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Variation in snow cover drives differences in frost resistance in seedlings of the alpine herb *Aciphylla glacialis*

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ABSTRACT

Snow cover protects alpine plants from winter frost damage, keeping them under warmer and more stable temperatures than if there were no snow. Future climate scenarios predict less snow cover and earlier snow melt due to warming, causing paradoxically colder winters in a warmer climate. We compared intraspecific variation in cold tolerance between early snow melt (ESM) and late snow melt (LSM) populations of *Aciphylla glacialis*. Seedlings grown under common conditions were found to differ in cold tolerance consistent with their habitat of origin. ESM seedlings were more frost resistant and had a greater capacity to increase frost resistance in response to low temperatures than LSM seedlings. These results emphasise the relevance of microclimatic heterogeneity in driving physiological differences that might buffer some effects of climate change. Loss of snow cover could increase vulnerability of *A. glacialis* to lethal freezing in LSM sites whereas plants with greater frost tolerance in adjacent colder habitats (ESM sites) would be protected. Thus, intraspecific differentiation in tolerance of climatic stresses in combination with microclimatic refuges provided by topographic variation could enhance survival of some alpine species as climate warming progresses.

1. Introduction

Climate change is affecting ecosystems worldwide, and alpine environments are categorised as highly vulnerable ecosystems under climate change projections (Byars et al., 2007; Theurillat and Guisan, 2001). Indeed, the most vulnerable species to climate change are likely to occur in the cool to cold climates, at high latitudes and high elevations, where seasonal temperatures and the length of the frost-free period are important determinants of the growing season (Chen et al., 1995). *In situ* warming experiments show that warming advanced bud burst and flowering phenology, increased vegetative growth in early years of warming, especially in herbaceous plants, and later increased reproductive effort (Arft et al., 1999). However, an increase of growing season temperature is not the only factor to consider under future climate scenarios.

One of the most important climate changes factors for alpine areas that arises as a result of warming is the decrease in the proportion of precipitation falling as snow (IPCC, 2007) leading to a thinner and less insulating snow cover, which will develop later and melt earlier (Wipf et al., 2009). The low thermal conductivity of snow can keep plants and soil temperatures close to 0 °C even when air temperatures are well below 0 °C (Körner, 2003; Mondoni et al., 2012; Neuner et al., 1999). Hence, alpine evergreen species rely on snow cover to survive the alpine winter. Reduction of both snow depth and duration will expose plants to extreme low temperatures that they may not be able to tolerate. Warmer conditions in the mountains have already caused early loss of snow cover and cold de-acclimation (loss of tolerance to cold conditions due to exposure to warmer temperatures) causing a reduction in shoot and canopy growth, death of stems, and reductions in flowering and fruit production (Bokhorst et al., 2009; Inouye, 2008). Lack of adequate snow cover can also make highly freeze tolerant trees more vulnerable to low winter temperatures (Schaberg et al., 2008). *In situ* warming experiments using open top chambers (OTCs) that increased temperature by 2–3 K have also shown that warming decreased the ability of plants to tolerate frost (Sierra-Almeida and Cavieres, 2012). Furthermore, elevated CO_2 concentrations amplify

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vulnerability to freezing injury by increasing the temperature at which ice forms (Barker et al., 2005; Lutze et al., 1998) while delaying acclimation to freezing temperatures in autumn (Barker et al., 2005; Loveys et al., 2006) and accelerating de-acclimation in spring (Woldendorp et al., 2008). Therefore paradoxically, frost is emerging as a major driver of change in cold climate vegetation in response to warming (Ball et al., 2011; Cannell and Smith, 1986).

Ice formation can occur extra or intracellularly; the latter is always lethal but plants can tolerate extracellular ice formation and the resulting dehydration (Larcher, 2003; Sakai and Larcher, 1987). Plants can avoid or resist intracellular ice formation in different ways. For instance, plants avoid freezing when they are fully hydrated by increasing solute concentrations which causes a decrease in ice nucleation temperatures (supercooling or freezing point depression). Supercooling has been described as a mechanism that allows plants to stay ice-free and is common in species inhabiting areas where the intensity of freezing events is moderate or of short duration (Larcher, 2003; Sakai and Larcher, 1987). On the other hand, plants can avoid/resist freezing by dehydration, storing water in places where is safe to have ice or losing water to the environment, such as in mosses (Lenne et al., 2010). The extent of supercooling is usually quantified by measuring changes in nucleation temperatures using thermocouples (Burke et al., 1976) or by visualising ice formation using infrared thermography (Hacker and Neuner, 2008; Wisniewski and Fuller, 1999). On the other hand, extracellular freezing tolerance can be quantified by measuring the functioning of the photosynthetic apparatus after the induction of external ice formation, commonly measured as percentage of photoinactivation (%PhI) or low temperature damage, LT_{50} (Bannister et al., 2005; Larcher, 2000; Sierra-Almeida and Cavieres, 2012). Quantification of visual tissue damage as well as electrolyte leakage is also a way to measure the effect of extracellular ice formation. Cold acclimation, a period of exposure to low but non-freezing temperatures, usually enhances cold tolerance as shown by decrease in LT_{50} and NT (Bravo et al., 2001).

