Variation in Thermal Tolerance and Leaf Morphology among Alpine *Ranunculus* Species in Kosciuszko National Park

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Abstract

As global temperatures increase under climate change, understanding the thermal tolerance of plant species and how this relates to other functional traits is becoming increasingly important. I investigated thermal tolerance and leaf morphology in six interfertile alpine *Ranunculus* species that occur in water-inundated and terrestrial habitats in Kosciuszko National Park, Australia. I sought to identify whether thermal tolerance and leaf morphology differed between species and between habitats, and whether leaf morphological traits were correlated with measures of thermal tolerance. The critical minimum and maximum temperatures at which photosystem II in leaves becomes irreversibly damaged; $T_{\text{crit (cold)}}$ and $T_{\text{crit (hot)}}$, respectively, were measured using chlorophyll fluorimetry techniques to represent plant thermal tolerance, and thermal tolerance breadth (TTB) calculated. Functional traits affecting plant responses to temperature change and inundation, including leaf dissection, effective leaf width, leaf area, leaf mass per unit area (LMA) and leaf thickness, were also assessed. Species differed in thermal tolerance and all leaf morphology characteristics measured. Plants from inundated habitats had lower heat tolerance and TTB than plants from drier, not inundated habitats, and exhibited more dissected leaves with lower leaf width, area, LMA and leaf thickness. Thermal tolerance characteristics were weakly related to most morphological traits, however strong positive correlations between TTB and both LMA and leaf thickness emerged in my data. These results provide insight into the ability of alpine *Ranunculus* species to cope with future climate extremes and how leaf morphology may contribute to thermal tolerance. Avenues for future research into predictors of thermal tolerance and drivers of leaf morphology in inundated and terrestrial alpine habitats are also identified.

Introduction

Thermal tolerance influences plant species' distribution and performance (Nievola et al., 2017). Environmental temperatures not only affect the rate of chemical reactions involved in photosynthesis, but exposure to extreme temperatures can cause irreversible damage to the photosynthetic apparatus that permanently impairs plant function (Geange et al., 2021, Curtis, 2017). Leaf shape and form affects the transfer of heat energy and thus leaf temperature, affecting plant thermoregulation and the ability to withstand temperature extremes (Leigh et al., 2017, Nicotra et al., 2011). In the Australian alps, predicted increases in average temperatures and decreases in the duration of seasonal snow cover will expose plants to greater and more frequent hot and cold temperature extremes (Pickering et al., 2008). As such, understanding thermal tolerance in alpine species and its relationship to other traits is becoming increasingly important.

A widely-used measure of thermal tolerance is T_{crit}, the temperature at which a leaf sustains irreversible damage to photosystem II (PSII; Geange et al., 2021). $T_{crit\ (hot)}$ and $T_{crit\ (cold)}$ reflect the maximum hot and cold temperature extremes, respectively, that a plant can withstand before growth is impaired (Curtis, 2017). T_{crit} can be measured through chlorophyll fluorimetry (Arnold et al., 2021). Up to 5% of light energy absorbed by chlorophyll pigments in a leaf is re-emitted as fluorescence, with almost all emitted by PSII (van der Tol et al., 2014, Strasser et al., 2004). Basal fluorescence, F_0, is the minimal fluorescence obtained when leaves are dark-adapted and maximum light energy reaches the reaction centres of PSII (Buonasera et al., 2011). In contrast, maximum fluorescence, F_M, is observed under saturating light, and the difference between F_M and F_0 is defined as variable fluorescence, F_V (Weis and Berry, 1987). F_V/F_M is a measure of photochemical efficiency and is often used as an indicator of leaf health, with healthy leaves exhibiting a non-stressed F_V/F_M of around 0.8 (Ogaya et al., 2011). Changing environmental temperatures affect the electron transport chain of PSII, altering the amount of energy reaching reaction centres and causing an increase in F_0 (Buonasera et al., 2011, Lambreva et al., 2014, Harris et al., 2023, Knight and Ackerly, 2002). When plants are exposed to high or low temperatures, temperature-F_0 curves reveal sharp increases in F_0 that signal the onset of irreversible damage to PSII (Arnold et al., 2021). T_{crit} is then the inflection point between the initial gradual rise in F_0 and the fast-rise phase (Arnold et al., 2021).

Several studies have identified regional and global-scale patterns in plant thermal tolerance. For example, species from warmer climates and higher latitudes can exhibit greater heat and cold tolerance, respectively (Lancaster and Humphreys, 2020, Zhu et al., 2018). Other studies found thermal tolerance to be influenced by microhabitat features; for example, Curtis et al. (2016) found water availability in a species' native microhabitat was a better predictor of thermal tolerance than broader scale climate or latitude.

Thermal tolerance is also highly plastic, and within a single plant or species can vary in response to growth conditions, exposure to heat and drought stress, and seasonal or interannual changes in environmental conditions (Geange et al., 2021). Plants grown under warmer and drier conditions or exposed to heat and drought treatments tend to exhibit greater thermal tolerances than those exposed to cool, well-watered conditions (Sumner et al., 2022, Seemann et al., 1986). To date, fewer studies have assessed variation in thermal tolerance between species of the same genus along environmental gradients on a small, local scale (Feeley et al., 2020). Additionally, particularly for alpine species, few previous studies have assessed tolerance to both heat and cold extremes, meaning we have little understanding of the range of temperatures over which these species can persist (Geange et al., 2021).

