trait means it is likely that thermal tolerance of *Ranunculus* species varies under different conditions and throughout the year. If rates of acclimation differ between species, the degree of difference between species may also vary. Future research should address uncertainties related to acclimation in response to sustained changes in growth temperature and seasonal variations in natural environments, which hinder the prediction of spatial and temporal variations in plant heat tolerance. Further research on the potential of alpine *Ranunculus* species to acclimate in response to drought and temperature extremes in controlled laboratory experiments could be beneficial to isolate soil moisture as a variable and assess acclimation capacity. Previous studies have shown contrasting results regarding PHT acclimation, with some indicating CT_{max} adjustment of approximately 0.3°C per 1°C increase in growth temperature (Ghouil et al. 2003). Increases in heat tolerance have also been reported to affect low temperature tolerance by decreasing freezing tolerance during warmer periods, a phenomenon called frost dehardening (Bannister 2007; Larcher et al. 2010). These findings indicate thermal tolerance acclimation varies by species and season.. The impact of seasonal environmental changes on critical temperature and its predictability across spatial and temporal patterns remain uncertain for *Ranunculus* plants, and necessitates additional data on seasonal variability in thermal tolerance.

Exploring the effects of more extreme temperature conditions and simulating drought effects on *Ranunculus* and other species will be crucial for understanding adaptive capacities in species and ecosystems, informing climate change research, and mitigation strategies. Another important metric in assessing the risk of temperature inhibition and

damage to photosynthesis occurring is the margin between leaf temperatures and critical temperatures. The collection of fine scale microclimatic data, including leaf temperature, is necessary to do this accurately (Cook et al. 2021) and was beyond the scope of this study. If resources and time allow for it, this would prove beneficial in future studies.

Conclusions:

We found differences between *Ranunculus* species in critical temperature and thermal tolerance breadth, and observed that the thermal tolerance of hybrids is often between those of their parent species. We also found that enhanced thermal tolerance is associated with moister soils along the soil moisture gradient studied. These findings highlight the threat posed to *Ranunculus* plants in the Australian Alps by predictions of increased temperature and aridity with climate change and the potential for these threats to affect species unequally. The incorporation of other species in the vegetation community to studies on photosynthetic heat tolerance would allow for comparison between species and growth types and give a broader perspective of how thermal tolerance is distributed when making conservation decisions for Australia's alpine ecosystems.

Acknowledgements:

We extend sincere gratitude to Professor Adrienne Nicotra, Megan Head, Thomas Hanley, James King, Joshua Coates and all other staff who participated in the Field Studies in Functional Ecology field trip from both the Australian National University and other institutions. Your support, expertise and guidance has been instrumental in the development of this project. We express particular gratitude to Tom for his leadership in this project, assistance with equipment set-up and operation, and for extracting the critical temperature values from the data. Additionally, we would like to thank Megan for her advice and help on statistical analyses and data transformation in R. We also would like to say thankyou to the editing team for their work on putting the journal together.

Finally, we would like to thank our peers that contributed to this project. We appreciate the time and effort put in by Julia D'Angelo, Esther Duve, Amanda Longhurst, Anita Ipalawatte, Ellie Jordan, Ariel Neidorf and Noah Tan. Whether it was through sample collection, unravelling the hybridisation complex, or setting up and performing the critical temperature analyses, this project would not have been achievable without your support, for which we are deeply grateful. To all the BIOL3303 students, thank you for your mentorship throughout the field trip, especially Ella Wishart and Kelvin Meng for their time and assistance with the collection of samples.

References:

Aitken SN, Whitlock MC (2013) Assisted Gene Flow to Facilitate Local Adaptation to Climate Change. *Annual review of ecology, evolution, and systematics* **44**, 367-388.

Armstrong TTJB (2003) Hybridization and adaptive radiation in Australian alpine *Ranunculus*. Thesis (PhD).

Arnold PA, Briceño VF, Gowland KM, Catling AA, Bravo LA, Nicotra AB (2021) A high-throughput method for measuring critical thermal limits of leaves by chlorophyll imaging fluorescence. *Functional plant biology* **48**, 634-646.