Freezing tolerance is closely related to the temperatures to which plants are exposed and varies with seasonal changes in temperature. Air temperature generally decreases with increasing elevation in alpine areas; hence plants from higher elevations tend to be more cold tolerant than plants from lower elevation (Sierra-Almeida et al., 2010; Squeo et al., 1996; Taschler and Neuner, 2004), but see (Sierra-Almeida et al., 2009).

The presence of snow, however, affects local thermal conditions. Lack of snow cover caused by early snow melt in low elevation sites exposes plants to more frequent and more severe frost events than plants growing in high elevation sites where snow persists for longer periods. For example, species inhabiting snowbanks are less frost tolerant than those in snow free areas (Bannister et al., 2005). This invites the question: does cold tolerance differ between populations of a single species co-occurring in early and late snow melt sites, and if so does acclimation state affect those differences? Though not widely researched, few studies have found differences in cold tolerance between populations of the same species living along elevation gradients (Loik and Redar, 2003; Melcher et al., 2000) or between ecotypes inhabiting contrasting habitats (Gianoli et al., 2004).

Here, we explored patterns of small scale variation in cold tolerance of a species of alpine herb, *Aciphylla glacialis*, which is distributed across alpine areas of early (ESM) and late snow melt (LSM) in Kosciuszko National Park (KNP), Australia. The snowpack duration has been shortened by 8.5 days in this area since 1954 (Sánchez-Bayo and Green, 2013). We grew seedlings under common conditions to determine whether seedlings from ESM habitats would be more freezing tolerant and have greater capacity to increase freezing tolerance in response to low temperatures than LSM progeny. By determining how snow duration generates intraspecific differences in cold tolerance, we can assess whether losses of upslope snow cover with a warming climate may adversely affect winter survival of species that rely on snow protection during winter.

2. Materials and methods

2.1. Species and study site

A. glacialis (Apiaceae) is a perennial dioecious herb with tough foliage 30–70 cm high and a tuberous root. Leaves are dark green, pinnately spreading and sharply pointed and crowded at the base of the plant. Inflorescences emerge from the base of the plant on stiff peduncles, and are comprised of a terminal compound white umbel bearing many lateral compound umbels. Leaf-like bracts are borne on the inflorescence (Costin et al., 2000). Individual plants are rarely seen in the field; instead large clumps of plants are common. Some of the leaves die while the plants are covered in snow in winter but a large proportion of the foliage remains intact while under snow. New leaves emerge when the snow melts in late spring, followed by flowers in early summer. Fruit maturation is rapid and seeds disperse by late summer. Seeds germinate under the snow after warm and cold stratification (Hoyle et al.unpublished data). In our study site at KNP, NSW, Australia, *A. glacialis* is distributed between an elevation of 1800–2228 m a.s.l, including the summit of Mt. Kosciuszko, the highest peak in Australia (2228 m a.s.l).

2.2. Environmental data

Air temperature data collected by the Bureau of Meteorology at Charlotte Pass (Kosciuszko Chalet; 36.43° S, 148.33° E, 1755 m a.s.l) and Thredbo (Thredbo AWS 36.49° S, 148.29° E, 1959 m a.s.l) (Bureau of Meteorology 2012, http://www.bom.gov.au) were used to describe patterns in air temperature at sites close to those of seed collection.

In order to describe the environments to which plants are exposed in ESM and LSM sites, leaf temperature was measured on four plants nearby ESM (36.27° S, 148.17° E, 1880 m a.s.l) and on four plants nearby LSM sites (36.45° S, 148.26° E 2228 m a.s.l) using Ibutton dataloggers (EDS, USA). Ibuttons were launched at the same date and time in both sites and attached to the plants under the leaf with surgical tape to avoid overheating by direct sunlight. Measurements were taken every 3 h for a period of one year (December 2011 till December 2012). Only the data for the coldest months were considered for this study (Mid-May–October 2012).