The ability to thermoregulate is influenced by leaf morphology, and thermoregulation strategies form one current hypothesis to explain diversity in leaf shape and form (Leigh et al., 2017, Nicotra et al., 2011). Leaf morphology affects heat exchange with the environment via sensible heat exchange, i.e. conduction or convection (Curtis, 2017). By influencing the width of the boundary layer (the region of still air around the leaf in which heat transfer is slower), and leaf thermal mass (which affects the rate of response to changes in ambient temperature) morphological traits including leaf area, dissection, effective width, and thickness alter the extent to which leaf temperatures track environmental temperatures (Leigh et al., 2012, Curtis, 2017). Leaves with smaller area, greater dissection, and smaller effective leaf widths have thinner boundary layers and are more efficient at convective heat transfer (Leigh et al., 2017). These traits are common in plants adapted to hot and dry environments where leaves need to dissipate heat without losing excessive water through the stomata (Leigh et al., 2017, Kullberg and Feeley, 2022). Greater leaf thickness increases leaf thermal mass, meaning leaf temperatures can remain low for longer when air temperatures spike, and is also regarded as an adaptation to hotter environments (Leigh et al., 2012, Curtis, 2017) . By influencing heat exchange regardless of changes in light or water availability, these traits could affect species' thermal tolerances (Kullberg and Feeley, 2022, Curtis, 2017). For example, Groom et al. (2004) found plants with thicker leaves in mallee-heathland environments were more likely to survive an extreme heatwave.

Plant strategies with regard to the Leaf Economics Spectrum may also be tied to thermal tolerance (Curtis, 2017, Knight and Ackerly, 2001). Leaf mass per unit area (LMA) represents investment of resources in leaf construction and robustness (Nicotra et al., 2011). Because leaves with greater LMA need to survive longer to see a return on investment, greater thermal tolerance may ensure their persistence over a broader range of environmental conditions (Knight and Ackerly, 2001). Negative correlations between specific leaf area (SLA, the inverse of LMA) and heat tolerance have been identified across biomes and growth forms to support this hypothesis (Münchinger et al., 2023, Valliere et al., 2023, Knight and Ackerly, 2003).

In environments subject to water inundation, abiotic conditions differ drastically to dry, terrestrial habitats, meaning selective pressures affecting leaf morphology and thermal tolerance may differ (Hamann and Puijalon, 2013). Submerged plants are subject to slower gas diffusion rates and drag from water movement (Hamann and Puijalon, 2013, Mommer and Visser, 2005). Under these conditions, a thinner boundary layer is advantageous to improve gas exchange at the surface of the leaf, while smaller surface areas and effective leaf widths minimise drag force experienced (Hamann and Puijalon, 2013, Hammer, 2019). Previous studies have identified greater leaf dissection and smaller leaf areas in plants from inundated habitats (Lynn and Waldren, 2002, Lynn and Waldren, 2001). Additionally, the higher heat capacity and specific heat of freshwater can mean temperatures in inundated environments are less variable and typically cooler during hot times of the year than terrestrial environments; meaning efficient thermoregulation may be less vital.

Six herbaceous *Ranunculus* species commonly occurring in Charlotte Pass, Kosciuszko National Park represent an interesting model system to investigate how thermal tolerance and leaf morphology vary on a localised spatial scale (Costin et al., 2000, Hammer, 2019). These species, *R. millanii, R. niphophilus, R. dissectifolius, R. muelleri, R. graniticola* and *R. acrophilus,* are regarded as morphologically distinct and yet are interfertile, creating an extensive hybridisation complex (Armstrong, 2003). Each species exhibits unique habitat preferences, particularly in regard to soil moisture (see Table 1; Hammer, 2019, Armstrong, 2003). In spring and summer, when snow cover is minimal, these species are distributed across water-inundated and drier, terrestrial habitats. *R. niphophilus* and *R. millanii* exist in inundated habitats, with the former found in running water courses largely fed by snowmelt while the latter is found in water-filled fens (Hammer, 2019, Costin et al., 2000). The remaining four species are found in increasingly drier habitats along a gradient roughly from *R. dissectifolius, R. muelleri, R. graniticola,* and *R. acrophilus*. Studying this species complex provides an opportunity to not only ascertain the thermal tolerance of multiple Australian alpine herb species, but to also identify whether patterns in thermal tolerance and leaf morphology exist among species of the same genus and between different habitats.

This study investigated thermal tolerance characteristics and leaf morphology in *Ranunculus* species in Kosciuszko National Park. I examined whether differences in thermal tolerance and leaf morphology exist between species and habitats, and whether any relationships between thermal tolerance and morphology exist. Specifically, I sought to determine:

a) Whether $T_{\text{crit (hot)}}$, $T_{\text{crit (cold)}}$, thermal tolerance breadth (TTB) and leaf morphology differ between *Ranunculus* species and between inundated and not inundated habitats, and;

b) Whether thermal tolerance characteristics correlate with leaf morphological traits.

Based on previous findings regarding leaf morphology in these species (Armstrong, 2003) and the ability for thermal tolerance to drive species distributions, I predicted species would differ significantly in both thermal tolerance characteristics and in leaf morphology, such that species found in inundated habitats would have lower $T_{\text{crit (hot)}}$, higher $T_{\text{crit (cold)}}$, and lower TTB as a result of the high heat capacity of water, along with greater leaf dissection and lower effective leaf width, area, LMA, and thickness than plants from drier environments. Within the constraints of a small, preliminary project, I describe here the general patterns observed in the species complex. My results offer some insight into the evolutionary ecology of thermal tolerance in an alpine herb species complex and identify potential avenues for future research.

