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Summer freezing resistance decreased in high-elevation plants exposed to experimental warming in the central Chilean Andes

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Abstract Alpine habitats have been proposed as particularly sensitive to climate change. Shorter snow cover could expose high-elevation plants to very low temperatures, increasing their risk of suffering damage by freezing, hence decreasing their population viability. In addition, a longer and warmer growing season could affect the hardening process on these species. Thus, understanding the ability of these species to withstand freezing events under warmer conditions is essential for predicting how alpine species may respond to future climate changes. Here we assessed the freezing resistance of 11 species from the central Chilean Andes by determining their low temperature damage (LT_{50}) and freezing point (FP) after experimental warming in the field. Plants were exposed during two growing seasons to a passive increase in the air temperature using open top chambers (OTCs). OTCs increased by ca. 3 K the mean air and soil daytime temperatures, but had smaller effects on freezing temperatures. Leaf temperature of the different species was on average 5.5 K warmer inside OTCs at midday. While LT_{50} of control plants ranged from −9.9 to −22.4, that of warmed plants ranged from −7.4 to −17.3°C. Overall, high-Andean species growing inside OTCs increased their LT_{50} ca. 4 K, indicating that warming decreased their ability to survive severe freezing events. Moreover, plants inside OTCs increased the FP ca. 2 K in some studied species, indicating that warming altered processes of ice crystal formation. Resistance of very low temperatures is a key feature of high-elevation species; our results suggest that current climate warming trends will seriously threaten the survival of high-elevation plants by decreasing their ability to withstand severe freezing events.

Keywords Alpine · Climate change · Freezing temperatures · Frost damage · Open top chambers

Introduction

The ability to resist freezing temperatures is an important determinant of a plant species' distribution (Woodward 1987), and it is the first environmental filter that species have to pass to inhabit high-elevation habitats (Körner 2003). Thus, plants inhabiting high-elevation environments are adapted to cope with the extreme low temperatures that characterize these habitats (Körner 2003). Studies dealing with the ability of high-elevation species to resist low temperature extremes have revealed that during summer many of these species can survive temperatures lower than −10°C, with some species being able to resist temperatures as low as −20°C (e.g. Sakai and Ötsuka 1970; Squeo et al. 1996; Bannister et al. 2005; Sierra-Almeida et al. 2009).

Based on the current climatic projections for the next 50 years (IPCC 2007), several authors suggested that the survival and reproduction of high-elevation plants will be seriously affected by the global increases in temperature (Björk and Molau 2007; Inouye 2008; Kudernatsch et al. 2008). On one hand, increases in the global temperature will lead to reductions in snow cover duration in high-elevation

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habitats (Körner 2000). Given that predicted increases in the average air temperatures are not expected to decrease the frequency and intensity of episodic freezing events (Inouye 2000, 2008), snow cover reductions will increase the exposure of plants to sudden freezing events during the beginning of the growing season (Menzel and Fabian 1999; Easterling et al. 2000; Cannone et al. 2007), which may cause severe damage by freezing in high-elevation plants (Neuner et al. 1999; Neuner and Taschler 2004). On the other hand, given that the ability to survive freezing temperatures (i.e. freezing resistance) is highly related to the ambient temperature that plants experience (Beck et al. 2004; Bannister et al. 2005), warmer daytime temperatures due to climate change may decrease the ability of high-elevation plants to resist freezing temperatures, making even the most freezing-resistant species more vulnerable to damage by freezing (Loveys et al. 2006; Woldendorp et al. 2008). However, little is known about the consequences of warming on the freezing resistance of high-elevation species. Recently, Klein et al. (2004) reported decreases in species richness during warming experiments in the Himalayan Plateau, and suggested that these could be related to decreases in the ability to resist freezing temperatures in those individuals growing under warmer conditions. Thus, it seems important to know the ability of high-elevation plants to withstand freezing conditions when they are exposed to warmer temperatures during the growing season.

A recent study reported that leaves of the high-elevation plants of the central Chilean Andes can resist freezing temperatures from −8.2 to −19.5°C during the growing season, where freezing tolerance was the most common freezing-resistance mechanism (Sierra-Almeida et al. 2009). Climate projections for this area predict increases of 4–5°C on the mean temperature during the growing season (CONAMA 2006). Thus, if warmer temperatures during the growing season affect the ability to withstand low temperature, then high-elevation plant species in this area will decrease their ability to resist freezing events.

In this study we experimentally assessed the effect of warmer temperatures during the growing season on the freezing resistance of 11 high-elevation species from the central Chilean Andes. Particularly, we assessed whether leaves of plants exposed to experimental warming during two consecutive growing seasons were less freezing resistant than those of non-exposed plants.