2.3. Seed collection, germination, and seedling growth and acclimation treatment

A. glacialis seeds were collected in the field at the point of natural dispersal from three plants at low elevation locations where early snow melt was observed (hereafter ESM sites, 1800, 1906 and 1913 m a.s.l) and from three plants at higher elevation locations where late snow melt was observed (hereafter late snow melt sites, 2192, 2202 and 2210 m a.s.l) in late summer 2010. Approximately 100 seeds were collected from each mother plant. In the laboratory seeds were sown on Petri dishes containing 1% water agar before being exposed to a cold stratification treatment (4 °C in the dark for 8 weeks) to alleviate physiological dormancy. Seeds were then exposed to 25/15 °C with a photoperiod of 12/12 h light/dark. After germination they were transferred to 68 mm square tubes with Native Planting Mix (Martins Fertilizer, Australia) and Yates Nutricote Grey pellets (16N 4.4P 8.3K) fertilizer. Seedlings were grown in glasshouse conditions (25 °C daily average temperature) for one year in order to accumulate enough biomass to perform the

experiment (on average six small leaves). After that period, twenty-four seedlings (12 from ESM, 12 from LSM sites, 4 from each of the 6 plants) were acclimated to each of two temperature conditions (warm, 20 °C day and 15 °C night and cold, 6 °C day and 4 °C night) in a growth chamber. Plants were arranged randomly in the two chambers. Light and photoperiod was kept the same for both chambers, 300 μmol of photons and 8 h photoperiod which corresponds to a late summer photoperiod in KNP. Plants were watered to saturation every day and maintained in these treatments for 60 days.

2.4. Sample collection

All measurements were conducted on mature, fully expanded seedling leaves. A leaflet of the pinnate leaf was cut and placed in wet floral foam in a cooler and transported to the laboratory. The leaf sample size was standardised (~3 mm × 10 mm) to avoid differences in nucleation temperatures due to sample area. In the laboratory, samples were placed in Petri dishes on wet filter paper to avoid desiccation. Samples were then transferred to a refrigerator (4 °C) where they were dark adapted for 30 min, after which time F_v/F_m (ratio of variable to maximum fluorescence, quantum efficiency of PSII) was measured to assess the effect of the temperature acclimation on the photosynthetic efficiency before exposure to low temperatures. Fluorescent measurements were done using an IMAGING PAM (Walz, Germany). Samples were transferred to 8 mL (10 mm internal diameter) Pyrex (Corning – Life Sciences, New York, NY, USA) test tubes and placed in a water bath to assess frost resistance.

2.5. Freezing treatments

We measured tolerance of extracellular ice formation (promoted by adding small pieces of ice in contact with the leaves) and resistance of intracellular ice formation (promoted by a fast cooling rate). Tolerance of extracellular ice nucleation was assessed by measuring the percentage of photoinactivation of photosystem II (%PhI), which is calculated using F_v/F_m before and after exposure to freezing temperatures at 15 and 60 days of acclimation. The temperature at which intracellular ice formed was measured using ice nucleation temperatures (NT) at 15 days of cold acclimation. Nucleation temperatures were used as a measured of resistance of intracellular ice formation.

2.5.1. Extracellular freezing test

Five samples per plant (total of 24 plants: 12 plants acclimated to cold and 12 plants acclimated to warm conditions, where 6 from ESM and 6 of them were from LSM sites) were suspended in the coolant bath (Julabo FP45 temperature-controlled water bath, Julabo Labortechnik, Seelbach, Germany) at 4 °C and temperature was reduced to −18 °C at 2 K h^{-1}, with plants held at −2 °C for 1.5 h after which ice was applied to each sample to simulate frost, promote extracellular ice nucleation and prevent supercooling (Webb and Steponkus, 1993). Cooling then resumed, and 24 leaf samples (6 samples per treatment) were removed at every target temperature (−2, −6, −10, −14, and −18 °C). After removal from the bath, samples were transferred to Petri dishes, exposed to 300 μmol PAR m^{-2} s^{-1} for 2 h at 4 °C to simulate thawing in light, as might occur on an alpine morning in the absence of snow. These conditions were deemed likely to demonstrate the damage of the photosynthetic apparatus. Leaf samples were subsequently dark adapted at 4 °C for 24 h at which time F_v/F_m after freezing was measured using an IMAGING-PAM (Walz, Germany). The same procedure was done after 60 days of acclimation to cold and warm conditions using the other 24 plants.