Methods

Study Site and Sample Collection:

This study took place in Kosciuszko National Park, NSW, Australia. This site forms part of Australia's alpine region and experiences a temperate climate with mean annual temperatures ranging from 1.3°C to 11°C and mean annual rainfall of 2171mm (data from Perisher Valley AWS; Bureau of Meteorology, 2023a). The region also experiences seasonal snow cover, mostly during the Australian winter (NSW Department of Planning Industry and Environment, 2020).

Five transects were established within the study site. Transects 1, 2, 3 and 4 extended from waterlogged bog in a valley floor near Spencer's Creek to Kosciuszko Road, Charlotte Pass (36.44°S, 144.33°E). Transect 5 extended along the Main Range walking track from Charlotte Pass to Carruthers Peak (36.40°S, 148.28°E, Table 1). Species were classified by habitat type, either 'inundated' or 'not inundated', based on the presence of surface water covering the site at the time of collection.

Species identification was based on flower colour, petal number, and leaf morphology as per Costin et al. (2000). At least three leaves were collected per plant and transported in a Ziplock bag containing a moist paper towel back to Kahane Lodge, Perisher Valley, for further analysis.

All samples were collected during the Australian spring of 2023 between the 21st and 28th of November. Approximately 58mm of rain fell during the sample period (data from Perisher Valley AWS; Bureau of Meteorology, 2023b), resulting in soils being saturated, as confirmed using a soil-moisture probe.

Table 1: Description of transects along which *Ranunculus* species were sampled and typical characteristics observed at collection sites for each species. Species are presented in order of soil moisture preference, from wettest to driest, based on the findings of Hammer (2019) and Armstrong (2003). Note the soil moisture preference of *R. acrophilus* has not been studied but is assumed to be low due to its preference for rocky, feldmark habitats (Hammer 2019).

Species	***Transects***	***Habitat Classification***	***Description of Collection Sites***
R. millanii	1, 2, 3, 4	Inundated	Waterlogged bogs at each transect. Samples completely or partially submerged in water.
R. niphophilus	5	Inundated	Within drainage lines along the transect. Most plants found in or on the edge of running water.
R. dissectifolius	1, 2, 3, 4	Not Inundated	Herb field near bogs, but never inundated.
R. muelleri	1, 2, 3, 4	Not Inundated	Tall alpine herb fields and heathland at the upper edge of each transect, closer to Kosciuszko Road on slightly steeper slopes.
R. graniticola	1, 2, 3, 4	Not Inundated	Tall alpine herb fields and heathland at the upper edge of each transect, closer to Kosciuszko Road on steeper slopes.
R. acrophilus	5	Not Inundated	Distributed along the transect up to the Carruthers Peak feldmark; away from waterlogged sites.

Thermal Tolerance Measurements:

T_{crit} was measured using a Pulse Amplitude Modulated (PAM) imaging system following methodologies of Arnold et al. (2021) and Harris et al. (2023). An 8x7 paper grid was mounted on top of a Peltier plate, with a fluorescence camera fixed 185mm above. Thermocouples were fixed to each grid cell and connected to a data taker recording temperature at five-second intervals. Leaf samples were cut to fit within a cell using a razor or leaf punch and placed over the thermocouple in a randomly assigned grid cell. A glass sheet was placed over the grid to keep samples flat against thermocouples.

The apparatus was covered by black fabric to exclude light and samples allowed to dark adapt for 20 minutes. F_0 was determined by applying a blue light of insufficient intensity to initiate photosynthesis, before exposing samples to a saturating light pulse to determine F_M. These values were used to calculate pre-stress F_V/F_M for each sample. Leaves with pre-stress F_V/F_M values below 0.5 were considered unhealthy and excluded from further analysis.

By changing the temperature of the Peltier plate, samples were either heated to approximately 60°C at a rate of 30°h^{-1} (for $T_{crit\ (hot)}$ measurements) or cooled to -20°C at -15°h^{-1} (for $T_{crit\ (cold)}$ measurements). F_0 measurements for each sample were recorded every 20 seconds and plotted against temperature. T_{crit} was then calculated according to Arnold et al. (2021) as the inflection point at which the rate of change in fluorescence transitioned to a sharp spike.

For both $T_{crit\ (hot)}$ and $T_{crit\ (cold)}$ measures, two runs with 54 samples and one run with 45 samples were completed. In some cases, the temperature-fluorescence curve was excessively noisy or did not follow a shape required to calculate a reasonable T_{crit} value, indicating poor contact with the plate or thermocouple, and so the sample was excluded from analysis. This resulted in uneven sample sizes between species and in differences in the number of $T_{crit\ (hot)}$ and $T_{crit\ (cold)}$ measurements obtained (see Table 2). For plants with both values, the range of temperatures over which PSII remained undamaged, referred to as the 'thermal tolerance breadth' (TTB), was calculated as the sum of $T_{crit\ (hot)}$ and the absolute value of $T_{crit\ (cold)}$.

Leaf Morphological Trait Measurements:

Leaf traits were measured for ten leaves per species. Following collection, leaves were placed in water-filled falcon tubes and allowed to rehydrate overnight. Hydrated leaf mass was measured using an analytical balance accurate to 0.001g. One-sided leaf area, perimeter, and effective leaf width were determined from digital photographs using ImageJ software v1.51 (Rasband, 1997-2018). Leaf dissection was calculated as leaf perimeter divided by the square-root of leaf area. Leaf thickness was measured in three places per leaf using digital calipers accurate to 0.01mm, attempting to avoid major veins, then averaged. Leaves were dried in a food dehydrator at 70°C for at least twelve hours and weighed to obtain leaf dry mass. *R. millanii* masses were too small to directly obtain individual dry mass, so were dried and weighed in groups of four and individual dry mass calculated by multiplying the combined dry mass by the proportion of the combined fresh mass made up by that individual. LMA was calculated as dry mass divided by hydrated leaf area (g m^{-2}).

