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Reduced early growing season freezing resistance in alpine treeline plants under elevated atmospheric CO_2

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Abstract

The frequency of freezing events during the early growing season and the vulnerability to freezing of plants in European high-altitude environments could increase under future atmospheric and climate change. We tested early growing season freezing sensitivity in 10 species, from four plant functional types (PFTs) spanning three plant growth forms (PGFs), from a long-term *in situ* CO_2 enrichment (566 vs. 370 ppm) and 2-year soil warming (+4 K) experiment at treeline in the Swiss Alps (Stillberg, Davos). By additionally tracking plant phenology, we distinguished indirect phenology-driven CO_2 and warming effects from direct physiology-related effects on freezing sensitivity. The freezing damage threshold (lethal temperature 50) under ambient conditions of the 10 treeline species spanned from $-6.7 \pm 0.3\,^{\circ}C$ (*Larix decidua*) to $-9.9 \pm 0.6\,^{\circ}C$ (*Vaccinium gaultherioides*). PFT, but not PGF, explained a significant amount of this interspecific variation. Long-term exposure to elevated CO_2 led to greater freezing sensitivity in multiple species but did not influence phenology, implying that physiological changes caused by CO_2 enrichment were responsible for the effect. The elevated CO_2 effect on freezing resistance was significant in leaves of *Larix*, *Vaccinium myrtillus*, and *Gentiana punctata* and marginally significant in leaves of *Homogyne alpina* and *Avenella flexuosa*. No significant CO_2 effect was found in new shoots of *Empetrum hermaphroditum* or in leaves of *Pinus uncinata*, *Leontodon helveticus*, *Melampyrum pratense*, and *V. gaultherioides*. Soil warming led to advanced leaf expansion and reduced freezing resistance in *V. myrtillus* only, whereas *Avenella* showed greater freezing resistance when exposed to warming. No effect of soil warming was found in any of the other species. Effects of elevated CO_2 and soil warming on freezing sensitivity were not consistent within PFTs or PGFs, suggesting that any future shifts in plant community composition due to increased damage from freezing events will likely occur at the individual species level.

Keywords: climate change, elevated CO_2, FACE, freezing resistance, LT50, temperature, treeline

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Introduction

Plants growing near treeline in the Alps experience 7–8 months of winter and have adapted to survive seasonal low temperatures and persistent snow cover. During autumn, plants change physiologically and undergo a hardening process to reach maximum freezing resistance in mid-winter. As a consequence, very low temperatures during the winter have not been found harmful for fully hardened plants. Additionally, with the exception of windswept areas, low-stature plants and sometimes even trees growing near treeline are thermally insulated by snow cover during winter, which protects them against cold temperatures (Sakai & Larcher, 1987; Körner, 2003). However, freezing conditions can occur year-round in high-elevation environments and are particularly common in temperate

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regions during the early part of the alpine growing season and in early autumn. Physiologically active (dehardened) plants, especially newly developed tissue, are comparatively vulnerable to freezing temperatures and often suffer damage from episodic freezing events during the early growing season. Damage caused by freezing can have lasting impacts on plant performance, as tissue damage means both a loss of stored carbon and nutrients and of the capacity for photosynthetic carbon gain.

The particular species assemblages occurring in alpine and treeline locations indicate that the present plant species are able to persist over the long term, despite occasional freezing events during the growing season. What is unclear, however, is how plants will cope with altered frequencies and temporal distributions of freezing temperatures in the future, given expected changes in climate and in plant phenology and physiology resulting from the progressive increase in atmospheric CO_2 concentration. Overall warmer conditions and an increase in high temperature extremes during summer relative to mean climatic conditions are expected (Schär *et al*., 2004; IPCC, 2007). However, there is also a realistic possibility of an increased frequency of freezing events during periods when plants are active in mountain environments, simply because a warmer climate might advance phenology in many taxa without a concurrent decline in the risk of freezing temperatures (Inouye, 2000, 2008). Photoperiodic sensitivity of many alpine plant species safeguards against premature dehardening during warm spells in early spring, but temperature often has a stronger influence on vegetative and reproductive phenology later in the spring when days are longer (Keller & Körner, 2003). Further, some opportunistic high-elevation species develop shortly after release from dormancy (snow cover), regardless of photoperiod (Keller & Körner, 2003). Declining snow depth and duration in the Alps due to climate warming, already evident during the last century, will likely cause earlier dehardening in these species (Keller *et al*., 2000; Beniston *et al*., 2003; Wipf *et al*., 2009). If late-spring freezing events remain constant or even increase, earlier plant development due to warmer temperatures and/or earlier snow melt will increase the probability of freezing damage to sensitive new plant tissue. On the other hand, mature leaves and shoots are typically less vulnerable to freezing conditions than newly formed tissue (Taschler *et al*., 2004; Sierra-Almeida *et al*., 2009), and plant tissue that is more developmentally advanced as a result of warmer growing conditions might actually be less susceptible to freezing damage as the growing season progresses.