Photoinactivation of PSII at every target temperature (−2, −6, −10, −14, and −18 °C), was measured as the effect of external ice formation. This parameter was calculated as the percentage of photoinactivation (100*PhI), where PhI = $(1 - F_T/F_{max})$, F_T is the F_v/F_m of the sample exposed to a freezing temperature T and F_{max} is the maximum value of F_v/F_m for all samples, measured before freezing (Larcher, 2000).

2.5.2. Intracellular freezing test

The temperature at which intracellular ice is formed was recorded after 15 days of acclimation on 8 plants per treatment from each of LSM and ESM habitats of origin (n = 32 leaf samples). Preliminary analysis of the %PhI data indicated no difference in acclimation state between the 15 and 60 day acclimation treatments, so intracellular freezing was only measured at 15 days. Samples were enclosed in an insulated box connected to a water bath (Julabo Labortechnik, Seelbach, Germany), temperature was decreased from 20 °C till −18 °C at a fast rate of 6 °C h^{-1}, which ensures intracellular ice formation. Copper-constant thermocouples were attached to every leaf sample and the leaf temperature was recorded every second using a Datataker DT500 [Biolab (Aust) Pty Ltd. trading as a Datataker, Scoresby, Australia]. The rise in leaf temperature (exotherm) produced by the heat released during the intracellular freezing process was used to determine the NT which corresponds to the lowest temperature before the exotherm and indicates the onset of ice crystal formation (Larcher, 2003). Samples were removed at the end of the cooling profile, and F_v/F_m was measured.

2.6. Statistical analysis

Differences in daily minimum air temperatures between the two nearby weather stations (see above) were assessed by comparing the mean ± stdev of the two sites; no formal statistical test was done because there were only two weather stations. Differences in mean minimum leaf temperature between ESM and LSM plants were analysed using REML analysis with an auto-regressive structure to account for repeated measurements. The model included site as a fixed term and incorporated random terms for plant and day. Mean frequency of subzero leaf temperatures from mid-May–October 2012 between plants from ESM and LSM habitats of origin was analysed using a generalised linear model with a Poisson distribution and Chi square statistic as appropriate for count data. %PhI was analysed using REML analysis with consideration of effects at different strata and incorporating the nested structure of the design. Fixed terms were treatments (cold and warm), site (ESM, LSM), days of acclimation (15, 60), and target temperature (−2, −6, −10, −14, and −18 °C); the random model incorporated terms for mother plant nested in site, and replicate seedling nested within mother and site. Nucleation temperatures were also analysed using a REML analysis with site and treatment as fixed effects and mother nested in site as the random model. All statistical analyses were done in GenStat (version 14.1.0.593, VSN International Ltd. London).

3. Results

3.1. Environmental differences between early and late snow melt habitats of origin

Although minimum air temperature (mid May–October) between the weather station close to the LSM (Thredbo) and near the ESM habitats of origin (Charlotte Pass) was similar (−3.6 °C ± 0.2 and −2.9 °C ± 0.2 respectively) daily minimum leaf temperature of plants from the ESM was significantly lower than for LSM plants (−1.6 °C ± 0.1 and 0.8 °C ± 0.06 respectively, P = 0.031, Fig. 1a and