Materials and methods

Study site

This study was carried out near the locality of Farellones, in the central Chilean Andes, 50 km east of Santiago. A total of 11 species were studied at two elevations. The low-elevation site was located at 2,900 m, on a northwest-facing slope, near La Parva ski resort (33°21′S, 70°19′W). This area has a plant cover of <50% and is characterized by the dominance of the cushion plant *Laretia acaulis* Gillies & Hook. (Apiaceae) and the presence of dwarf shrubs, annuals and perennial herbs (Cavieres et al. 2000). At this elevation, the growing season usually starts with the snowmelt in October and finishes in April with the first snowfalls. The high-elevation site was located at 3,600 m, on a northeast-facing slope (33°19′S, 70°15′W), near Valle Nevado ski resort. This site is characterized by a low plant cover (<10%) and the scarce vegetation is dominated by the cushion plant *Azorella madreporica* Clos (Apiaceae), with the presence of some rosette-forming and prostrate perennial herbs (Cavieres et al. 2000). The growing season at this high-elevation site usually starts in December and finishes in March.

Target species

At the low-elevation site, eight plant species were taken among the most abundant species within the study area (Table 1). The selected species were three native dwarf shrubs, *Haplopappus anthylloides* Meyen & Walp (Asteraceae), *Nassauvia looseri* Cabrera (Asteraceae), and *Senecio polygaloides* Phil. (Asteraceae), the cushion plant *Laretia acaulis*, and four perennial herbs, the natives *Euphorbia collina* Phil. (Euphorbiaceae), *Perezia carthamoides* Hook & Arn. (Asteraceae), *Phacelia secunda* J.F. Gmel (Hydrophyllaceae) and the non-native *Taraxacum officinale* (L.) Weber. *T. officinale* (dandelion) is native to Europe, but now it is naturalized along the central Chilean Andes (Matthei 1995). At the high-elevation site, the cushion plant *Azorella madreporica* and four abundant perennial herbs were selected: *Hordeum comosum* (J. Presl) Löve (Poaceae), *Phacelia secunda*, *Pozoa coriacea* Lag. (Apiaceae) and *T. officinale* (Table 1).

Experimental warming

Soon after snowmelt of 2007 (16 November and 17 December 2007 for low- and high-elevation sites, respectively), ten mature individuals per target species were randomly selected in both elevation sites. For each species, five individuals were randomly assigned to a warming treatment. For this, on each of these individuals we placed a hexagonal chamber of transparent Plexiglass®, with walls of 50 cm height and 120 cm in diameter and open at the top. Open top chamber (OTC) walls were punched with 25 holes of 1.5 cm diameter each to allow some wind to pass through and hence avoid an excessive increase in the air temperature (see Molina-Montenegro et al. 2009 for further

Table 1 Morphological attributes of the species considered for summer freezing resistance determinations

Species[a]	Family	Growth form	Foliage height (cm)	Leaf area (cm^2)
Low-elevation site				
Euphorbia collina	Euphorbiaceae	Forb	10.2 ± 0.4	1.47 ± 0.33
Haplopappus anthylloides	Asteraceae	Dwarf shrub	5.4 ± 0.2	0.99 ± 0.16
Laretia acaulis	Apiaceae	Cushion	4.5 ± 0.6	3.75 ± 0.55
Nassauvia looseri	Asteraceae	Dwarf shrub	9.6 ± 0.1	0.40 ± 0.11
Perezia carthamoides	Asteraceae	Rosette	3.8 ± 0.3	3.53 ± 0.76
Phacelia secunda	Hydrophyllaceae	Forb	9.1 ± 0.8	2.84 ± 0.32
Senecio polygaloides	Asteraceae	Dwarf shrub	8.3 ± 1.2	0.37 ± 0.06
Taraxacum officinale	Asteraceae	Rosette	3.9 ± 0.2	3.46 ± 0.46
High-elevation site				
Azorella madreporica	Apiaceae	Cushion	3.2 ± 0.3	0.28 ± 0.08
Hordeum comosum	Poaceae	Grass	10 ± 1.2	0.97 ± 0.14
Pozoa coriacea	Apiaceae	Rosette	4.3 ± 0.1	2.01 ± 0.29
Phacelia secunda	Hydrophyllaceae	Forb	6.2 ± 0.4	0.31 ± 0.04
T. officinale	Asteraceae	Rosette	3.6 ± 0.1	2.60 ± 0.94

[a] Species nomenclature follows Marticorena and Quezada (1985)

details). These OTCs are passive warming systems that have been widely used in warming experiments in alpine and arctic tundras (Henry and Molau 1997; Marion et al. 1997). At each site 14 OTCs were positioned because in some cases a single OTC enclosed more than one individual but of a different target species. The other five individuals per species grew under natural conditions, and were at least 2 m distant from the nearest OTC. Plants were exposed to experimental warmer conditions during two entire growing seasons (first growing season from 16 November 2007 to 1 April 2008 and from 17 December 2007 to 29 March 2008 at low- and high-elevation sites, respectively; second growing season from 20 November 2008 to 28 March 2009 and from 31 December 2008 to 28 March 2009 at low- and high-elevation sites, respectively). Plant samples for freezing resistance determinations (see below) were collected near the end of the second growing season.