Australian Government Bureau of Meteorology (2023). *November 2023 Daily Weather Observations*. Station: Perisher Valley AWS (071075). Latitude: -36.41, Longitude: 148.41, Elevation: 1738.0 m. Accessed on 3 Sep 2024. Available from: http://www.bom.gov.au/climate/dwo/202311/html/IDCJDW2112.202311.shtml.

Baker NR (2008) Chlorophyll Fluorescence: A Probe of Photosynthesis In Vivo. *Annual review of plant biology* **59**, 89-113.

Bannister P (2007) A touch of frost? Cold hardiness of plants in the Southern Hemisphere. *New Zealand journal of botany* **45**, 1-33.

Becker M, Gruenheit N, Steel M, Voelckel C, Deusch O, Heenan PB, McLenachan PA, Kardailsky O, Leigh JW, Lockhart PJ (2013) Hybridization may facilitate in situ survival of endemic species through periods of climate change. *Nature climate change* **3**, 1039-1043.

Beniston M, Diaz HF, Bradley RS (1997) Climatic change at high elevation sites : An overview: Climatic change at high elevation sites. *Climatic change* **36**, 233-251.

Berry J, Bjorkman O (2003) Photosynthetic Response and Adaptation to Temperature in Higher Plants. *Annu. Rev. Plant Physiol.* **31**, 491-543.

Brauer CJ, Sandoval-Castillo J, Gates K, Hammer MP, Unmack PJ, Bernatchez L, Beheregaray LB (2023) Natural hybridization reduces vulnerability to climate change. *Nature climate change* **13**, 282-289.

Briggs BG (1962) Interspecific Hybridization in the *Ranunculus* lappaceus Group. *Evolution* **16**, 372-390.

Buchner O, Neuner G (2003) Variability of Heat Tolerance in Alpine Plant Species Measured at Different Altitudes. *Arctic, antarctic, and alpine research* **35**, 411-420.

Buchner O, Neuner G (2009) low-temperature freezing system to study the effects of temperatures to -70 °C on trees in situ. *Tree physiology* **29**, 313-320.s

Buckley LB, Huey RB (2016) How Extreme Temperatures Impact Organisms and the Evolution of their Thermal Tolerance. *Integrative and comparative biology* **56**, 98-109.

Chunco AJ (2014) Hybridization in a warmer world. *Ecology and evolution* **4**, 2019-2031.

Cook AM, Berry N, Milner KV, Leigh A (2021) Water availability influences thermal safety margins for leaves. *Functional ecology* **35**, 2179-2189.

Coyne JA, Orr HA (2004) 'Speciation.' (Sinauer Associates, Inc. Publishers: Sunderland, Mass)

Cowan T, Purich A, Perkins S, Pezza A, Boschat G, Sadler K (2014) More Frequent, Longer, and Hotter Heat Waves for Australia in the Twenty-First Century. *Journal of climate* **27**, 5851-5871.

CSIRO and Bureau of Meteorology (2016) State of the Climate 2016. https://www.csiro.au/en/research/environmental-impacts/climate-change/State-of-the-Climate/Previous/State-of-the-Climate-2016

Curtis EM, Gollan J, Murray BR, Leigh A (2016) Native microhabitats better predict tolerance to warming than latitudinal macro-climatic variables in arid-zone plants. *Journal of biogeography* **43**, 1156-1165.

Dai A, Zhao T, Chen J (2018) Climate change and drought: A precipitation and evaporation perspective. *Current Climate Change Reports* **4**, 301–312.

Danzey LM, Briceño VF, Cook AM, Nicotra AB, Peyre G, Rossetto M, Yap J-YS, Leigh A (2024) Environmental and Biogeographic Drivers behind Alpine Plant Thermal Tolerance and Genetic Variation. *Plants (Basel)* **13**(9), 1271. doi:10.3390/plants13091271

Dickopp J, Lengerer A, Kazda M, (2018) Relationship between groundwater levels and oxygen availability in fen peat soils. *Ecological Engineering* **120**, 85-93.

Frank D, Reichstein M, Bahn M, Thonicke K, Frank D, Mahecha MD, Smith P, Velde M, Vicca S, Babst F, Beer C, Buchmann N, Canadell JG, Ciais P, Cramer W, Ibrom A, Miglietta F, Poulter B, Rammig A, Seneviratne SI, Walz A, Wattenbach M, Zavala MA, Zscheischler J (2015) Effects of climate extremes on the terrestrial carbon cycle: concepts, processes and potential future impacts. *Global change biology* **21**, 2861-2880.

