

Aciphylla glacialis mortality, growth and frost resistance: a field warming experiment

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Abstract. Decreasing snow depth and earlier snowmelt in alpine regions are expected to expose plants to a greater range of thermal extremes. Thus, paradoxically, in addition to increasing mean temperatures, aseasonal frost is likely to emerge as a major determinant of plant survival and development under a warming climate. Through the use of open top chambers, we assessed the effects of simulated warming on seedlings of the alpine herb *Aciphylla glacialis* (F.Muell.) Benth, sourced from provenances that vary in thermal characteristics. We hypothesised that seedlings grown at elevated temperature would have reduced survival and lower freezing resistance, but that individuals that did survive would show increased growth. Further, we hypothesised that seedlings sourced from the lower-elevation sites, where temperatures are more variable, would exhibit lower mortality at warmed conditions than those from sites with narrower thermal ranges. Warmed conditions significantly increased seedling mortality but those that survived grew slightly taller than their ambient counterparts, with no impact on leaf production or photosynthetic efficiency. Although the warming treatment did not influence temperature minimums, which would have allowed us to assess the impact of aseasonal frosts, there was no effect on freezing resistance. Contrary to our expectations, there was little evidence of variation among provenances. Our results indicate that a warmer climate with more extreme events may lead to a reduction in seedling establishment and survival; however, seedlings that do survive and establish are unlikely to express any lingering detrimental effects.

Additional keywords: ice nucleation, intraspecific variation, open top chambers, phenotypic plasticity, transplant experiment.

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Introduction

Climate change will bring a warmer future for most ecosystems, but for some, such as alpine ecosystems, increasing temperatures may paradoxically increase the risk of frost (Inouye 2000; Ball *et al.* 2012). This is because snow acts as an insulating blanket protecting vegetation from freezing winter temperatures, and providing a source of water during spring and early summer (Billings and Bliss 1959; Körner 2003). Predictions of increased temperatures and variable patterns of precipitation may substantially reduce snow pack development and persistence, resulting in earlier snow melt (Inouye 2008; Rixen *et al.* 2012; Luca *et al.* 2018). In an ecosystem where plant growth is constrained by low temperatures and a truncated growing season, increased mean temperatures and earlier snow melt may present an opportunity for increased plant growth (Galen and Stanton 1993, 1995). However, models of projected climate change also predict increased risk of temperature extremes (IPCC 2014; Harris *et al.* 2018), which may instead limit growth through damage to the photosynthetic apparatus, or in extreme cases, result in the loss of leaves, or plant death. These

apparent contradictions, along with the increased risk of otherwise aseasonal freezing events suggests a need to better understand how resilient alpine plants are to both warming and frost (Inouye 2000; Beniston 2003; Wipf *et al.* 2009; Larcher *et al.* 2010; Sierra-Almeida and Cavieres 2012; Gerdol *et al.* 2013).

The ability of a plant to persist across variable spatial or temporal landscapes may arise through phenotypic plasticity, which is the capacity for a given genotype to express different phenotypes across heterogeneous conditions (Schlichting 1986; Bradshaw and Hardwick 1989; Bradshaw 2006; Bradshaw and Holzapfel 2006). Within subalpine, alpine and arctic landscapes, plasticity in phenological traits has been widely documented (Anderson *et al.* 2012; Vitasse *et al.* 2013; Prev y *et al.* 2017; Richardson *et al.* 2017). However, we know much less about how underlying traits of growth and thermal tolerance vary across the alpine environment or influence plant survival. Furthermore, the extent of plasticity may vary not only among traits but also between populations of a given species as a function of both

environmental conditions and evolutionary history (van Kleunen and Fischer 2005; Ghalambor *et al.* 2007). This makes it difficult to generalise about the capacity of a species to respond to environmental change.

In the highly heterogeneous alpine environment, locations in which snow melts earlier may be characterised by greater exposure to spring freezing events, summer heat events, longer growing seasons, and more growing degree days than sites where snow typically melts later (Briceno *et al.* 2014). For these populations, historical exposure to greater temperature ranges may have equipped them with the capacity to respond to future climatic conditions. Indeed, within the Australian Alps, seedlings sourced from locations with greater thermal ranges, be they low elevation or early snow melt sites, have been shown to exhibit greater plasticity in functional traits in response to warming temperatures (Briceno 2014; Nicotra *et al.* 2015). Furthermore, for the native herb *Aciphylla glacialis* both adults and their glasshouse grown progeny sourced from thermally variable provenances had a greater capacity to acclimate to cold conditions (Briceno 2014; Briceno *et al.* 2014). Within species variation in trait expression may reflect current growing conditions, genetic differentiation, and also parental environmental condition during seed development (Auge *et al.* 2017). Thus, predicting the impact of climate warming in the heterogenous alpine environment will likely be complicated by several underlying drivers of plant response.

Temperatures within the Australian alps have increased by 0.8°C since the 1950s (Hennessy *et al.* 2008), and precipitation, snowfall and snow cover are becoming increasingly variable. In addition, there has been a decrease in the duration of snow cover by 8.5 days during this period (Osborne *et al.* 1998; Hughes 2003; Nicholls 2005; Hennessy *et al.* 2008; Green and Pickering 2009; Davis 2013; Harris *et al.* 2016). In the context of these changes we conducted an open top chamber (OTC) study to determine how simulated warming affects survival, growth, photosynthetic efficiency and freezing resistance in an Australian alpine herb, *Aciphylla glacialis*.