Microclimatic measurements

To characterize the microclimatic changes produced by the OTCs, air and soil temperatures inside and outside them were measured at the two elevations (Table 2). Soil temperatures were monitored using temperature sensors connected to a mini-logger (HOBO-H8; Onset, Bourne, Mass.). Soil sensors were placed at 5 cm depth from the soil surface, both inside and outside OTCs ($n = 5$). Air temperature was recorded with mini-loggers HOBO mini-loggers (Onset) that were placed at 15 cm above the soil surface, both inside and outside OTCs ($n = 2$), and protected with plastic shelters from direct exposure to the sun. Air sensors were place at this distance from the soil surface because no differences in thermal conditions around the plants have been found at distances ≤ 15 cm (Sierra-Almeida et al., unpublished results). All plant species were <15 cm height (Table 1); therefore, air sensors were able to capture thermal conditions for all studied species, regardless plant height differences among rosettes, dwarf shrubs and cushion plants. The mini-loggers were programmed to record the temperature every hour during each growing season.

Soil moisture was also measured inside and outside OTCs at each elevation. For this, a psychrometer (PST-55; Wescor, Utah) was buried at 20 cm depth (below soil surface) at the centre of each of six randomly selected OTCs and other six points randomly located on the vegetation without OTCs. Soil water potential (MPa) was monthly monitored using a data-logger (Psypro; Wescor), and data for plant collection date (28 March 2009) are shown in Table 2.

Leaf temperature

At each elevation, for each species we measured their leaf temperature within and outside OTCs. At each elevation, for each target species we randomly selected a single leaf in those individuals growing inside OTCs and its temperature was recorded with an IR thermometer (Extech Instrument, Waltham, Mass.). Simultaneously, the temperature of air surrounding the plant foliage was measured with a digital thermometer (Omega Engineering, Stamford, Conn.). This same procedure was carried out on individuals of each species growing under natural conditions outside

Table 2 Microclimatic conditions measured during the second growing season of the experimental warming in the central Chilean Andes

	Elevation			
	2,900 m		3,600 m	
	Control	Warming	Control	Warming
Length of the growing season (days)	128		87	
Mean air temperature (°C)	10.0 ± 1.2 a	13.2 ± 1.2 b	7.0 ± 1.6 a	10.8 ± 1.7 b
Minimum air temperature (°C)	2.7 ± 0.3 a	2.6 ± 0.3 a	−1.3 ± 0.4 a	−3.1 ± 0.4 b
Maximum air temperature (°C)	20.5 ± 0.6 a	30.6 ± 0.9 b	17.8 ± 0.6 a	30.8 ± 0.9 b
Daily temperature range (°C)	17.8 ± 0.5 a	28.0 ± 0.8 b	19.2 ± 0.7 a	33.9 ± 1.0 b
Intensity of freezing events (°C)	−0.6 ± 0.2 a	−0.6 ± 0.3 a	−2.2 ± 0.3 a	−3.3 ± 0.4 b
Number of freezing events	10 a	12 a	61 a	82 b
Duration of freezing events (h)	3.0 ± 1.0 a	2.7 ± 1.2 a	5.9 ± 0.6 a	6.5 ± 0.5 a
Mean soil temperature (°C)	16.5 ± 0.3 a	18.6 ± 0.3 b	12.0 ± 0.3 a	16.7 ± 0.4 b
Minimum soil temperature (°C)	6.3 ± 0.3 a	9.1 ± 0.3 b	2.2 ± 0.3 a	6.3 ± 0.5 b
Soil Ψ_{H_2O} in March (MPa)	−2.2 ± 0.3 a	−3.5 ± 0.3 b	−0.7 ± 0.3 a	−0.3 ± 0.1 a

Data were measured from 20 November 2008 to 28 March 2009 at the low- and from 31 December 2008 to 28 March 2009 at the high-elevation site. Values correspond to mean ± 2SE. Different *lower-case letters* indicate significant differences ($P < 0.05$)

OTCs. A leaf temperature record taken inside OTCs was followed by a record taken outside them, and the entire round of records took less than 30 min. Leaf temperatures were recorded on 28 December and 29 December 2009 at high and low elevation, respectively. Measurements were done at 2 times during the day: predawn (7 a.m.) and midday (1 p.m.). These times of the day represent the moments where the lowest and highest air temperatures are recorded in the field.