Geange S, Arnold P, Catling A, Coast O, Cook AM, Gowland K, Leigh A, Notarnicola R, Posch B, Venn SE, Zhu L, Nicotra A (2021) The thermal tolerance of photosynthetic tissues: a global systematic review and agenda for future research. *The New phytologist* **229**, 2497-2513. doi: 10.1111/nph.17052.

Ghouil H, Montpied P, Epron D, Ksontini M, Hanchi B, Dreyer E (2003) Thermal optima of photosynthetic functions and thermostability of photochemistry in cork oak seedlings. *Tree physiology* **23**, 1031-1039.

Gutschick VP, BassiriRad H (2003) Extreme Events as Shaping Physiology, Ecology, and Evolution of Plants: Toward a Unified Definition and Evaluation of Their Consequences. *The New phytologist* **160**, 21-42.

Hammer JM (2019) The relationship between functional trait variation and water availability in hybridising Australian alpine buttercups. *Field Studies in Ecology* **2**.

Hanley T. (2023). Patterns of thermal tolerance in the Australian Alpine *Ranunculus* species complex [Abstract]. Unpublished Manuscript.

Hansen MM, Olivieri I, Waller DM, Nielsen EE (2012) Monitoring adaptive genetic responses to environmental change. *Molecular ecology* **21**, 1311-1329.

Harris RMB, Beaumont LJ, Vance TR, Tozer CR, Remenyi TA, Perkins-Kirkpatrick SE, Mitchell PJ, Nicotra AB, McGregor S, Andrew NR, Letnic M, Kearney MR, Wernberg T, Hutley LB, Chambers LE, Fletcher MS, Keatley MR, Woodward CA, Williamson G, Duke NC, Bowman DMJS (2018) Biological responses to the press and pulse of climate trends and extreme events. *Nature climate change* **8**, 579-587.

Huang W, Yang Y-J, Hu H, Zhang S-B (2016) Responses of Photosystem I Compared with Photosystem II to Fluctuating Light in the Shade-Establishing Tropical Tree Species Psychotria henryi. *Frontiers in plant science* **7**, 1549-1549.

Intergovernmental Panel on Climate (2023) Technical Summary. In 'Climate Change 2021 – The Physical Science Basis: Working Group I Contribution to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change'. (Ed. C Intergovernmental Panel on Climate) pp. 35-144. (Cambridge University Press: Cambridge)

Intergovernmental Panel on Climate Change (2014) Climate Change 2014: Synthesis Report., Geneva, Switzerland. .

Intergovernmental Panel on Climate Change (2018) Global warming of 1.5° C: an IPCC special report on the impacts of global warming of 1.5° C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty. Geneva, Switzerland.

Knight CA, Ackerly DD (2003) Evolution and Plasticity of Photosynthetic Thermal Tolerance, Specific Leaf Area and Leaf Size: Congeneric Species from Desert and Coastal Environments. *The New phytologist* **160**, 337-347.

Korner C (2021) 'Alpine Plant Life: Functional Plant Ecology of High Mountain Ecosystems.' (Springer International Publishing AG: Cham)

Krause GH, Weis E (1984) Chlorophyll fluorescence as a tool in plant physiology. II. Interpretation of fluorescence signals. *Photosynthesis research* **5**, 139-157.

Kulmuni J, Wiley B, Otto SP (2024) On the fast track: hybrids adapt more rapidly than parental populations in a novel environment. *Evolution letters* **8**, 128-136.

Lancaster LT, Humphreys AM (2020) Global variation in the thermal tolerances of plants. *Proceedings of the National Academy of Sciences - PNAS* **117**, 13580-13587.

Larcher W, Kainmüller C, Wagner, J (2010) Survival types of high mountain plants under extreme temperatures. *Flora. Morphologie, Geobotanik, Oekophysiologie* **205**, 3-18.