To determine whether within species variation was important to those responses, we compared seed sourced from low elevation sites with larger thermal ranges to those from high elevation sites. We hypothesised that seedlings of *Aciphylla glacialis* grown under simulated warming would display higher rates of mortality, as a direct result of warming or indirect result of increased evaporative demand. In contrast, warmer conditions under OTCs could also promote growth and plant health (as indicated by photosynthetic efficiency, F_v/F_m), but also reduce freezing resistance in the surviving, warm-acclimated seedlings. Thus, we also hypothesised that seedlings sourced from the lower-elevation sites, where thermal variation over days and seasons was greater, would display greater trait plasticity resulting in both higher freezing and heat resistance, and thus have higher overall survival, growth and photosynthetic performance in the field.

Methods and materials

Study species and study sites

Aciphylla glacialis (F.Muell.) Benth (Apiaceae) is a common perennial, dioecious, Australian alpine herb found primarily in

grassy herbfields above the treeline (1800–2228 m a.s.l.) (Costin *et al.* 2000). Individuals often form clumps in the field, with individuals around 30–70 cm in height when flowering. The species is easy to recognise and propagate, and thus was selected as a good representative of the broader flora of perennial forbs in grassy herbfields, which is the dominant vegetation type in this alpine region.

Aciphylla glacialis seeds were collected in Autumn 2012 from Kosciuszko National Park from four populations that were chosen to reflect differences in thermal regimes, arising due to differences in elevation and snow duration patterns during the seed formation period (January–April 2012) (Briceno 2014; Supplementary Material table S1 available at the journal's website). These collections are hereafter referred to as provenances and called: Charlotte Pass (1842 m), Snowy River (1972 m), Seamans Hut (2030 m) and Kosciuszko Summit (2225 m). In brief, the summer-time conditions of the two lower elevation sites, Charlotte Pass and Snowy River, had greater temperature ranges and longer growing seasons than the two higher elevation sites. Specifically, Charlotte Pass was characterised by higher mean and maximum temperatures, frequent high temperature events, and a relatively longer growing season (based on growing degree-days), whereas Snowy River had the greatest frequency of frosts, and also more severe frost events, as well as lower minimum temperatures. The higher elevation sites, Seamans Hut and Kosciuszko Summit, were exposed to a narrower thermal range, both within days and across seasons and generally shorter growing seasons. A prolonged snow pack presence at Seamans Hut resulted in fewer growing degree-days than at Kosciuszko Summit (Briceno 2014; table S1).

Seed collection and germination

Seeds were collected at the stage of natural dispersal from 10 mature plants of a similar size for each provenance (hereafter designated as maternal lines). Plants were at least 3 m apart. Collected seeds from each maternal line (plant) were kept separate, cleaned and stored in a seed-drying room at ~15% relative humidity before cold stratification. Seeds were sown into 9-cm Petri dishes containing 1% agar. Germination in *A. glacialis* occurs after exposure to winter conditions (Briceno 2014; Hoyle *et al.* 2014). Therefore, we alleviated morphological and physiological dormancy by exposing seeds to conditions that mimicked the natural progression of post-dispersal temperatures through summer, early autumn, late autumn and winter. Following germination, seeds were transferred to 68 mm square pots with native planting mix (Martins Fertilizers, Yass, NSW) and Yates Nutricote grey pellets (16:4.4:8.3, N/P/K) fertiliser (Briceno 2014). Seedlings were then left to grow in a glasshouse for 2 years under warm summer alpine conditions 25/15°C day/night temperature regime until they had four true leaves and were ~25 mm in height and so large enough to establish and survive transplant into the field. By allowing the seedlings to establish under controlled conditions we sought to minimise the potential for maternal provisioning effects to be expressed as differences among provenances. As these small plants were pre-reproductive and still would not flower for several years, we refer to them as seedlings throughout, and

each seedling was allocated a unique individual plant identification (ID).

Open top chamber field trial

In early autumn (March 2014), seedlings were transplanted into the field at Kosciuszko National Park, near Merritt's Creek (1950 m above sea level, -36.462431 , 148.305765). Small polycarbonate open top chambers (OTC) were used to simulate climate warming (Cochrane *et al.* 2015). Chambers were circular with a 45 cm basal diameter, 32 cm opening diameter, and 15 cm central height. The experimental design consisted of five blocks, each composed of 10 plots (Supplementary Material fig. S1). A plot included one open top chamber and one equivalently sized ambient control subplot (five blocks by 10 plots by two subplots (one per treatment) = 100 subplots; 50 OTC and 50 ambient). Within each OTC or paired ambient subplot, four individual *A. glacialis* seedlings were planted, one per provenance. Maternal lines were randomised within each provenance using a stratified design across blocks, plots and subplots. Within each OTC or ambient subplot, seedlings with intact glasshouse soil were randomly planted at the cardinal directions. At planting each seedling was well watered. Seedlings were located ~15 cm away from one another, and encroaching vegetation was removed at each visit. Paired subplots were orientated in east/west pairings and separated from adjacent subplots by at least one meter. Plots as a whole were arranged in a generally north/south orientation, though positions and orientations were modified as dictated by the topography (e.g. large rocks or dense existing vegetation). We controlled as best as possible for constant slope and aspect across blocks and plots.

Field measurements

Seedling survival, growth and photosynthetic efficiency were assessed up to 11 times during the growing seasons between March 2014 and April 2016. Growth was measured as the height of the plant with the longest leaf extended (mm). We also counted the number of fully expanded leaves. The maximum quantum efficiency of photosystem II (PSII) (F_v/F_m) was used as an indicator of photosynthetic health of the seedlings throughout the experiment (Murchie and Lawson 2013). F_v/F_m was measured using a photosynthetic efficiency analyser (PEA Meter, Hansatech Instruments, King's Lynn, UK). Measurements were taken after 20 min of dark adaptation on a single leaf per seedling.