Plant material collection

At each elevation, for each species we collected plant material from the different individuals growing inside and outside the OTCs. Plant samples corresponded to small twigs with mature leaves for dwarf shrubs and cushion plants, and modules with at least two mature leaves or complete individuals for herbaceous plants. Immediately after collection, plant samples were placed into a cooler to avoid changes in tissue water status, and then transported to a field laboratory less than 10 min away by car.

Thermal analyses

For each species, and for each microhabitat (i.e. inside or outside OTCs) five expanded mature leaves were removed from the different plant samples taken in the field and each leaf was attached to a thermocouple (Gauge 30 copper-constantan thermocouples; Cole Palmer Instruments, Vernon Hills, Ill.), and immediately enclosed in a small, tightly closed cryotube. The cryotubes were placed in a cryostat (MGW LAUDA RC 20; Lauda-Königshofen, Germany), and the temperature was decreased from 0 to −18°C at a cooling rate of 2°C h^{-1}. The individual temperature of leaves was monitored every second with a Personal Daq/56 multi-channel thermocouple USB data acquisition module (IOtech, Cleveland, Ohio). The sudden rise in leaf temperature (exotherm) produced by the heat released during the extracellular freezing process was used to determine the freezing point (FP), which corresponds to the highest point of the exotherm, indicating freezing of water in the apoplast, including symplastic water driven outwards by the water potential difference caused by the apoplastic ice formation (Larcher 2003).

Low temperature damage

For each species, and for each microhabitat, five samples were introduced into separate, hermetically sealed plastic bags, and incubated in a previously cooled cryostat. The cryostat was programmed separately at six freezing temperatures: −6, −9, −12, −15, −18 and −22°C. Samples were kept at each temperature for 2 h to ensure homogeneous cooling. Then, the plastic bags were removed from the cryostat and left at 4°C in the dark for 24 h. The control treatment consisted of samples placed in plastic bags and directly kept at 4°C in the dark for 24 h (unfrozen samples). As visual damage was not immediately obvious for all species, leaf damage was assessed after thawing using a chlorophyll fluorimeter (Plant Efficiency Analyzer, Hansatech, Germany; Neuner and Buchner 1999) to determine the ratio of variable to maximum fluorescence (F_v/F_m) of dark-adapted photosynthetic organs of each sample (Maxwell and Johnson 2000). As dead material effectively had a F_v/F_m of zero, damage was calculated as percentage of photoinactivation (100 × PhI), where PhI is the photoinactivation ratio described by Larcher (2000): PhI = $(1-F_{fT}/F_{max})$, where F_{fT} 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 of each tested species. The temperature producing 50% damage (LT_{50}) was determined by linear interpolation using the temperature causing the highest PhI of <50% and the temperature causing the lowest PhI of >50% (Bannister et al. 1995, 2005).

Statistical analyses

Microclimatic conditions (i.e. mean, maximum and minimum temperatures and soil moisture) inside and outside OTC were compared with t tests, while the frequency of freezing events (air temperature below 0°C) was compared with a χ^2 test. For each species, the effect of warming on leaf temperature, FP and LT_{50} was assessed using $t$tests. Data were log transformed before statistical analyses when assumptions of normality and homoscedasticity were not met (Dytham 2003).

Results

Microclimatic conditions

At both elevations OTCs increased mean temperatures of air and soil by ca. 3 K (Table 2). At the lower elevation, there were no differences between inside and outside OTCs in the minimum air temperature recorded ($t = 0.493$, $P = 0.622$). In addition, the intensity, frequency and duration of the freezing events were similar inside and outside OTCs ($P > 0.05$; Table 2). In contrast, at the higher elevation, the minimum air temperature was 1.8 K lower inside OTCs ($t = 5.87$, $P < 0.0001$) and the OTCs affected the freezing events (Table 2). For example, freezing events were 1.1 K lower ($t = 3.71$, $P < 0.0002$) and 24% more frequent ($\chi^2 = 14.97$, $P < 0.001$) inside than outside OTCs, although their duration did not differ ($t = -1.62$, $P = 0.106$). Regarding soil temperature, at the lower site OTCs increased the minimum soil temperature by 2.7 K ($t = -9.63$, $P < 0.0001$; Table 2) and no freezing temperatures were recorded at the soil level. At the higher elevation site minimum soil temperature increased 4.1 K inside OTCs ($t = -8.35$, $P < 0.0001$).

Whilst at the lower elevation warming affected soil moisture by decreasing soil Ψ_{H_2O} by 1.3 MPa ($t = 5.65$, $P = 0.0002$; Table 2), the higher elevation there were no differences in soil moisture between inside and outside OTCs ($t = 2.21$, $P = 0.052$; Table 2).