Data Analysis:

Data analysis was completed using R software v4.1.2 (R Core Team, 2021). Due to limited sample sizes, the transect, sample number and sample date; representing the area and plant each leaf was sampled from and the day it was collected, were not included in statistical models. Exploratory analysis of these variables prior to analysis indicated there were not systemic differences among days or transects (plots not shown).

To assess whether species and habitats differed in thermal tolerance characteristics and leaf traits, two one-way ANOVAs were performed for each response variable; one in which species was the explanatory variable and one in which habitat (inundated vs non-inundated) was the explanatory variable. Pairwise differences between habitats and species were tested using Tukey's Honest Significant Difference (HSD) test. Results were considered significant where $p<0.05$.

Correlations between thermal tolerance characteristics and leaf traits were investigated by plotting species' thermal tolerance means against their leaf trait means and estimating linear lines of best fit. As statistical models fitted to the data tended to fit poorly due to small sample sizes, relationships were visually assessed and described qualitatively.

Results

Differences in Thermal Tolerance

Ranunculus species differed significantly in thermal tolerance characteristics (Figure 1, Table 2). One-way ANOVAs indicate species differed in $T_{crit\ (hot)}$ (Figure 1a; df=5, *F*=10.05, *p*<0.001), $T_{crit\ (cold)}$ (Figure 1b; df=5, *F*=4.80, *p* = 0.02), and TTB (Figure 1c; df=5, *F*=4.80, *p*=0.002). Several pairwise differences were identified between species. *R. muelleri* had significantly greater $T_{crit\ (hot)}$ than all other species except *R. dissectifolius*. *R. millanii, R. niphophilus* and *R. acrophilus* did not differ significantly in $T_{crit\ (hot)}$, and *R. graniticola* was similar to both this group and *R. dissectifolius* (Figure 1a). *R. millanii, R. niphophilus,* and *R. dissectifolius* exhibited significantly lower $T_{crit\ (cold)}$ values than *R. graniticola,* while *R. muelleri* and *R. acrophilus* were similar to both *R. graniticola* and the remaining three species (Figure 1b). *R. muelleri* also had a significantly greater TTB than *R. millanii, R. niphophilus* and *R. graniticola* (Figure 1c).

Plants from habitats that were not inundated exhibited higher $T_{crit\ (hot)}$ and a wider TTB on average than plants from inundated habitats (Figure 1a,c). $T_{crit\ (hot)}$ and TTB varied significantly between habitats (Figure1a,c; one-way ANOVAs; df=1, *F*=19.74, *p* < 0.001 and df=1, *F*=6.82, *p*=0.01 respectively), while $T_{crit\ (cold)}$ did not (Figure 1b; one-way ANOVA; df=1, *F*=0.02, *p*=0.90).

Table 2: Sample sizes (n), means ($\bar{x}$) and standard deviations (SD) in thermal tolerance and leaf morphological traits for a) six *Ranunculus* species and b) two habitat types in Kosciuszko National Park. Habitat data are averaged across species.

Species/Habitat	**Thermal Tolerance**						**Leaf Morphology**					
		$T_{crit\ (hot)}$		**$T_{crit\ (cold)}$**		**TTB**		**Leaf Dissection**	**Effective Leaf Width**	**Leaf Area**	**LMA**	**Leaf Thickness**
	n	$\bar{x} \pm SD$ (°C)	n	$\bar{x} \pm SD$ (°C)	n	$\bar{x} \pm SD$ (°C)	n	$\bar{x} \pm SD$	$\bar{x} \pm SD$ (cm)	$\bar{x} \pm SD$ (cm^2)	$\bar{x} \pm SD$ (g/m^2)	$\bar{x} \pm SD$ (mm)
a) Species data												
R. millanii	9	41.70±1.64	4	-7.33±2.15	4	46.70±5.00	10	21.27±2.92	0.08±0.02	0.38±0.13	90.18±38.01	0.20±0.02
R. niphophilus	12	41.00±1.54	10	-8.82±3.33	10	49.40±3.65	10	13.89±2.53	0.38±0.07	1.95±0.64	112.27±18.66	0.32±0.04
R. dissectifolius	13	44.61±2.18	4	-8.72±1.27	4	53.40±2.26	10	19.57±2.43	0.30±0.06	1.88±0.54	191.73±48.69	0.36±0.03
R. muelleri	12	45.30±2.01	10	-8.62±1.83	10	54.00±3.22	10	5.40±1.25	0.97±0.20	1.56±0.58	154.00±42.81	0.45±0.06
R. graniticola	11	42.70±1.79	4	-4.34±2.92	4	47.00±3.31	10	10.68±2.45	0.72±0.13	3.69±0.64	107.69±16.14	0.31±0.03
R. acrophilus	10	42.04±1.81	11	-9.26±1.67	9	51.20±2.91	10	5.67±1.18	1.10±0.30	2.05±0.50	171.94±41.77	0.49±0.11
b) Habitat data												
Inundated	21	41.30±1.59	14	-8.40±3.04	14	48.60±4.06	20	17.58±4.62	0.23±0.16	1.17±0.92	101.23±31.27	0.26±0.07
Not Inundated	46	43.80±2.33	29	-8.29±2.41	27	51.90±3.72	40	10.33±6.10	0.80±0.36	2.29±1.00	156.34±49.24	0.40±0.10