Meier JI, Marques DA, Mwaiko S, Wagner CE, Excoffier L, Seehausen O (2017) Ancient hybridization fuels rapid cichlid fish adaptive radiations. *Nature communications* **8**, 14363-14363.

Murchie EH, Lawson T (2013) Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. *Journal of experimental botany* **64**, 3983-3998.

Neuner G, Buchner O, Braun V (2000) Short-Term Changes in Heat Tolerance in the Alpine Cushion Plant Silene acaulis ssp. excapa [All.] J. Braun at Different Altitudes. *Plant biology (Stuttgart, Germany)* **2**, 677-683.

Neuner G, Pramsohler M (2006) Freezing and high temperature thresholds of photosystem 2 compared to ice nucleation, frost and heat damage in evergreen subalpine plants. *Physiologia plantarum* **126**, 196-204.

Nievola CC, Carvalho CP, Carvalho V, Rodrigues E (2017) Rapid responses of plants to temperature changes. *Temperature (Austin)* **4**, 371-405.

O'Sullivan OS, Heskel MA, Reich PB, Tjoelker MG, Weerasinghe LK, Penillard A, Zhu L, Egerton JJG, Bloomfield KJ, Creek D, Bahar NHA, Griffin KL, Hurry V, Meir P, Turnbull MH, Atkin OK (2017) Thermal limits of leaf metabolism across biomes. *Global change biology* **23**, 209-223.

Ottenburghs J (2021) The genic view of hybridization in the Anthropocene. *Evolutionary applications* **14**, 2342-2360.

Perez, TM, Feeley, KJ, Tjoelker, M (2020) Photosynthetic heat tolerances and extreme leaf temperatures. *Functional ecology* **34**, 2236-2245.

Pérez-López AV, Lim SD, Cushman JC (2023) Tissue succulence in plants: Carrying water for climate change. *Journal of plant physiology* **289**, 154081-154081.

Posit team (2023). RStudio: Integrated Development Environment for R. Posit Software, PBC, Boston, MA. http://www.posit.co/.

Potvin C (1985) Effect of leaf detachment on chlorophyll fluorescence during chilling experiments. *Plant physiology (Bethesda)* **78**, 883-886.

R Core Team (2023). _R: A Language and Environment for Statistical Computing_. R Foundation for Statistical Computing (Vienna, Austria) <https://www.R-project.org/>

Reyer CPO, Leuzinger S, Rammig A, Wolf A, Bartholomeus RP, Bonfante A, de Lorenzi F, Dury M, Gloning P, Abou Jaoudé R, Klein T, Kuster TM, Martins M, Niedrist G, Riccardi M, Wohlfahrt G, de Angelis P, de Dato G, François L, Menzel A, Pereira M (2013) A plant's perspective of extremes: terrestrial plant responses to changing climatic variability. *Global change biology* **19**, 75-89.

Scherrer D, Körner C (2010) Infra-red thermometry of alpine landscapes challenges climatic warming projections. *Global change biology* **16**, 2602-2613.

Schreiber U, Berry JA (1977) Heat induced changes of chlorophyll fluorescence in intact leaves correlated with damage of the photosynthetic apparatus. *Planta* **136**, 233-238.

Schreiber U, Bilger W (1987). 'Rapid assessment of stress effects on plant leaves by chlorophyll fluorescence measurements.' In: Tenhunen, J.D., Catarino, F.M., Lange, O.L., Oechel, W.C. (eds) Plant Response to Stress. *NATO ASI Series*, **15**. (Springer: Berlin, Heidelberg)

Schreiber U, Colbow K, Vidaver W (1976) Analysis of temperature-jump chlorophyll fluorescence induction in plants. *Biochimica et biophysica acta. Bioenergetics* **423**, 249-263.

Seemann JR, Berry JA, Downton WJS (1984) Photosynthetic response and adaptation to high temperature in desert plants. A comparison of gas exchange and fluorescence methods for studies of thermal tolerance. *Plant physiology (Bethesda)* **75**, 364-368.

Smillie RM, Oequist G, Nott R, Hetherington SE (1987) Chilling injury and recovery in detached and attached leaves measured by chlorophyll fluorescence. *Physiologia plantarum* **69**, 419-428.