Overall, at both elevations the leaf temperature of the different species was higher inside OTCs than outside them (Table 3). At the low-elevation site, the average leaf temperature of the different species was 1.5 and 4.5 K higher in plants growing inside OTCs, at predawn ($t = -10.32$, $P < 0.0001$) and midday ($t = -9.86$, $P < 0.0001$), respectively. At the high elevation, leaf temperatures were ca. 6 K higher in plants growing inside OTCs at midday ($t = -6.19$, $P < 0.0001$), but no differences were detected at predawn ($t = -1.49$, $P = 0.134$; Table 3).

Freezing resistance

The summer freezing resistance of the majority of the studied species was negatively affected by warming (Figs. 1, 2). At the low-elevation site, mean LT_{50} of plants growing inside OTCs was 4.4 K higher than that of plants growing under natural conditions ($t = -3.71$, $P = 0.001$). For example, *Laretia acaulis*, *Nassauvia looseri* and *Phacelia secunda* decreased their freezing resistance in ca. 3.5 K, while in *Haplopappus anthylloides* this decreased was ca. 9 K. Likewise, at the high-elevation site, plants inside OTCs showed a mean LT_{50} 3.3 K higher than control plants ($t = -4.53$, $P < 0.001$). For example, *Azorella madreporica* and *Hordeum comosum* decreased their freezing resistance in 2 and 3.4 K, respectively, while in *P. secunda* this decreased more than 4.6 K. In other words, at both elevations plants growing under warmer conditions were damaged at higher freezing temperatures (i.e. less negative temperatures) than plants growing under natural conditions.

Warming also affected the FP of some of the studied species (Figs. 1, 2). At low elevation, the dwarf shrub species *H.* anthylloides, *Nassauvia looseri* and *Senecio polygaloides,* and the herbaceous species *Euphorbia collina* showed a mean FP 1.6 K higher in plants growing inside OTC than under natural conditions ($t = -5.13$, $P < 0.0001$; Fig. 1). This indicates that the temperature at which ice crystals were formed in the apoplast was higher (less negative) in plants growing under warmer conditions (inside OTC). At the high-elevation site, the herbaceous species *Phacelia secunda* and *Pozoa coriacea* showed FP 2.2 K higher in plants growing inside OTCs than those of plants growing under natural conditions ($t = -7.28$, $P < 0.0001$; Fig. 2).

Discussion

In both elevations, the studied plant species growing within the OTCs were exposed to ca. 3 K higher temperature than non-exposed plants. This increase in temperature is in agreement with current predictions on future increases in

Table 3 Leaf temperature (°C) measured in plants under natural and warming conditions during 28 December and 29 December 2009 at high and low elevation, respectively

Species	Leaf temperature (°C)			
	Predawn		Midday	
	Control	Warming	Control	Warming
Low-elevation site				
E. collina	−0.7 ± 0.5 a	0.2 ± 0.7 b	7.4 ± 1.3 a	13.2 ± 1.3 b
Haplopappus anthylloides	−0.8 ± 0.4 a	0.4 ± 0.5 b	12.9 ± 0.8 a	15.0 ± 1.1 b
L. acaulis	−2.4 ± 0.1 a	−0.4 ± 0.1 b	10.6 ± 1.2 a	17.3 ± 1.6 b
N. looseri	−0.9 ± 0.4 a	0.7 ± 0.2 b	11.8 ± 0.9 a	19.0 ± 2.1 b
Perezia carthamoides	−0.7 ± 0.3 a	0.7 ± 0.5 b	13.5 ± 0.8 a	15.5 ± 0.6 b
Phacelia secunda	−1.3 ± 0.4 a	0.1 ± 0.5 b	9.3 ± 1.2 a	14.0 ± 0.9 b
S. polygaloides	−0.9 ± 0.6 a	1.2 ± 0.2 b	10.4 ± 0.9 a	15.6 ± 1.3 b
T. officinale	−0.7 ± 0.5 a	0.7 ± 0.4 b	12.2 ± 1.5 a	15.5 ± 1.2 b
Air temperature (°C)	0.2 ± 0.1 a	0.6 ± 0.1 b	10.2 ± 0.5 a	13.7 ± 0.8 b
High-elevation site				
A. madreporica	−0.1 ± 0.4 a	0.1 ± 0.3 a	19.3 ± 0.7 a	24.1 ± 0.8 b
Hordeum comosum	1.6 ± 0.2 a	2.1 ± 0.9 a	18.5 ± 1.7 a	29.2 ± 1.9 b
Phacelia secunda	0.3 ± 0.9 a	1.0 ± 1.1 a	19.1 ± 1.5 a	24.1 ± 1.3 b
T. officinale	2.6 ± 0.3 a	2.9 ± 0.4 a	26.7 ± 1.2 a	32.4 ± 1.2 b
Air temperature (°C)	1.8 ± 0.2 a	2.3 ± 0.1 b	9.1 ± 0.2 a	13.6 ± 1.0 b