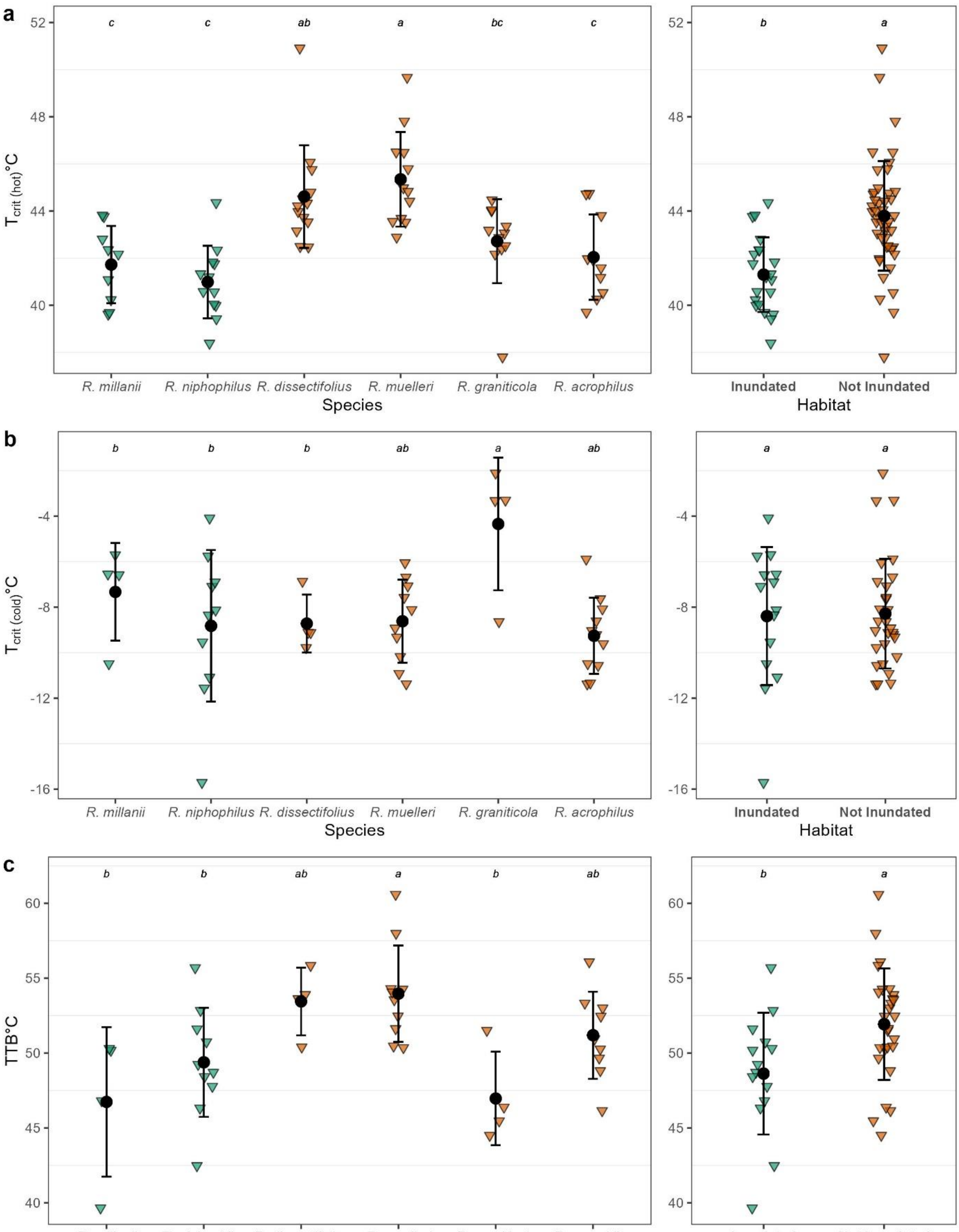

Figure 1: Thermal tolerance characteristics for six *Ranunculus* species in Kosciuszko National Park. a) and b) show $T_{crit\ (hot)}$ and $T_{crit\ (cold)}$ values for each species and habitat type, respectively, while c) shows the thermal tolerance breadth (TTB) of each species and habitat. For each plot, species differences are depicted in the left panel and habitat differences in the right panel. Blue and orange triangles represent raw data points for plants from inundated and not inundated habitats, respectively, while solid black dots and error bars represent species or habitat averages and standard deviations. Compact letter displays reflect significance; species or habitats that do not share a common letter differed significantly in that thermal tolerance trait according to Tukey's HSD test.

Differences in Leaf Morphology

Significant variation in all leaf morphological traits tested was identified between *Ranunculus* species (Figure 2, Table 2). One-way ANOVAs indicate species differ significantly in leaf dissection (Figure 2a; df=5, F=92.24, p<0.001), effective leaf width (Figure 2b; df=5, F=61.57, p<0.001), leaf area (Figure 2c; df=5, F=39.51, p<0.001), LMA (Figure 2d; df=5, F=20.76, p<0.001) and leaf thickness (Figure 2e; df=5, F=33.22, p<0.001).