Squeo FA, Rada F, Azocar A, Goldstein G (1991) Freezing tolerance and avoidance in high tropical Andean plants: is it equally represented in species with different plant height? *Oecologia* **86**, 378-382.

Steffen WL (2009) 'Australia's biodiversity and climate change.' (CSIRO Pub.: Collingwood, Vic)

Sukhov V, Gaspirovich V, Mysyagin S, Vodeneev V (2017) High-Temperature Tolerance of Photosynthesis Can Be Linked to Local Electrical Responses in Leaves of Pea. *Frontiers in physiology* **8**, 763-763.

Sumner EE, Williamson VG, Gleadow RM, Wevill T, Venn SE (2022) Acclimation to water stress improves tolerance to heat and freezing in a common alpine grass. *Oecologia* **199**, 831-843.

Theurillat J, Guisan A (2001) Potential impact of climate change on vegetation in the European Alps: A review. *Climatic change* **50**, 77-109.

Vogel S (2009) Leaves in the lowest and highest winds: temperature, force and shape. *The New phytologist* **183**, 13-26.

Yamane Y, Kashino Y, Koike H, Satoh K (1997) Increases in the fluorescence Fo level and reversible inhibition of Photosystem II reaction center by high-temperature treatments in higher plants. *Photosynthesis research* **52**, 57-64.

Zhu L, Bloomfield K, Hocart C, Egerton J, O'Sullivan O, Penillard A, Weerasinghe L, Atkin O (2018) Plasticity of photosynthetic heat tolerance in plants adapted to thermally contrasting biomes. *Plant, cell and environment* **41**, 1251.

Appendix:

Appendix A: List of the species sampled in this study and the key categorical characteristics used in identifying and differentiating between them.

Species	Degree of dissection (High/ Low)	Leaf shape (Broad/ Narrow)	Presence of hair (yes/ no)	Leaf thickness (thick/ thin)	Flower petal colour (white/ yellow)	Soil conditions (wet/ dry)	Other notes
R. millanii	High	Narrow	No	Thin	White	Wet	
R. dissectifolius x R. millanii	High	Narrow	No	Thin	Yellow	Wet	
R. dissectifolius	High	Narrow	Yes	Thin	Yellow	Dry	Leaflets slightly broader than *R. gunnianus*
R. dissectifolius x R. graniticola	High	Broad	Yes	Thin	Yellow	Dry	
R. graniticola	Low	Broad	No	Thin	Yellow	Dry	
R. graniticola x R. muelleri	Low	Broad	Yes	Thick	Yellow	Dry	Slight depressions in leaf
R. muelleri	Low	Broad	Yes	Thick	Yellow	Dry	No depression in leaf
R. dissectifolius x R. muelleri	High	Broad	Yes	Thick	Yellow	Dry	
R. gunnianus	High	Narrow	Yes	Thin	Yellow	Dry	Thin, coral looking leaflets and very large flower petals

Appendix B: Total number of samples collected for each species and hybrid type, and the distribution of these samples across the four transects.

Species	Total of samples taken from each transect				Total number of each species sampled
	Transect 1	Transect 2	Transect 3	Transect 4	
R. millanii	6	10	2	6	24
R. dissectifolius x R. millanii	6	6	4	6	22
R. dissectifolius	6	8	6	6	26
R. dissectifolius x R. graniticola	6	5	5	6	22
R. graniticola	6	8	4	6	24
R. graniticola x R. muelleri	6	6	4	4	20
R. muelleri	10	8	0	6	24
R. dissectifolius x R. muelleri	7	7	6	6	26
R. gunnianus	8	8	6	8	30
Total number of samples	61	66	37	54	218

Appendix C: Tukey's HSD post hoc test results for upper critical temperature (CT_{max}) showing differences between Ranunculus species and hybrids, with significant differences in bold text (significance codes: P < 0.001***, P < 0.01**, P < 0.05*).