Air temperature (°C) was measured simultaneously with leaf temperatures. Values correspond to mean ± 2SE. Different *lower-case letters* indicate significant differences ($P < 0.05$)

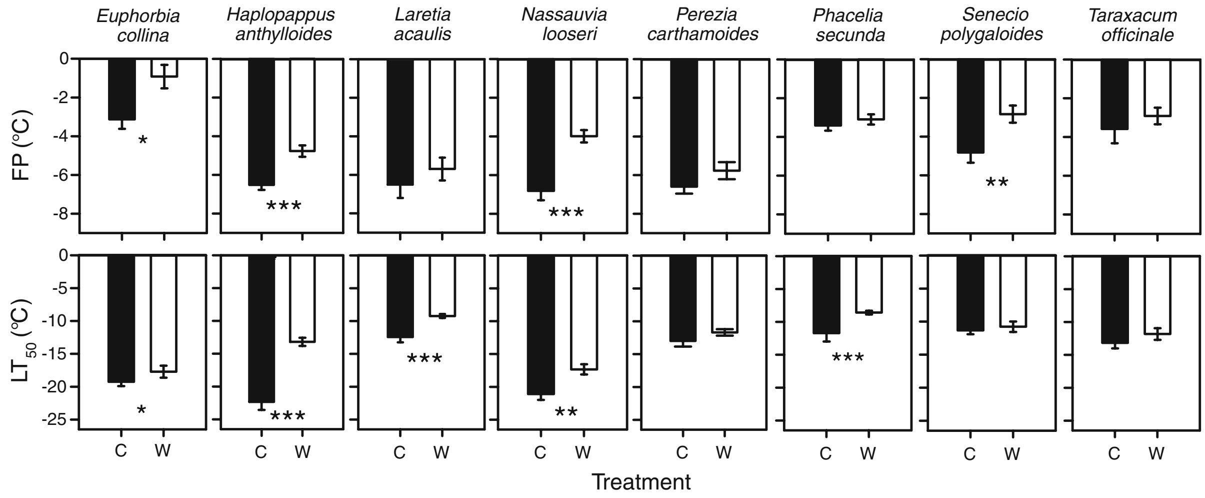

Fig. 1 Summer freezing resistance of high-elevation species from central Chilean Andes, measured at 2,900 m elevation. Data for eight species are shown as mean ± 2SE, $n = 5$. *Top panels* correspond to freezing point (*FP*; °C), and *bottom panels* correspond to the temperature producing 50% damage (LT_{50}; °C). *C* Unwarmed plants, *W* warmed plants. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (based on *t* tests)

the mean temperature for the entire world (IPCC 2007) and for the Andean region of central Chile in particular (CONAMA 2006). These warmer air conditions inside OTCs generated higher leaf temperatures compared to outside them, particularly at midday where the highest irradiance is received. Nonetheless, our warming treatment had small effects on freezing temperatures. For instance, whilst the frequency, intensity and duration of freezing events at the lower elevation did not differ between microhabitats (i.e. inside vs. outside OTCs), at the higher elevation, OTCs increased by 24% the frequency, and by 1.1 K the intensity of freezing events, but not their duration