Environmental characteristics

We monitored air temperature throughout the experiment. Air temperature was measured for each subplot at three-hourly intervals from March 2014 to April 2016 using iButtons (Maxim Integrated, San Jose, CA, USA). iButtons were hung from stakes within each subplot 5 cm above the soil. Freeze-thaw activity caused some stakes to sink closer to the soil but the iButtons were 1–5 cm above the soil, at roughly the same height range as the seedlings. The buttons were held on plastic clips that partially shaded them while hanging, but the possibility remains that some were exposed to direct sunlight for portions of the experiment. At each return visit, we carefully checked and re-

orientated/positioned each iButton. We screened the iButton data and excluded values greater than 50°C in both treatments which indicated periods of direct radiation on the buttons rather than accurate measures of air temperature. It was not possible to separate the relative effects of soil temperature and air temperature from our iButton data, and we recognise that soil temperature is likely to be a major determinant of the thermal microclimate of these small plants given their short stature.

To assess differences between our treatments, we divided the iButton temperature data into day (09:00, 12:00, 15:00 and 18:00 hours) and night (21:00, 24:00, 03:00 and 06:00 hours) readings during the growing season. The growing season was defined as any period of time without snowpack formation, where the date of snow pack formation was defined as four consecutive days of iButton data with a mean daytime temperature of 0°C. Date of snow melt was defined as the date when mean temperature exceeded 0°C. We focus on the growing season because when the plots were under snow there were no differences in the temperatures between warmed and ambient treatments.

We also measured soil moisture as a covariate. Soil relative water content was measured to 10 cm depth in each of the 100 subplots using a Delta-T Thetaprobe (Delta-T Devices, Cambridge, UK) at each site visit ($n = 9$).

Ice nucleation

Freezing resistance was assessed as the temperature at which intra-cellular ice formation occurs in leaves (Taschler and Neuner 2004). These measurements were conducted in late summer/early autumn just over 1 year after planting (14–15 April 2015) and followed the protocol of Briceno *et al.* (2014). Seedlings were considered too small for measurements in the first spring (2014). For each treatment, leaf tissue was sampled from five plants per treatment per provenance, giving a total of 40 samples (two treatments by four provenances by five replicates = 40). Due to high mortality during the previous summer, there was insufficient replication to entirely balance blocks or maternal lines within sites. As each treatment and provenance was represented in the freezing assay, the removal of a single blade was determined unlikely to have biased any comparisons made at the final harvest. The largest healthy leaf from each plant was collected in the field and kept damp at a temperature of 2–4°C in the dark during transportation back to the laboratory in Canberra for use within the next 10–48 h, depending upon whether the leaf was included in the first or second run of the freezing assay.

Leaves were trimmed to a standard size of 2 cm length before each sample was placed into an individual chamber in an insulated polystyrene box connected to a water bath (JULABO Labortechnik, Seelbach, Germany). Temperature within the insulated box was reduced from 20°C to -25°C at a rate of 2°C per 20 min following Briceno *et al.* (2014). Temperatures for each leaf sample were recorded at 1 s intervals using copper-constant thermocouples and a Datataker DT500 (Biolab Australia Pty Ltd, trading as Datataker, Scoresby, Vic.). The point of freezing was indicated by detecting an exotherm, or sudden rise in leaf temperature, which is a result of the heat produced through

the intracellular freezing process. Nucleation temperature was defined as the lowest temperature reached before the occurrence of the exothermic reaction. The insulated box held 20 samples, so the 40 samples were randomly allocated to each of two runs in a structured random design so that each run contained an equal representation of treatments and sites.

Harvest measurements

In late April 2016 all surviving seedlings were harvested to assess biomass allocation and specific leaf area (SLA). Seedlings were carefully excavated and all loose dirt shaken from root systems. Plants were stored in resealable plastic bags with damp paper towel and transported back to the laboratory in Canberra. Seedlings were separated into above- and below-ground components. Roots were washed to remove any fine soil particles. Leaf size (cm^2) of the three largest leaves for each individual was determined using a flatbed scanner and analysed using ImageJ (Schneider *et al.* 2012). Above- and below-ground biomass and leaf area samples were then oven-dried for 72 h at 60°C and then weighed using an analytical balance (Mettler-Toledo, Greifensee, Switzerland). SLA was calculated as the total leaf area (cm^2)/mass (g) for the three scanned leaves (Pérez-Harguindeguy *et al.* 2013).

Statistical analysis

Comparisons of iButton air temperature between OTCs and ambient plots were conducted using an ANOVA, with fixed terms treatment, season (spring, summer, autumn, winter), year and their interactions. Terms for block and plot were not included due to iButton loss throughout the experiment (10 ambient and 11 OTC remained). Tukey's *post-hoc* testing was used to compare OTC and ambient treatments. Only those subplots for which continuous data were available were analysed, e.g. excluding plots in which iButtons failed. Day- and night-time temperatures were assessed independently.

Soil moisture was measured to be used as a covariate in the analyses. Thus, we explored the pattern of variation in soil moisture among treatments using an initial analysis with treatment and trip as fixed effects and subplot nested within block as random factors. Comparisons were conducted using a 'lmer' model from lme4 (Bates *et al.* 2015). Although overall there was a significant main effect for soil moisture between treatments (average soil moisture for Ambient was 32.1% and OTC 29.4%, mean difference in percent soil moisture $\text{CI} = 1.79\text{--}3.67$, $P = <0.001$), this relationship varied from significantly wetter to significantly drier for the warmed treatment among dates, such that there was a significant interaction between trip and treatment. Given these results we have included soil moisture as a fixed effect as appropriate (see details below).