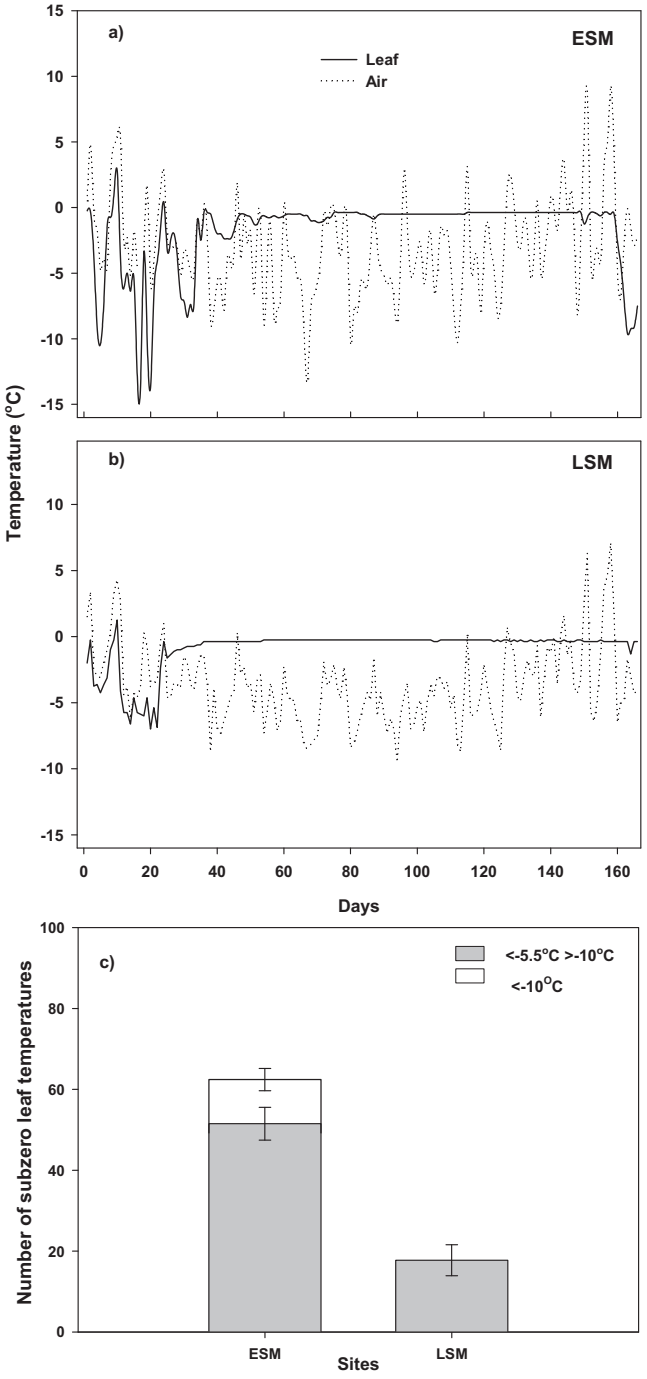

Fig. 1. Daily minimum leaf temperatures (solid lines) for plants living in (a) early snow melt (ESM) and (b) late snow melt habitats (LSM) near our seed collecting sites for the coldest months (mid-May–October) in Kosciuszko National Park (NSW, Australia) and daily minimum air temperature (dotted lines) of weather stations nearby ESM and LSM sites for the same period of time, (c) number of temperature events below −5.5 °C but higher than −10 °C (grey) and number of subzero events under −10 °C (white) measured on leaves of plants from ESM and LSM habitats, near seed collecting sites.

b). Furthermore, the lowest daily minimum leaf temperature registered on ESM plants was −15 °C, while it was only −8 °C in LSM plants. Differences in snow cover duration between these sites caused much greater frequency of subzero temperatures for ESM than LSM plants. ESM and LSM plants were exposed to 51 and 19 freezing events of <−5.5 < −10 °C magnitude respectively (Chi pr. <0.001). In addition, ESM plants were exposed to 13 subzero events under −10 °C while such low temperatures were never registered on LSM plants (Chi pr. <0.001, Fig. 1c).

3.2. Differences between seedlings from early and late snow melt sites

As expected, acclimation to cold temperatures significantly decreased photochemical efficiency. Mean value of F_v/F_m was 0.82 ± 0.008 and 0.75 ± 0.031 for warm and cold acclimated plants respectively ($P < 0.001$).

The effect of extracellular ice formation on the photosynthetic apparatus of ESM and LSM progeny was evaluated by assessing extent of photoinactivation (%Phi) caused by frost after 15 and 60 days of acclimation to warm or cold conditions. As temperature decreased, %PhI significantly increased as expected ($P < 0.001$, Fig. 2a). The effect of freezing on the photosynthetic apparatus did not differ depending on acclimation temperature (cold/warm), habitat of origin (ESM/LSM), or days of acclimation (Table 1). Seedlings exposed to −6 °C or higher showed minimal levels of photoinactivation, while seedlings exposed to −18 °C showed the highest mean photoinactivation but even at −18 °C (lower than the minimum temperature detected in the field), there was only 37.21% photoinactivation. In addition, samples taken out of freezing tolerance treatment were green with no evident tissue damage even at −18 °C. F_v/F_m values were also still high (0.8) and reduction of F_v/F_m was observed only after samples were exposed to light. The temperature at which 50% of potential damage caused by frost occurs (LT_{50}) was not possible to calculate because of the low levels of photoinactivation shown in this species. Perhaps testing temperatures lower than −18 °C, leaves would show damage, although temperatures below −18 °C do not occur in our study site, therefore we did not consider a further decreased on temperature in our freezing treatment.