Species	R. dissectifolius	R. dissectifolius x R. graniticola	R. dissectifolius x R. millanii	R. dissectifolius x R. muelleri	R. graniticola	R. graniticola x R. muelleri	R. gunnianus	R. millanii	R. muelleri
R. dissectifolius	-	0.249	0.934	0.913	0.336	0.999	1.000	0.060	0.976
R. dissectifolius x R. graniticola		-	0.976	0.967	1.000	0.189	0.069	0.999	**0.023***
R. dissectifolius x R. millanii			-	1.000	0.993	0.780	0.687	0.753	0.391
R. dissectifolius x R. muelleri				-	0.990	0.745	0.616	0.699	0.319
R. graniticola					-	0.251	0.101	0.993	**0.034***
R. graniticola x R. muelleri						-	1.000	0.055	1.000
R. gunnianus							-	**0.012***	1.000
R. millanii								-	**0.004****
R. muelleri									-

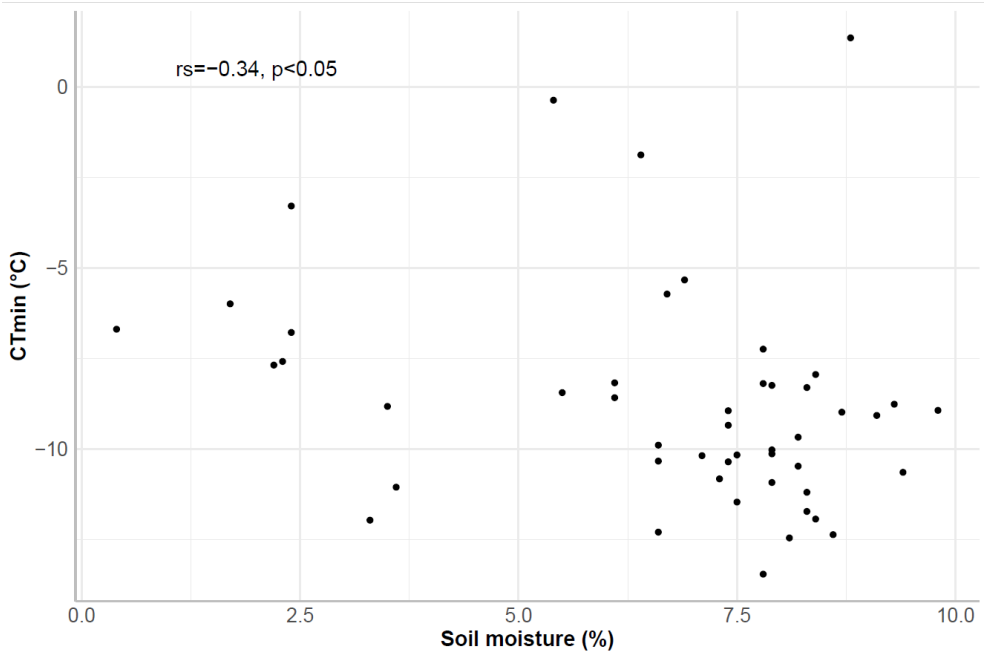
Appendix D: Tukey's HSD post hoc test results for lower critical temperature (CT_{min}) showing differences between Ranunculus species and hybrids, with significant differences in bold text (significance codes: P < 0.001***, P < 0.01**, P < 0.05*).

Species	R. dissectifolius	R. dissectifolius x R. graniticola	R. dissectifolius x R. millanii	R. dissectifolius x R. muelleri	R. graniticola	R. graniticola x R. muelleri	R. gunnianus	R. millanii	R. muelleri
R. dissectifolius	-	0.659	0.677	0.802	**0.008****	0.957	1.000	**0.005****	0.941
R. dissectifolius x R. graniticola		-	1.000	1.000	0.556	0.998	0.834	0.540	0.992
R. dissectifolius x R. millanii			-	1.000	0.311	1.000	0.859	0.278	0.998
R. dissectifolius x R. muelleri				-	0.112	1.000	0.946	0.084	1.000
R. graniticola					-	0.136	**0.012***	1.000	0.053
R. graniticola x R. muelleri						-	0.996	0.113	1.000
R. gunnianus							-	**0.008****	0.995
R. millanii								-	**0.036***
R. muelleri									-