Analysis of mortality over the experimental duration was conducted using the R package 'survival' (Therneau 2015). We used a binomial restricted maximum likelihood model to test for differences among treatments and provenances using the function 'glmer' within the R package 'lme4' (Bates *et al.* 2015). To explore what factors might contribute to mortality we similarly applied a binomial restricted maximum likelihood

model with treatment and its interaction with soil moisture or plant trait (including plant height, leaf number, or F_v/F_m) from the preceding visit as fixed factors to predict mortality at the following trip. Block and individual plant ID (nested within provenance) were included in the model to account for variation in a given plant through time. The high mortality meant that maternal line could not be included in the analyses.

Differences in ice nucleation temperatures were examined using a restricted maximum likelihood model (REML) and the function 'lmer' within the R package 'lme4' (Bates *et al.* 2015). Treatment, provenance and their interaction were considered as fixed factors, and block and maternal line (nested within provenance), were treated as random factors. As we chose to select samples according to maternal line, samples were not paired at a plot level, and thus plot was not considered as a random factor.

To assess variation in plant height, leaf number, and F_v/F_m throughout the experiment, several restricted maximum likelihood models (REML) were developed using a mixed effects model approach. Comparisons were constructed using the fixed factors treatment, provenance, sample date, and soil moisture, along with possible interactions. Random factors were block with plot nested within block, and individual plant ID which was nested within provenance. Plant height and F_v/F_m were assessed using 'lmer', while leaf number was assessed using 'glmer' and a Poisson distribution. Model comparisons were then conducted and the best model was identified based on AICc scores using the package 'AICcmodavg' (Mazerolle 2016). In cases where multiple models fell within four AICc values, the model with the least number of parameters (most parsimonious) was chosen (final models within Supplementary Materials table S2). Note, this analysis was conducted only upon individuals that survived the experimental duration (table S2).

Analysis of final trait measurements for treatment and provenance effects were conducted using a restricted maximum likelihood models (REML) in 'lmer'. For leaf number a Poisson distribution was used. Treatment and provenance were fixed factors. We included block with plots nested within block and maternal line as random effects. Data were natural log-transformed when they did not meet the assumptions of normality.

Results

Open top chambers significantly increased the average daytime temperature at plant height during the growing season by 2.5 K ($\text{CI} = 1.6\text{--}3.4$, Fig. 1; Table 1). Night-time temperatures were also warmed on average by 1.1 K ($\text{CI} = 0.58\text{--}1.57$), though this was not a statistically significant difference (Fig. 1; Table 1). The onset of snowpack development was not influenced by the presence of OTCs. However, mid-season melts, and earlier spring thaw did occur under the OTCs, resulting in a reduction of 11 days during the first year and 44 in the second (Table 2). Daytime temperatures in excess of 30°C occurred on 20–30 more days under OTC than ambient conditions. There was no apparent influence of the OTCs on the frequency of frost events below 0°C ($P = 0.253$) or below -5°C ($P = 0.760$).

Few seedlings died in the first autumn or winter post transplanting, however, during the first summer period, high

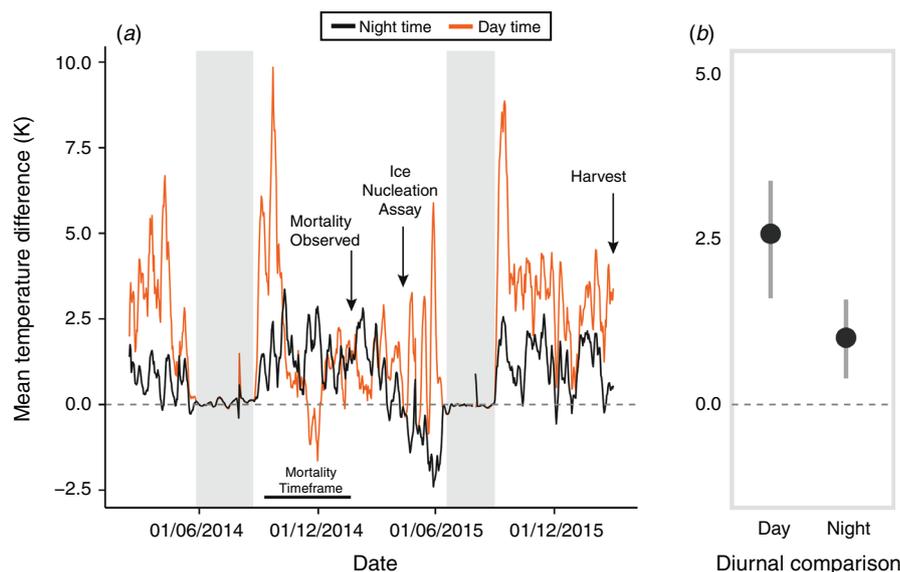


Fig. 1. (a) Illustration of the mean temperature difference (K) between ambient and warmed (open top chamber, OTC) treatments over the course of the experiment from April 2014 to April 2016 at Merritt's Creek, Kosciuszko National Park, NSW, Australia. Daytime temperature differences shown as an orange line, while night-time temperature differences are shown as a black line. Temperature readings were taken 3–5 cm above ground level. Grey bars represent periods under snow cover. (b) Average temperature difference between ambient and warmed (OTC) plots during the growing seasons for day- and night-time temperatures.