The temperature at which intracellular ice nucleates in the leaf (NT) was measured in ESM and LSM progeny after 15 days of acclimation to cold and warm treatments. Nucleation temperature differed as a function of both origin (ESM/LSM) and acclimation treatment (cold/warm, Table 1). ESM progeny were able to supercool (decrease NT) and thus prevented intracellular ice formation to a greater extent than those from LSM origin. In addition, ESM progeny showed significant decreases in nucleation temperatures following cold acclimation (Fig. 2b), whereas LSM progeny showed no change in nucleation temperature with cold acclimation. Notably, intracellular ice nucleation occurred at much warmer temperatures than the temperature at which significant decrease of %PhI was observed: ∼−4 °C for seedlings from LSM provenances and ∼−6 °C or ∼−8 °C for warm and cold acclimated seedlings from ESM origin respectively. Furthermore, after measurements of nucleation temperatures (at the end of the cooling profile, −18 °C) samples showed marked evidence of tissue damaged (dark and mushy) and F_v/F_m values were very low (about 0.2), which indicates damage caused by intracellular ice formation.

4. Discussion

Our results demonstrate that small scale environmental variation, such as differences in duration of snow cover, can underlie fundamental physiological differentiation within a species over fine spatial scales. Our measurements of leaf temperature *in situ* showed, as expected, that snow decoupled leaf temperature from air temperature, causing contrasting thermal environments of plants in ESM and LSM sites. Furthermore, our results showed that *A. glaciallis* seedlings have a high level of tolerance to dehydration

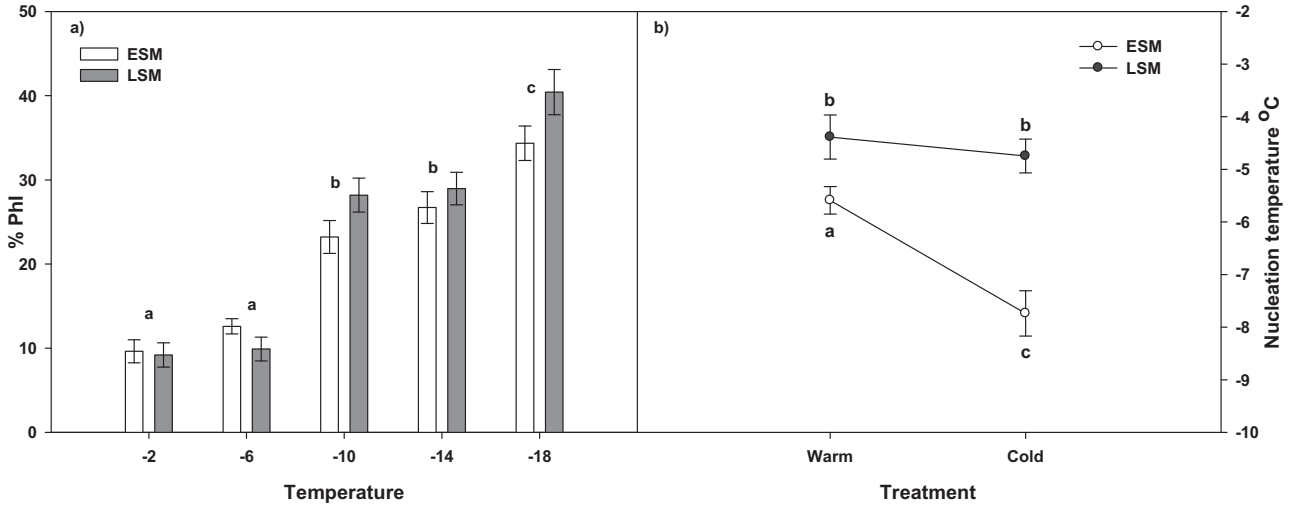

Fig. 2. (a) Percentage of photoinactivation (%PhI) for ESM (white bars) and LSM (grey bars) progeny calculated for each freezing temperature. (b) Nucleation temperatures for ESM and LSM progeny acclimated to warm and cold conditions. Different letters indicate significant differences between treatments.

Table 1
REML analysis for percentage of photoinactivation (%PhI) and nucleation temperature (NT). Analysis of %PhI included treatment (warm, cold), site (ESM, LSM), days of acclimation (15, 60) and target temperature (−2, −6, −10, −14, and −18 °C) as fixed terms. Mother plant (seed source) nested in site and replicate seedling nesting within mother and site were considered as random factor. Analysis of NT considered site and treatment as fixed effect and mother nested in site as random factor. Differences on %PhI were only significant between target temperatures. NT was significantly different between sites and treatments.