Several significant pairwise differences between species were also identified (Figure 2). In leaf dissection, *R. dissectifolius* and *R. graniticola* were similar to one another, as were *R. millanii* and *R. dissectifolius*, while all other pairwise combinations were significantly different (Figure 2a). *R. millanii, R. niphophilus* and *R. dissectifolius* did not differ significantly in leaf width, nor did *R. muelleri* and *R. acrophilus*, but all other pairwise comparisons differed (Figure 2b). In leaf area, while *R. niphophilus, R. dissectifolius, R. muelleri* and *R. acrophilus* did not differ significantly from one another, all other pairwise combinations were significantly different (Figure 2c). *R. millanii, R. niphophilus,* and *R. graniticola* did not differ significantly in LMA, nor did *R. dissectifolius, R. muelleri* and *R. acrophilus* when compared to one another. *R. niphophilus* and *R. muelleri* were also not significantly different. All other pairwise LMA comparisons were significant (Figure 2d). In leaf thickness, *R. niphophilus, R. dissectifolius* and *R. graniticola* were similar, as were *R. muelleri* and *R. acrophilus*. All other pairwise comparisons between species in leaf thickness were significant (Figure 2e).

Habitats differed in leaf dissection (df=1, F=21.89, p<0.001), effective leaf width (df=1, F=40.93, p<0.001), leaf area (df=1, F=17.83, p<0.001), LMA (df=1, F=20.76, p<0.001) and leaf thickness (df=1, F=32.34, p<0.001). Pairwise comparisons show plants from inundated habitats had significantly higher leaf dissection and significantly lower leaf width, leaf area, LMA and leaf thickness than those from drier, not inundated habitats (Figure 2).

Correlations between Thermal Tolerance and Leaf Morphology

The majority of correlations between species' average thermal tolerance characteristics and leaf morphological traits identified were weak, with high variation observed (Figure 3). Two apparently strong correlations were identified, however. TTB increased with increasing LMA, with the nature of this relationship consistent overall and when plants from inundated and not inundated habitats were considered individually (Figure 3l). TTB also positively correlated with leaf thickness, with the relationship consistent across both habitat types and overall (Figure 3o).