Table 1. ANOVA output for mean daytime and night-time temperatures during the snow free growing seasons between April 2014 and April 2016

Model structure included treatment (ambient and warmed; OTC), field year (i.e. Year 1 or Year 2), and season (spring, summer, autumn, winter). d.f., degrees of freedom; SS, sum of squares; RSS, residual sum of squares; AIC, Akaike information criterion. Significant differences are indicated by bolded *P*-values

	d.f.	SS	RSS	AIC	<i>F</i> -value	<i>P</i> -value
<i>Daytime temperature model</i>						
Treatment	1	405	63862	4526	6.96	0.008
Field year	2	1319.1	64776	4539.8	11.33	>0.000
Season	3	9135.5	72593	4664	52.33	>0.000
Treatment × field year	2	112.1	63569	4518.9	0.96	0.382
Treatment × season	3	205.3	63663	4518.6	1.18	0.318
Field year × season	3	61.2	63518	4516	0.35	0.789
Treatment × field year × season	3	203.3	63660	4518.5	1.16	0.322
<i>Night-time temperature model</i>						
Treatment	1	22.5	19420	3210.8	1.26	0.261
Field year	2	56.6	19904	3236.1	14.30	>0.000
Season	3	1931.3	21329	3310.8	36.24	>0.000
Treatment × field year	2	0.5	19398	3207.5	0.01	0.985
Treatment × season	3	15.4	19413	3206.4	0.29	0.833
Field year × season	3	239.2	19637	3219.1	4.49	0.004
Treatment × field year × season	3	5.3	19403	3205.8	0.09	0.961

levels of seedling mortality were observed (December 2014–February 2015). Mortality was higher under open top chambers at 76% (surviving $n = 48$), compared with 56% (surviving $n = 88$) under ambient conditions ($P = 0.012$, Fig. 2). Comparing environmental conditions and plant traits from the measurement period preceding mass mortality, we found that mortality was high where soil moisture was comparatively low. There was not, however, a significant ‘treatment × soil moisture’ interaction for this period,

indicating that the effect of soil moisture was not driven by OTCs but rather varied among blocks and plots (Table 3). Likewise, seedlings that were either small, had few leaves, or had low F_v/F_m were more likely to die (Table 3). These patterns were consistent irrespective of treatment or provenance. Following the mortality event instances of mortality stabilised as did F_v/F_m (Fig. 4c; Table 5), and most plants that survived the initial event persisted until the end of the experiment (Fig. 2).

Table 2. Comparison of days of: snow duration, maximum temperatures exceeding 30°C and minimum temperatures below 0°C for each year of the field experiment for either ambient or warmed (open top chamber, OTC) conditions

	Year 1		Year 2	
	Ambient	Warm	Ambient	Warm
Snow duration (days)	114	104	158	115
Days with max. exceeding 30°C	130	169	155	172
Days with min. below 0°C	253	232	276	277

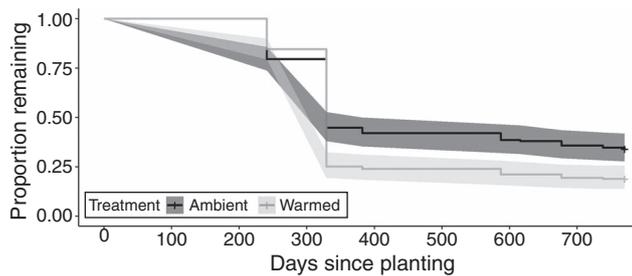


Fig. 2. Kaplan–Meier mortality curve for *Aciphylla glacialis* seedlings grown under ambient (A) and warmed (W) open top chamber (OTC) conditions. The solid line represents means for each treatment averaged across the four provenances and the shaded bands are the 95% confidence intervals.

We assessed freezing resistance in the autumn following the high mortality event (April 2015), and found that *A. glacialis* seedlings were highly freezing resistant. On average, intracellular ice formation was initiated at -9.4°C (Fig. 3). There were no significant differences in freezing resistance between treatments or provenances (Table 4).

At the end of the experiment there were no significant effects of treatment or provenance on SLA, leaf number, total biomass, or above and below ground biomass (Table 5). However, surviving seedlings grown under the OTCs were significantly taller at harvest, and Charlotte Pass seedlings were significantly taller than the other provenances at harvest (Fig. 4; Table 5).

Discussion

The Australian alps are predicted to warm by 0.4–2.0 K by 2030 and 1–6 K by 2070 (Hennessy *et al.* 2008). To simulate a warmer future, we used open top chambers and successfully increased mean daytime temperature by 2.5 K. The experimental warming increased mortality, but there was no detrimental reduction in growth or change in physiological freezing resistance for surviving seedlings. Below we discuss potential mechanisms that could explain differences and similarities in key traits, and what this may mean for how alpine plant species respond to future changes in the climate.