Fixed term	Wald statistic	n.d.f.	*F* statistic	d.d.f.	*F* pr
%PhI					
Treatment	0.02	1	0.02	8.1	0.900
Site	1.92	1	1.92	8.0	0.203
Days	2.16	1	2.16	32.4	0.152
Temperature	414.76	4	103.69	152.3	<0.001
Treatment site	0.54	1	0.54	8.1	0.483
Treatment days	0.05	1	0.05	32.6	0.820
Site days	0.42	1	0.42	32.5	0.521
Treatment temperature	6.34	4	1.59	152.8	0.181
Site temperature	9.54	4	2.38	152.5	0.054
Days temperature	7.43	4	1.86	152.8	0.121
Treatment site days	0.32	1	0.32	32.7	0.577
Treatment site temperature	1.76	4	0.44	153.1	0.780
Treatment days temperature	1.69	4	0.42	153.3	0.792
Site days temperature	7.66	4	1.92	152.9	0.111
NT					
Site	10.53	1	10.53	3.8	0.033
Treatment	17.07	1	17.07	23.9	<0.001
Site treatment	8.66	1	8.66	23.9	0.007

caused by extracellular ice formation (showed by low photoinactivation even at −18 °C). ESM and LSM progeny differed in frost resistance (avoidance of intracellular ice formation) and in their ability to acclimate to cold, when grown under common conditions. Strikingly, cold acclimation decreased NT in ESM but not LSM progeny. ESM sites are generally those at lower elevations, where maximum air temperature is warmer than at high elevations and thus lower frost resistance at lower elevation is often assumed. In contrast, we found that ESM progeny have a greater capacity to survive frost than LSM progeny, consistent with greater exposure of the ESM plants to lower temperatures. Here we describe the contrasting ESM and LSM environments, the functional differences between ESM and LSM progeny, the possible sources of these differences, and discuss implications of these results in the context of climate change.

4.1. Contrasting environmental conditions between ESM and LSM sites

As shown here, snow can create contrasting environmental conditions over small differences in elevation (~400 m). Although minimum air temperatures between ESM and LSM are not significantly different, plants living in LSM site were protected by snow for longer, and hence leaf temperatures remained higher than those of ESM plants (Fig. 1). Plants in ESM habitats deal with more intense and frequent freezing events than LSM plants. ESM plants were exposed to 13 temperature events below −10 °C, while such low temperatures were not recorded for LSM plants. These results are consistent with previous studies showing that sufficient snow cover thermally insulates plants from low air temperature extremes and reduces both the daily leaf temperature amplitude

and the number of freeze–thaw cycles (Neuner et al., 1999). It follows that such contrasting environmental conditions could drive fundamental and lasting differences in the progeny of ESM and LSM plants.

4.2. Differences between ESM and LSM progeny

When cellular dehydration by extracellular ice formation was induced by placing small ice crystals in contact with the leaves, the resulting response (%PhI) did not differ between ESM and LSM progeny; indeed the change in %PhI was very low for both (Fig. 2a). Interestingly, damage could not be observed either visually or through decreased F_v/F_m immediately after treatments, but developed over time. After 24 h at 4 °C in the dark followed by 30 min at 4 °C under a PAR of 300 μmol m^{-2} s^{-1}, the maximum %PhI was still only about ~40% and samples remained green with little visible damage. LT_{50} in seedlings of Andean species ranged from -7.3 ± 0.8 °C to -12.8 ± 0.5 °C (Sierra-Almeida and Cavieres, 2012), whereas *A. glaciallis* seedlings did not reach 50% photoinactivation of PSII even at −18 °C. Thus, freeze-induced dehydration is not a major risk for *A. glaciallis* seedlings, especially if freezing temperatures are followed by snow that might protect them from high irradiance.

In contrast, when intracellular ice nucleation was induced by rapid decrease in leaf temperature (6 °C h^{-1}), the damage was evident immediately and F_v/F_m decreased drastically (~75% decreased in F_v/F_m) after the freezing treatment. Furthermore, differences between ESM and LSM progeny were substantial. Intracellular ice formation, which is lethal for plants, occurred at ~−4 °C in LSM progeny and at ~−6 °C in ESM progeny acclimated to warm conditions. Hence, ESM progeny withstand lower temperatures without intracellular freezing than LSM seedlings, even in the absence of cold acclimation. This difference would be of biological significance in early autumn hen extreme frosts are common. In summer, freezing events of less than −4 °C are unlikely to occur at our study sites; thus both ESM and LSM progeny could be expected to resist summer freezing events.