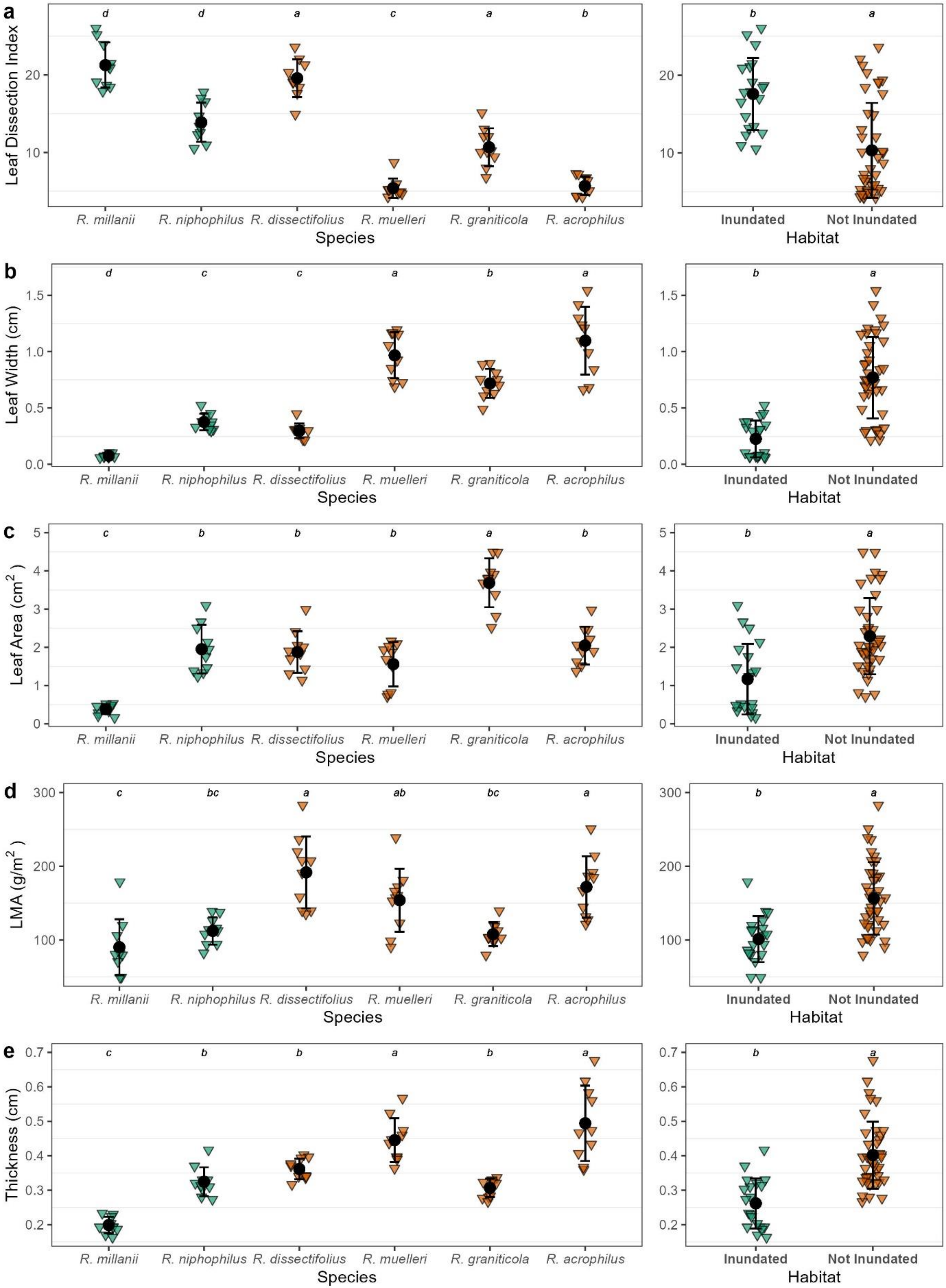

Figure 2: Leaf morphological traits a) leaf dissection, b) effective leaf width, c) hydrated leaf area, d) LMA and e) average leaf thickness observed in *Ranunculus* species in Kosciuszko National Park. For each trait, the left panel depicts differences between species while the right panel depicts differences between habitat types. Blue and orange triangles represent raw data points for inundated and not inundated habitats, respectively, with n=10 leaves measured per species. Solid black points and errorbars represent means and standard deviations, respectively. Species or habitats that do not share a common compact letter differed significantly in that trait according to Tukey's HSD test.

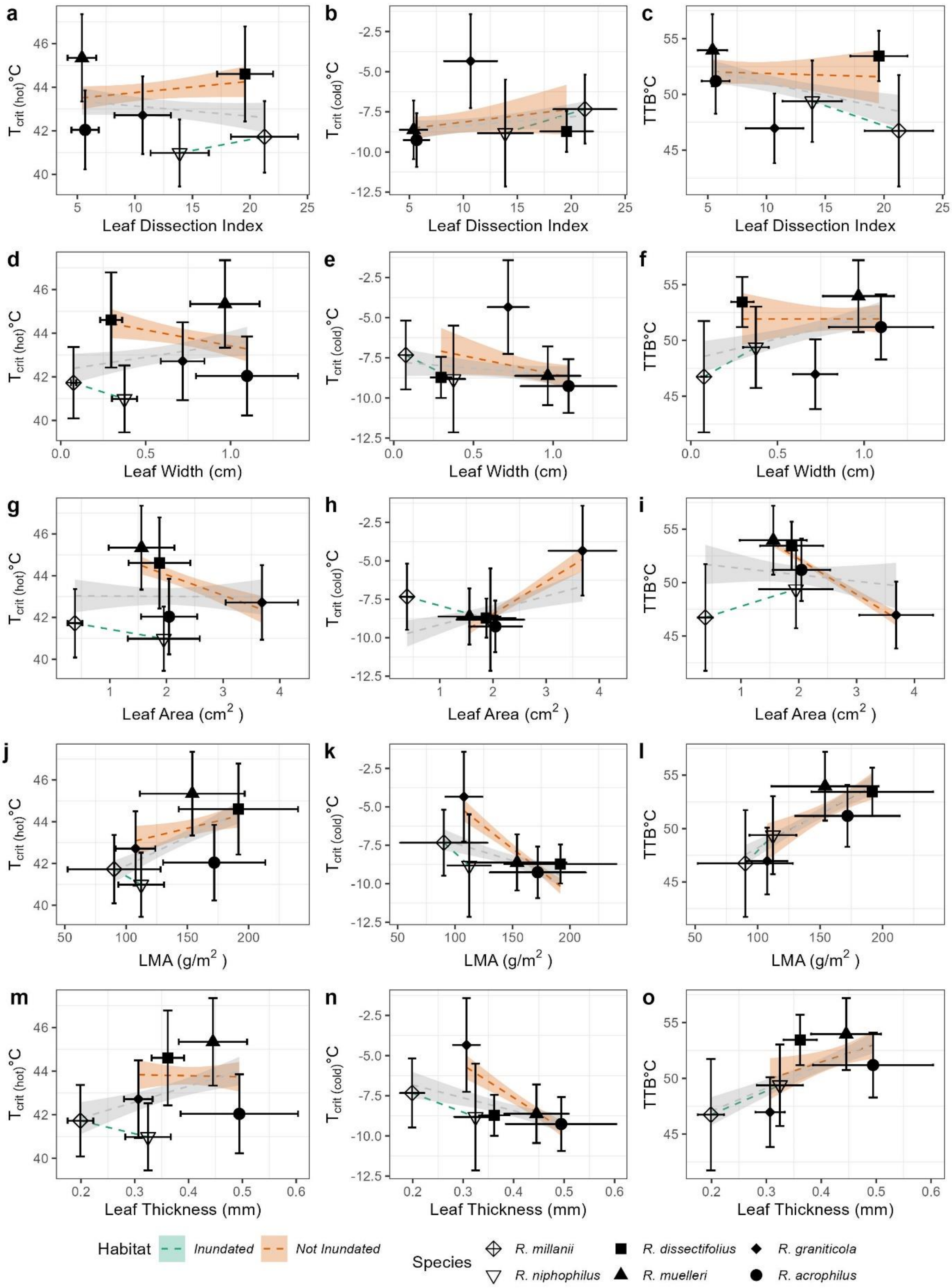

Figure 3: Relationships between leaf morphological traits and thermal tolerance characteristics in alpine *Ranunculus* species. Individual points represent the average trait value and thermal tolerance value for each species. Horizontal error bars represent standard deviation in morphological traits and vertical error bars the standard deviation in thermal tolerance. Linear lines of best fit are represented by dashed lines, with grey lines based on all data points, orange lines based on values for not inundated habitats only, and blue lines based on inundated habitats only. Shaded areas represent 95% confidence intervals.