Mortality rates during seedling establishment are generally high, but may vary substantially depending on the frequency and magnitude of extreme events or species-specific characteristics among other factors (Walck *et al.* 2011; Williams *et al.* 2015). In our experiment, seedling mortality was highest under warmed conditions in the first summer (~6–9 months after planting), which is consistent with prior research exploring artificial

Table 3. Survival analysis for *Aciphylla glacialis* seedlings

Analysis of deviance table (Type II Wald Chi-Squared tests) for survival of *A. glacialis* seedlings at the point of mass mortality (November 2014–January 2015) compared with plant traits measured at the preceding time point (October 2015): plant height (mm), leaf number, F_v/F_m , soil moisture and the treatment, either warmed (open top chamber, OTC) or ambient. d.f., degrees of freedom. Significant values are indicated by bolded *P*-values

Factors	Chi-Squared	d.f.	<i>P</i> -value
Height	0.034	1	0.854
Leaf number	13.410	1	0.000
F_v/F_m	23.540	1	0.000
Treatment	6.569	1	0.010
Provenance	4.630	3	0.201
Soil moisture	6.659	1	0.009
Treatment \times provenance	0.924	3	0.819
Treatment \times soil moisture	0.480	1	0.488

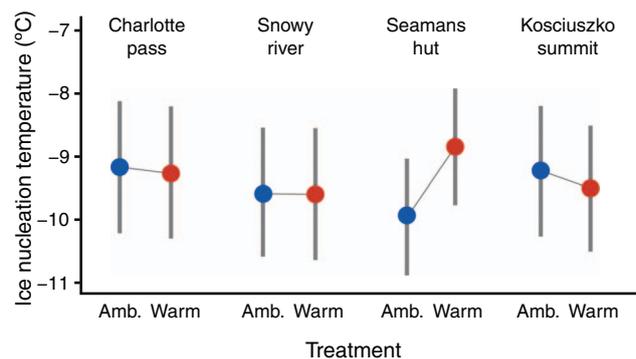


Fig. 3. The temperature of ice nucleation (the formation of internal ice) for *Aciphylla glacialis* seedlings grown under ambient (blue) and warmed (open top chamber, OTC) conditions (orange). For completeness, each provenance is displayed as a panel, and ordered from left to right in ascending elevation. Error bars represent 95% confidence intervals.

Table 4. Statistical output of ice nucleation temperatures for *Aciphylla glacialis* across treatments and provenances

Restricted likelihood estimate (REML) comparing the temperature at which ice nucleation occurred (intracellular freezing) in leaves harvested in April 2015 from warmed (open top chamber, OTC) or ambient conditions across the four provenances. SS, sum of squares; MS, mean of squares; d.f., degrees of freedom; Den. d.f., denominator degrees of freedom. Significant values are indicated by bolded *P*-values

Factors	SS	MS	Num d.f.	Den d.f.	<i>F</i> -value	<i>P</i> -value
Provenance	0.615	0.205	3	26	0.204	0.893
Treatment	0.232	0.232	1	26	0.231	0.635
Treatment \times provenance	2.701	0.910	3	26	0.906	0.452

warming (Cochrane *et al.* 2015); though forested habitats may show contrasting responses (e.g. see Piper *et al.* 2013). In conjunction with warmer temperatures, low soil moisture has previously been shown to increase the incidence of mortality (Aragon-Gastelum *et al.* 2017). In our case, we found that both warming and low soil moisture were associated with increased risk of mortality, but the lack of a significant interaction between these factors indicates that soil moisture effects were not

Table 5. Statistical comparisons of final harvest traits (plant height, leaf number, $F_{\sqrt{F_m}}$, specific leaf area (SLA), total biomass, total above ground biomass (ABG, log-transformed), and total below ground biomass (BG, log-transformed), for *Aciphylla glacialis*

Comparisons include treatment (Trt), provenance and their interaction. SS, sum of squares; MS, mean of squares; d.f., degrees of freedom; Den d.f., denominator degrees of freedom. Significant values are indicated by bolded *P*-values

Trait	Factors	SS	MS	Num d.f.	Den d.f.	<i>F</i> -value	<i>P</i> -value
Height	Treatment	2338.56	2338.56	1	69.46	17.33	0.000
	Provenance	1106.24	368.75	3	72.77	2.73	0.050
	Trt × provenance	747.35	249.12	3	67.16	1.85	0.147
Leaf number	Treatment	2.67	2.67	1	69.78	1.43	0.236
	Provenance	6.00	2.00	3	74.31	1.07	0.366
	Trt × provenance	12.24	4.08	3	68.41	2.19	0.097
$F_{\sqrt{F_m}}$	Treatment	0.00	0.00	1	66.82	0.09	0.762
	Provenance	0.00	0.00	3	27.21	1.05	0.386
	Trt × provenance	0.01	0.00	3	63.59	1.24	0.304
SLA	Treatment	141.08	141.08	1	54.79	0.75	0.390
	Provenance	1330.88	443.63	3	57.76	2.36	0.081
	Trt × provenance	631.60	210.53	3	51.57	1.12	0.349
Total biomass	Treatment	0.76	0.76	1	44.23	1.69	0.201
	Provenance	0.20	0.07	3	27.22	0.15	0.930
	Trt × provenance	2.08	0.69	3	40.55	1.54	0.220
ABG(log)	Treatment	1.51	1.51	1	51.55	2.48	0.122
	Provenance	0.16	0.05	3	27.16	0.09	0.966
	Trt × provenance	3.16	1.05	3	47.71	1.73	0.174
BG(log)	Treatment	0.19	0.19	1	38.64	0.51	0.477
	Provenance	0.37	0.12	3	27.27	0.33	0.801
	Trt × provenance	1.52	0.50	3	35.18	1.37	0.265

specifically modified by the presence of the OTCs. As soil moisture data were collected episodically every 3 months, we can only infer patterns of soil moisture in the intervening period between measurements but nearby weather station data for the relevant period when mortality occurred indicated that minimum temperatures were warmer than usual and precipitation was substantially lower than average (Bureau of Meteorology 2019).