When seedlings were acclimated to cold, as they would be in autumn and winter, ESM progeny had an even lower nucleation temperature than were warm-acclimated (~−8 °C). LSM progeny in contrast showed no change in ice nucleation temperature following cold acclimation. NT in seedlings of Andean species also ranged from -4.9 ± 0.5 to -8.4 ± 0.7 °C (Sierra-Almeida and Cavieres, 2012) although, within species differences in NT and supercooling in relation to habitats have not been reported previously. The capacity to increase supercooling by decreasing nucleation temperature in response to cold acclimation would allow ESM progeny to survive at lower temperatures than LSM progeny. We note that temperatures below −8 °C occurred often before snow fall and after snow melt at ESM sites (Fig. 1), suggesting that even the acclimated nucleation temperature in the ESM progeny would not be sufficient to prevent intracellular ice in ESM seedlings. It is possible that a longer and colder period of acclimation in the field leads to even lower nucleation temperatures than measured in the present study. In addition it is likely that temperatures below −8 °C would generally be associated with the formation of frost on the leaf surface, thereby facilitating dehydration and prevention of intracellular ice formation. In contrast, plants inhabiting LSM sites remain insulated by snow for longer periods and such a mechanism may not be needed. The duration of snow cover is influenced by slope, aspect and elevation and therefore can vary at small topographic scales along a narrow elevation range, but the pattern of snow duration is generally consistent between years. We suggest that this consistency in snow duration is sufficient to drive differentiation of *A. glaciallis* populations to local melting patterns and hence lasting differences can be found in the progeny of ESM and LSM *A. glaciallis* plants.

4.3. The sources of these differences

Interestingly, the fundamental differences described above occurred in seedlings germinated in the laboratory and grown under common garden conditions. Hence these seedlings never experienced the alpine environment and were not acclimated to these contrasting field environments. There are several potential sources for the observed differences between seedlings ESM and LSM habitats of origin. Local adaptation or genetic differentiation driven by the existence of microhabitat could be one explanation, as also demonstrated for *Poa hiemata* (Poaceae) populations on a narrow elevation gradient in the Australian Alps (Byars et al., 2007) and in *Festuca eskia* (Poacea) in the Pyrenees in spite of high gene flow between populations (Gonzalo-Turpin and Hazard, 2009). We think that ecotype or genetic differentiation in *A. glaciallis* might be reinforced by phenological differences between plants from in ESM and LSM sites. Plants in ESM sites flower earlier (mid-December) than LSM plants (mid-January, V.B.R. pers obs), this asynchrony in flowering time might restrict gene flow between these populations, hence generating ecotype/genotype differentiation even over short spatial scale. It is also possible that seeds developed from different environments in the field could have been epigenetically imprinted in response to different environmental cues at the time of seed development, or that seeds have been provisioned by the mother plant in ways that had lasting effects on their early growth and physiology. For instance, female flowering environment (especially temperature and day length) influence progeny frost hardiness and other adaptive traits in *Picea abies* (Johnsen et al., 1996, 2005a,b). In addition, maternal environments influence seed nitrogen and starch concentrations (Charest and Potvin, 1993), which has been associated with higher seedling cold tolerance later on (Biodner et al., 2007). Disentangling the various factors that underlie differences between LSM and ESM project would require genetic as well as freezing resistance tests on the progeny of several maternal lines from ESM and LSM sites.

4.4. Implications for small scale differences under rapidly changing climate

If plants from LSM sites are unable to adapt rapidly, loss of snow cover predicted by climate change could increase the frost vulnerability of plants that rely on the snow cover for longer periods. However, the existence of plants with a higher level of frost tolerance in adjacent habitats with less persistent snow cover would protect against freeze-induced local extinction as climate warming progresses. Previous studies have mentioned the relevance of microclimate refugia in the persistence of species under climate warming (Moritz and Agudo, 2013; Scherrer and Körner, 2010, 2011). Here we emphasise the potential importance of environmental heterogeneity in creating a population of *A. glacialis* with the physiological resources to persist in their current range. Thus, we conclude that habitat-dependent variation in frost tolerance, whether due to genetic, epigenetic or maternal effects, results in *A. glacialis* having a greater environmental response envelope than might be expected from a species occurring over such a narrow elevation range. Paradoxically, the breadth in capacity to cope with low temperature extremes may contribute to survival in a warming climate as reduction in snow cover exposes plants to more frequent and more severe frost.

5. Conclusion

The alpine environment can be viewed as a mosaic of habitats rather than a simple or linear thermal gradient from low to high elevation. This diversity of habitats may result in a diversity of ecotypes/genotypes that are often ignored in comparisons between species, but are relevant to maintaining species diversity under future climates. Projections of the responses of alpine plant species to future warming should consider this diversity of habitats in order to have a more realistic scenario for species persistence.

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