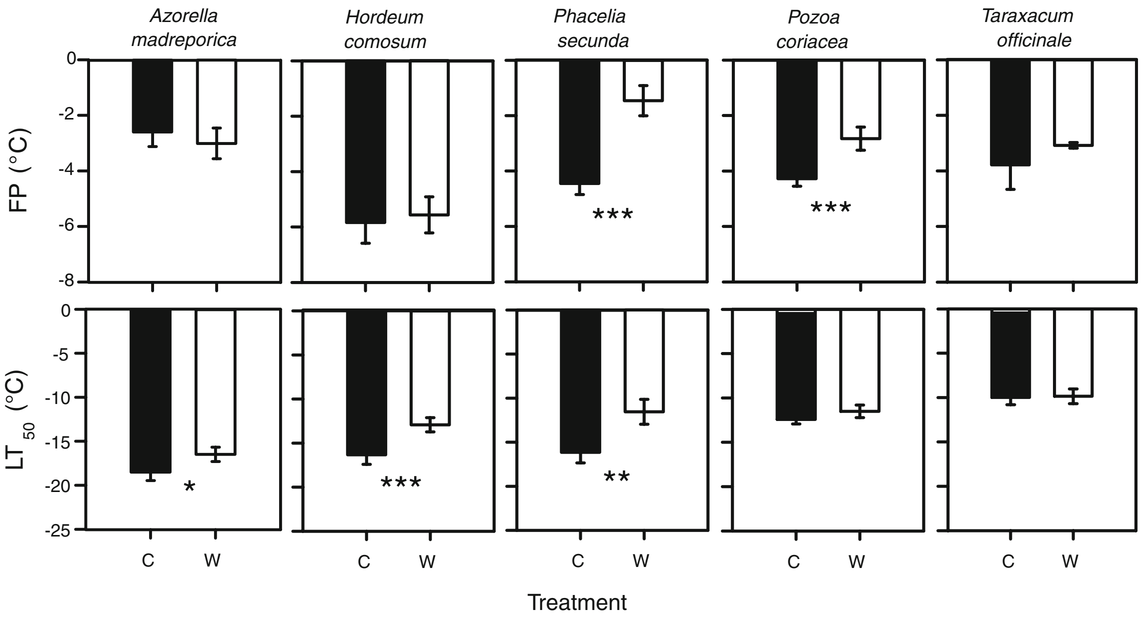

Fig. 2 Summer freezing resistance of high-elevation species from central Chilean Andes, measured at 3,600 m elevation. Data for five species are shown as mean ± 2SE, $n = 5$. *Top panels* correspond to FP(°C), and *bottom panels* correspond to LT_{50} (°C). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (based on *t* tests). For abbreviations, see Fig. 1

(Table 2). Slightly lower freezing temperatures inside OTCs have also been observed at other arctic and alpine sites (Marion et al. 1997; Danby and Hik 2007), a phenomenon likely related to the fact that the OTCs cause a layer of still air within the chambers, allowing greater infrared radiation to a cold nighttime sky.

The warmer temperatures experienced by plants growing inside the OTCs during two consecutive growing seasons decreased the extreme low temperature that the studied species can resist. At the lower elevation, warming increased leaf temperature by 1.5 and 4.6 K at predawn and midday, respectively, and LT_{50} by 4.4 K. At the higher elevation, warming increased leaf temperature by 6.5 K at midday, while it did not affect leaf temperatures at predawn. LT_{50} increased by 3.3 K in warmed plants. Although we have leaf temperature data for only a single day at two different hours, they are representative of most of the growing season conditions, particularly for the low-elevation site. For the high-elevation site, the measurements were made during clear-sky conditions which are less frequent compared to the low site. Data taken during overcast conditions at the higher site indicated an average increase of ca. 2 K for the leaf temperatures inside OTCs (data not shown). Thus, these results suggest that the magnitude of the decrease in the ability to resist freezing temperatures is related to the magnitude of the increase in both air and leaf temperature.

High-Andean species are among the high-elevation species with the highest summer freezing resistance reported so far (Bannister et al. 2005; Sierra-Almeida et al. 2009). However, the negative effect of warming on summer freezing resistance suggests that even these highly resistant species might suffer severe damage by freezing if future climate conditions are characterized by warmer mean temperatures during the growing season, but with no change in the intensity of extreme low temperatures (Inouye 2000, 2008). Given that there were no significant effects of OTCs on the intensity, frequency and duration of freezing events, the negative effects of the experimental warming on the summer freezing resistance of high-Andean species seems to be related to the overall warmer conditions experienced within OTC during daytimes. Ambient temperature itself is an important cue that regulates the ability of plants to survive freezing events (Sakai and Larcher 1987; Körner 2003). Some studies have reported that warm temperatures modified processes of cold hardening and dehardening in the plants (see Bertrand and Castonguay 2001 for review). For instance, needles of *Pinus sylvestris* exposed to warm temperatures decreased by ca. 10 K their cold hardiness during the spring, triggering the burst of buds about 20 days before that of unwarmed buds (Repo et al. 1996). In addition, Loveys et al. (2006) found that the exposure of *Eucalyptus pauciflora* seedlings to warm temperatures delayed the start of cold hardening by 2–3 weeks, increasing the vulnerability of seedlings to damage by freezing temperatures. Similar results have been also reported by some studies where elevated CO_2 affected the freezing resistance of plants through indirect effects on leaf temperature (Lutze et al. 1998; Beeling et al. 2001; Barker et al. 2005). In our case, the warmer air conditions inside the OTCs generated warmer leaf temperatures which could affect the normal start of cold hardening processes on the studied species.