Seedlings that were short, had few leaves or had low $F_{\sqrt{F_m}}$ in the period before the mortality sampling point had a greater chance of mortality. These smaller seedlings may not have established deep enough root systems to enable them to persist under drier and warmer conditions (Aragon-Gastelum *et al.* 2017). The higher mortality of *A. glacialis* under warmed conditions could reflect one driver of the predicted shift from tall-alpine herb communities to shrub-land. For example, Camac *et al.* (2017) demonstrated that shrubs suffered little mortality in general, and have much greater growth rates under OTC conditions.

A critical component of alpine plant establishment and persistence is freezing resistance. Frosts may occur within the Australian alpine area throughout the year, and not surprisingly many Australian alpine plants exhibit relatively high levels of freezing resistance year-round (Bannister 2007; Venn *et al.* 2013). Low-profile plants, such as *A. glacialis*, are not only exposed to high daytime leaf temperatures, due to the decoupling from ambient air temperature (Körner 2003), but also possibly cooler than ambient night-time temperatures as, particularly on calm days, long wave energy is dissipated from the surface (Jordan and Smith 1994). We found that *A. glacialis* exhibited

high levels of freezing resistance, with intracellular ice nucleation occurring at approximately -9°C , which is comparable to both *A. glacialis* adults in the field (Briceno 2014) and glasshouse grown seedlings (Briceno *et al.* 2014). Freezing resistance was not affected by our OTC treatment or provenance of origin. The seedlings were grown in the glasshouse for 2 years to ensure that maternal and transgenerational environmental effects were minimal before transplant and this may have led to loss of pre-existing provenance effects. The lack of an OTC effect is perhaps more surprising. We allowed a full year for the plants to recover from transplant shock and measured freezing resistance in late summer/early autumn before plants had a chance to acclimate to severe frosts. Thus, the measurements target differences in freezing resistance between treatments in plants before being completely cold hardened when we predicted that any differences would be most pronounced. The OTCs exposed our seedlings to significantly warmer days, but not significantly warmer nights. Perhaps the lack of treatment effects reflects the similarity of night-time low temperatures. In addition, the upright leaf angle (Körner and Hiltbrunner 2018), waxy leaves (Körner 2003; Wisniewski *et al.* 2014), and pinnatifid or finely dissected leaf structure of *A. glacialis* may contribute further to resistance to freezing under natural conditions (Hacker and Neuner 2008). Consequently, it seems unlikely that frost damage is a major risk for this species, either when the plants are cold-hardened or when aseasonal frosts occur and regardless of global warming.

In terms of the potential benefits of warmer temperatures, we predicted that seedlings that did establish would be larger

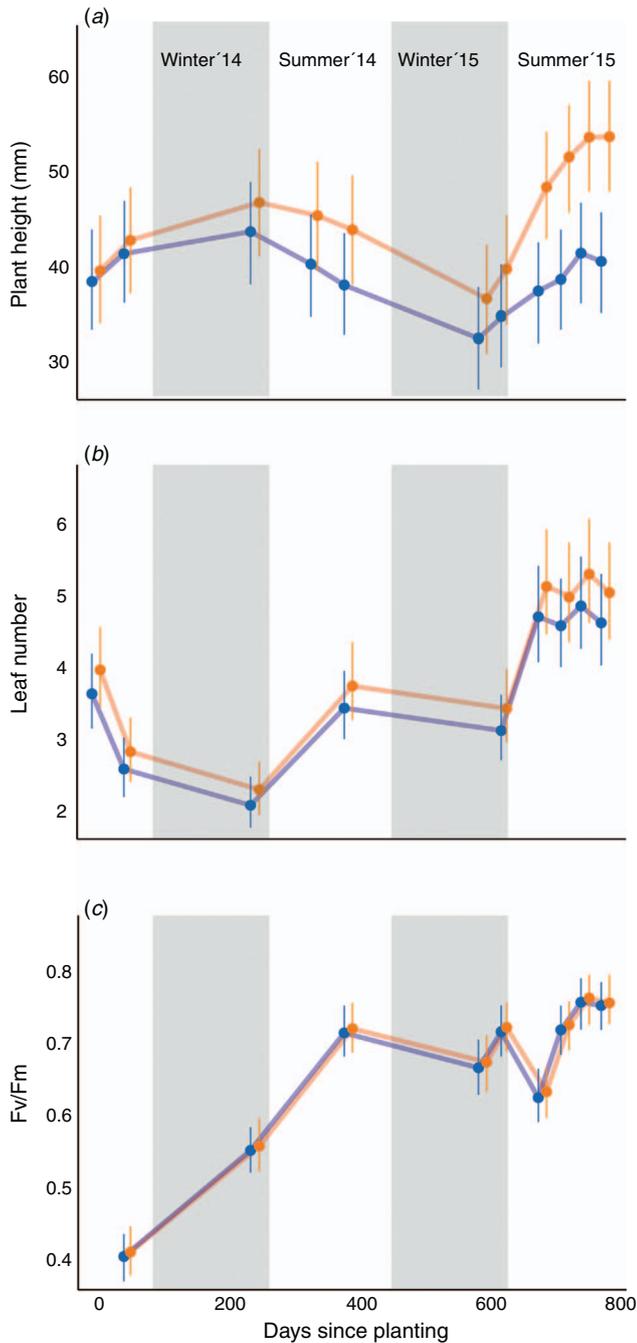


Fig. 4. *Aciphylla glacialis* plant traits observed during the growing season on 10 visits between March 2014 and April 2016 for all surviving seedlings, grown under either ambient (blue) conditions or warmed OTC conditions (orange). Means and error bars (CI) represent modelled values of (a) plant height (mm), (b) the number of expanded leaves and (c) photosynthetic efficiency (F_v/F_m). Points are slightly offset horizontally for clarity. Lines between means are to help visualise trends over time. Grey shading in the background represents the winter and summer seasons, respectively, for each year of the study.