Discussion

This study aimed to determine whether six species of alpine *Ranunculus* in Kosciuszko National Park differed in thermal tolerance and leaf morphology, and whether these traits were correlated with one another. Differences in thermal tolerance characteristics and in leaf morphology were identified between species and more broadly between inundated and drier, not inundated habitats in all measured traits except $T_{crit\ (cold)}$. Species from drier habitats exhibited greater thermal tolerance than those from inundated habitats. While thermal tolerance characteristics were weakly related to most morphological traits, positive correlations between TTB and both LMA and leaf thickness emerged.

Differences in Thermal Tolerance

Thermal tolerance thresholds observed in this study were similar to values reported in previous studies of alpine plants (e.g. Buchner and Neuner, 2003, Geange et al., 2021). Species differed significantly in all thermal tolerance characteristics, supporting previous findings that thermal tolerances may influence species distributions (Nievola et al., 2017, Geange et al., 2021). However, pairwise species differences did not follow a simple correspondence with distribution along an elevation or soil moisture gradient. For example, *R. muelleri*, which exhibited the highest average $T_{crit\ (hot)}$ and TTB, was collected from an intermediate elevation and was not distributed at the extremities of a soil-moisture gradient as reported by Hammer (2019). Similarly, *R. graniticola,* whose distribution typically bordered that of *R. muelleri,* had $T_{crit\ (hot)}$ and TTB more similar to *R. millanii* and *R. niphophilus* than *R. muelleri.* These results show that thermal tolerance varies between species of the same genus within a localised area, but, at least in the case of a single point in time study like this one, not in a simple manner over an elevational or soil-moisture gradient.

That said, species collected from inundated habitats had lower $T_{crit\ (hot)}$ and lower TTBs than those from habitats that were not inundated in this study. This supported the hypothesis that inundated environments constitute a more stable and cooler environment than terrestrial environments, meaning adaptation to a wide range of temperatures is less important in inundated environments.

While the thermal tolerance values observed in this study are realistic, they may not capture the full range of extremes these species can tolerate due to the timing of sampling. T_{crit} is known to be highly plastic and varies on a seasonal basis. Sampling here was completed in late spring when exposure to hot or cold temperature extremes is unlikely. Further, rainfall occurring in the area during sampling meant plants were unlikely to be water stressed even in the drier microsites, and able to keep stomata open as needed; potentially affecting leaf temperatures reached and thus T_{crit} values observed. Further studies over a broader range of environmental conditions are needed to obtain a comprehensive understanding of thermal tolerance in this system. To better explore the mechanisms underlying these apparent patterns, further investigation with larger sample sizes and repeated sampling over the growing season is warranted.

Differences in Leaf Morphology

The substantial differences in leaf morphology between species observed here supports multiple previous studies that described these *Ranunculus* species as highly morphologically distinct (Costin et al., 2000, Armstrong, 2003, Hammer, 2019). The exception to this was *R. muelleri* and *R. acrophilus,* which differed only in leaf dissection. These species were collected from different transects, with *R. acrophilus* typically at higher elevations than *R. muelleri.* These results prompt some questions as to whether they are distinct species or plastic variations along an elevational gradient.

The differences in morphology between habitats also aligned with predictions and with previous studies such as Lynn and Waldren (2001)'s finding that plants subject to inundation possess smaller and more dissected leaves. Inundated leaves likely had thinner boundary layers and lower thermal mass than those from not inundated habitats, which could be associated with the need to improve gas exchange in water or the need to minimise drag from moving water rather than thermoregulation.

Relationships Between Thermal Tolerance and Leaf Morphology

Few studies to date have assessed relationships between leaf morphology and thermal tolerance. Of these, Knight and Ackerly (2003) and Valliere et al. (2023) both reported negative correlations between SLA and heat tolerance. My results support these findings, with TTB increasing with increasing LMA among *Ranunculus* species from both habitats. I additionally identified a positive correlation between leaf thickness and TTB, which suggests greater thermal mass could confer broader thermal tolerance. This finding somewhat aligns with Kullberg and Feeley (2022), who found leaf thickness was correlated with maximum environmental temperatures, which in turn were correlated with thermal tolerance.

In several cases, the direction of correlation between thermal tolerance and leaf morphology traits differed between habitats. For example, the direction of relationship between $T_{crit\ (cold)}$ and leaf area and the relationship between TTB and leaf area was seemingly opposite for plants from inundated habitats and those from drier habitats that were not inundated. Though these relationships were not statistically significant it is interesting to consider: Do smaller leaf areas confer thinner boundary layers, which is beneficial for faster convective heat transfer in terrestrial environments but in aquatic environments instead result in slower gas exchange and inhibition of growth, leading to differences in the direction of selection of leaf areas between habitats and a difference in relationship?

Although care was taken to avoid intermediate leaf forms, the extensive hybridisation complex formed by alpine *Ranunculus* species in Kosciuszko National Park makes it difficult to ensure plants sampled in this study were true parent species and not hybrids based on morphology alone. Future studies could incorporate genetic tests to confirm species identification, and additionally could investigate whether the thermal tolerance characteristics and leaf morphology of hybrids lie between values found for parent species and follow the patterns observed here.

The high variation seen in my results combined with a limited sample size means that much of my discussion regarding the relationship between thermal tolerance and leaf morphology is speculative. Further studies with larger sample sizes that enable fitting of robust statistical models are required to confirm whether the correlations identified here are robust. This research does, however, suggest avenues for future research to ascertain whether plants occupying different habitat types differ in their susceptibility to future climate change, and whether leaf morphology could provide a useful predictor of vulnerability to temperature extremes.

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