Most of the studies assessing the impacts of global warming on high-elevation plant species have focused on vegetative growth and reproductive success (see Arft et al. 1999 for review), with negative or positive impacts of warming depending on the time frame of the study (Arft et al. 1999; Hollister et al. 2005; Kudernatsch et al. 2008). Direct assessments of the effects of warming on the ability to survive extreme temperature events are scarce. For instance, Marchand et al. (2006) observed a clear decay of plant performance in four Arctic plant species after their exposure to consecutive heat waves. These authors suggested that exposure to warmer conditions decreased cold acclimation in plants, resulting in damage after the exposure to the low temperatures that characterize arctic habitats. Loik et al. (2004) working in the subalpine zone of the Rocky Mountains in Colorado, found that warming increased the photosynthetic freezing tolerance of the shrub *Artemisia tridentata*, but did not have effects on the herbaceous *Erythronium grandiflorum*. As far as we are aware, there are no studies carried out on truly alpine habitats (i.e. away above tree line). Thus, our study seems to be one of the first experimental assessments of the direct consequences of warming on the freezing resistance of high-elevation alpine plants. The scarce evidence available so far indicates that most of the species decreased their ability to survive freezing temperatures after growing in warmer conditions, which has important implications with respect to species withstanding future climate changes (Inouye 2000).

An increasing body of literature has reported that global warming may increase the CO_2 assimilation rate and growth of high-elevation plants (Kudo and Suzuki 2003; Loik et al. 2004; Takahashi 2005; Danby and Hik 2007; Kudernatsch et al. 2008). However, potential gains in plant productivity because of an increase in the daytime temperature and in the length of the growing season may be reduced by a decrease in the ability of high-elevation plants to resist freezing temperatures as we found in this study. Hence, some high-elevation species may become unable to cope with more frequent and more severe freezing events due to climate change.

In our study, warming also affected the apoplastic FP of some of the studied species, where six out of 11 studied species increased their FP inside OTCs. Although warming decreased the soil moisture at the lower elevation, it is unlikely that it was related to the increase in the FP observed in these species. FP depends on specific properties of the plant tissues and it may vary according to the cell sap concentration and/or the accumulation of water-binding substances inside the cell (Sakai and Larcher 1987). For instance, water-stressed plants accumulate a higher concentration of solutes (i.e. sugars, proteins) in their tissues that depress FP (Chen et al. 1977; Goldstein et al. 1985; Anisko and Lindstrom 1996). However, we found that FP increased in the leaves of plants growing inside OTCs, suggesting that plants growing under warmer conditions modify the processes of ice formation in their tissues rather than adjust the osmotic potential of their tissues in response to an increase in the soil water deficit. In addition, most of those substances produced by plants to cope with freezing temperatures are mobilized and/or removed from plant tissues during cold hardening and dehardening processes (Sakai and Larcher 1987). Thus, the previously reported effects of warming on cold hardening (Repo et al. 1996; Loveys et al. 2006), and our finding of lower freezing resistance with warming, may be mediated by changes in the synthesis and accumulation of these cryoprotectant substances. In our study, four out of six species where FP increased with experimental warming also showed higher LT_{50} (i.e. *Euphorbia collina*, *Haplopappus anthylloides* and *Nassauvia looseri* at low, and *Phacelia secunda* at high elevation), indicating that warming reduces the freezing resistance of the plants by both increasing the temperature at which their leaves are damaged, and the temperature at which ice crystals start to form. This suggests that those species where both parameters changed may be even more vulnerable to global warming.

Some authors have suggested that freezing resistance assessed in attached and detached leaves varies depending on the techniques employed (see Bannister 2007 for a complete review). In particular, Taschler and Neuner (2004) found that the freezing resistance of Austrian Alp species measured on detached leaves was underestimated with respect to that found for attached leaves. Our estimations were based on detached plant materials, suggesting that the actual freezing resistance of the high-Andean species may be underestimated. However, it seems unlikely that the decrease in the ability to resist freezing observed after experimental warming can be reversed with measures taken in attached leaves.

Finally, *Laretia acaulis* and *Azorella madreporica* are two cushion plant species dominating low- and high-elevation sites, respectively. Both species have been reported to facilitate the survival of several high-elevation species, becoming key species in the maintenance and functioning of these habitats (Cavieres et al. 2006, 2007). Our results indicate that both cushion species decreased their freezing resistance under warmer conditions, suggesting that global warming could have detrimental consequences for high-elevation plant communities both by direct negative effects (i.e. lowering freezing resistance) and by indirect effects (i.e. plant interactions) through negative impacts on key species as the nurse cushion plants of the high-Andes of central Chile.

In conclusion, we showed that the summer freezing resistance of high-Andean species decreases under warmer ambient temperatures, providing a likely mechanism by which the decreases in species richness observed in warming experiments in high-elevation habitats occurs (e.g. Klein et al. 2004). The ability to withstand freezing conditions is a key feature of high-elevation species, hence if current climate warming trends continue, the survival of high-elevation plants will be seriously threatened, as is already being reported in both empirical and theoretical studies (e.g. Guisan and Theurillat 2000; Gottfried et al. 2002; Dirnböck et al. 2003; Wilson and Nilsson 2009).

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