and more vigorous as a result of either the direct impact of increased mean temperatures under the OTCs, or indirectly through the extension of the growing season (Galen and

Stanton 1993; Galen and Stanton 1995). Growth promotion has been found in other studies utilising OTCs (Kudo and Suzuki 2003; Kudernatsch *et al.* 2008; Camac *et al.* 2017), and similarly with the use of infrared lamps (Rustad *et al.* 2001) and underground heating; but see Wheeler *et al.* (2014). We observed modest increases in height, but not in other traits, though we certainly did not find any evidence of further detrimental effects of warming, such as might be expected given the higher mortality.

Prior studies indicate that survival, of both leaves and individuals, may not necessarily rely on an intrinsic capacity to withstand thermal events outright, but rather an ability to sacrifice, regenerate and/or repair (Körner 2003; Taschler and Neuner 2004). Our plants may have undergone significant replacement of leaves as our estimation of plant growth in *A. glacialis* did not account for leaf turnover. Therefore, assessing leaf longevity and turnover might have provided a more accurate measure of biomass investment (Venn *et al.* 2009). Furthermore, herbaceous species, such as *A. glacialis*, are known to display a greater lag time in response to simulated warming than graminoids or grasses (Kudernatsch *et al.* 2008). We conducted this study over 2 years, but the plants were still immature. We thus recognise, as many others have (e.g. Lindenmayer *et al.* 2012), that the establishment of even longer term monitoring of field based climate manipulation studies is required to fully assess the impacts of warming on long-lived organisms and their communities.

At the outset, we hypothesised that provenances from sites exposed to more frequent high and low temperature events (our lower elevation sites) would be more plastic in their trait responses, in line with previous studies on this species (Briceno 2014; Briceno *et al.* 2014), and others in the region (Bannister *et al.* 2005; Nicotra *et al.* 2015). However, we found no evidence of such intra-specific variation. Although this contrasts with recent literature for this species, our result is in line with results of several other studies conducted on alpine plants, which have shown similar degrees of freezing resistance in individuals growing along elevation, or snowmelt gradients (Bannister *et al.* 2005; Marquez *et al.* 2006; Sierra-Almeida *et al.* 2009; Wheeler *et al.* 2014). It is possible our capacity to detect these effects was reduced by high mortality, but the number of surviving seedlings was similar to those in other experiments that have demonstrated significant within species differentiation. Thus, we consider this null result is most likely biologically genuine, and therefore interesting for several reasons. There exists the potential for an enduring developmental effect to be present, whereby during the seed and early seedling developmental stages maternal provisioning may contribute to provenance differentiation (Bischoff and Müller-Schärer 2010). We aimed to remove growing plants under controlled conditions in early life. Therefore, the work on seedlings by Briceno (2014) may reflect parental imprint in early life stages, and at the adult stage perhaps reflects acclimation to local conditions (Briceno *et al.* 2014). Our study may likewise indicate that differences among provenance reflect acclimation more than local adaptation. The glasshouse growth period may have mitigated the provisioning influence as we intended, and subsequent acclimation to the variable field conditions may have

overwhelmed any lingering small differences that would indicate ecotypic differentiation among provenances. Such differences might be apparent under controlled conditions, but harder to detect against the noise of real field circumstances.

Understanding if, and when, intraspecific variation may play an important role in species establishment, growth, and survival is becoming increasingly relevant to conservation in view of climate change (Parmesan 2006). Modelling efforts that do not incorporate either intra-specific variation, or a relevant scale of micro-habitat variability, may overestimate species loss into the future (New *et al.* 2002; Nogués-Bravo *et al.* 2007; Randin *et al.* 2009; Moran *et al.* 2016) or underestimate it (Valladares *et al.* 2014). It is possible that micro-habitats of suitable scale and stability may act as refugia, promoting in-situ persistence and possibly the evolution of locally adapted ecotypes, particularly within topographically complex alpine regions, or where species have restricted dispersal (Armbruster *et al.* 2007; Scherrer and Körner 2010, 2011; Opedal *et al.* 2015; Lenoir *et al.* 2017); as is the case for many Australian alpine species (Morgan and Venn 2017). Defining microhabitat variability may, however, be challenging because it requires an understanding of the interplay between abiotic and biotic drivers over spatial and temporal scales as well as information about their relative contribution towards the development of the phenotype. Thus, the potential for within-species variation in establishment and growth traits should neither be assumed nor ignored, but must be considered under biologically realistic conditions.

Seedling establishment and growth is a crucial, but often understudied aspect of alpine plant ecology. We have demonstrated that warming elicited high levels of mortality indicating that *A. glacialis* seedlings might be susceptible to the negative impacts of climate warming. Continued monitoring revealed that despite the mortality, surviving seedlings showed high levels of resilience to the altered growing conditions, regardless of provenance. Our seedlings were sourced along the entire elevational range of the species within this area: from tree-line to summit and all provenances showed equivalent capacity to tolerate warming and associated frost events. Thus, the effects of climate warming are likely to vary throughout seedling development. Understanding how in-situ persistence will influence alpine plant distributions into the future will require a greater acknowledgement of not only the role of micro-habitats, but also the rate at which acclimation occurs.

Conflicts of interest

The authors declare no conflict of interest.

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