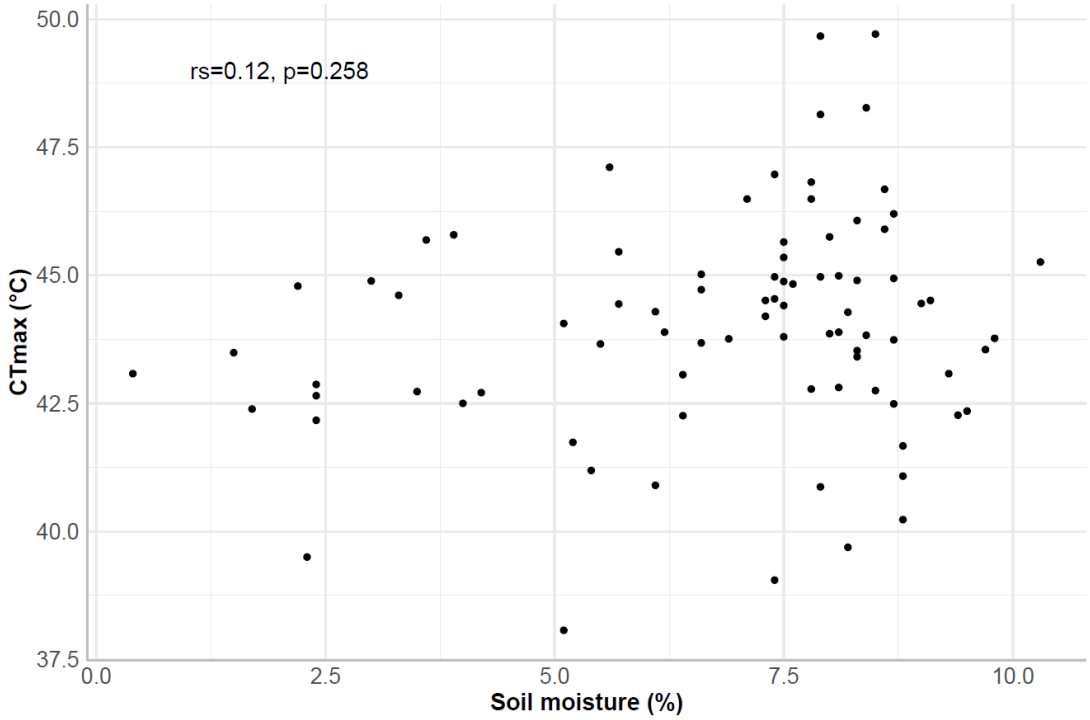
Appendix E: Tukey's HSD post hoc test results for thermal tolerance breadth (TTB) showing differences between Ranunculus species and hybrids, with significant differences in bold text (significance codes: P < 0.001***, P < 0.01**, P < 0.05*).

Species	R. dissectifolius	R. dissectifolius x R. graniticola	R. dissectifolius x R. millanii	R. dissectifolius x R. muelleri	R. graniticola	R. graniticola x R. muelleri	R. gunnianus	R. millanii	R. muelleri
R. dissectifolius	-	0.517	0.828	0.940	**0.009****	1.000	1.000	**0.008****	1.000
R. dissectifolius x R. graniticola		-	1.000	0.965	0.694	0.770	0.426	0.566	0.286
R. dissectifolius x R. millanii			-	1.000	0.273	0.964	0.763	0.210	0.605
R. dissectifolius x R. muelleri				-	0.056	0.996	0.898	**0.048***	0.756
R. graniticola					-	**0.033***	**0.004****	1.000	**0.001****
R. graniticola x R. muelleri						-	1.000	**0.027***	1.000
R. gunnianus							-	**0.004****	1.000
R. millanii								-	**0.002****
R. muelleri									-

Appendix F: Scatterplot graph of soil moisture and lower critical temperature (CT_{min}) of Ranunculus species sampled from Kosciuszko National Park in November 2023. Results from Spearman's rank correlation test results (sample size: n = 48) are written in the top left-hand corner.



Appendix G: Scatterplot graph of soil moisture and upper critical temperature (CT_{max}) of Ranunculus species sampled from Kosciuszko National Park in November 2023. Results from Spearman's rank correlation test results (sample size: n= 84) are written in the top left-hand corner.



Appendix H: Scatterplot graph of soil moisture and thermal tolerance breadth (TTB) of Ranunculus species sampled from Kosciuszko National Park in November 2023. Results from Spearman's rank correlation test results (sample size: n = 44) are written in the top left-hand corner.