More frequent and more serious freezing damage has been observed in alpine *Delphinium barbeyi* in the American Rocky Mountains during springs with early snow melt (Inouye *et al*., 2002) and in dwarf shrubs experiencing experimentally advanced snow melt in the Swiss Alps (Wipf *et al*., 2009). Similarly, several studies have found that warmer temperatures impact growing season freezing sensitivity through changes in springtime development. Accelerated tissue dehardening, and therefore reduced freezing resistance during spring, has been observed after experimental warming throughout winter (without snow cover manipulation) in subarctic *Vaccinium myrtillus* (Taulavuori *et al*., 1997a, b), in boreal *Pinus sylvestris* saplings (Repo *et al*., 1996), and in *Betula pubescens* seedlings (Taulavuori *et al*., 2004). However, no effect of experimental warming during the growing season was found for subarctic *Empetrum hermaphroditum* (Ögren, 2001). To our knowledge, no studies have previously investigated the possibility of warming-induced shifts in the dehardening process of alpine treeline plants.

Atmospheric CO_2 enrichment has also been found to reduce freezing resistance but, unlike warming, this phenomenon has only rarely been observed in conjunction with advanced phenology (but see Repo *et al*., 1996). Reduced growing season freezing resistance in response to elevated CO_2 has been documented for forbs and grasses grown in a native temperate grassland (Obrist *et al*., 2001) and for field-grown dwarf shrubs from a subarctic heath community (Beerling *et al*., 2001). The same result was found for saplings of chamber-grown *Pseudotsuga menziesii* (Guak *et al*., 1998) and *Ginkgo biloba* (Terry *et al*., 2000), and for *Eucalyptus pauciflora* seedlings in an OTC field experiment (Lutze *et al*., 1998; Barker *et al*., 2005; Loveys *et al*., 2006). Although mechanisms for increased sensitivity of CO_2-enriched plants have not been determined, several authors have speculated that previously documented physiological and chemical changes in response to elevated CO_2 are involved. No shift in freezing resistance under elevated CO_2 has been found for *Picea abies* saplings (Wiemken *et al*., 1996), for subarctic *Vaccinium vitis-idaea* (Taulavuori *et al*., 2001), or for *Picea mariana* seedlings (Bigras & Bertrand, 2006). Enhanced freezing resistance has even been observed for dormant winter buds of *Betula alleghaniensis* seedlings (Wayne *et al*., 1998) and for *Yucca* seedlings from the Mojave Desert (Loik *et al*., 2000).

The contrasting freezing resistance responses to elevated CO_2 reported in previous studies may be related to species-specific effects, to differences in growing conditions or in duration of CO_2 exposure, or to different ontogeny, which is particularly important for trees (Sakai & Larcher, 1987). It is also possible that results were influenced by methodological differences including detached or attached plant tissue, rate and duration of freezing, and damage assessment technique (Taschler

& Neuner, 2004; Bannister, 2007). Finally, shifts in freezing resistance under elevated CO_2 have consistently been reported as <2 K (e.g. Lutze *et al*., 1998; Beerling *et al*., 2001; Obrist *et al*., 2001), and studies reporting no CO_2 effect often used freezing temperature increments too large to detect such subtle differences (e.g. Wiemken *et al*., 1996; Taulavuori *et al*., 2001; Bigras & Bertrand, 2006).

Plant growth form (PGF) and plant functional type (PFT) classification systems can be valuable tools for predicting plant responses to climate change in cases where differences among groups remain consistent under changing environmental conditions. For example, Chapin *et al*. (1996) found that, for a range of arctic species, those from the same PFT showed similar long-term responses to changes in nutrient availability and soil moisture. Also, similar phenological and growth responses to experimental warming have been reported within PFTs (Arft *et al*., 1999; Dunne *et al*., 2003). However, shifts in the freezing resistance of co-occurring species under atmospheric and climate change have not yet been compared at the PGF or PFT level. As plant height (Squeo *et al*., 1991) as well as deciduousness and woodiness (Taschler & Neuner, 2004) have been found to influence freezing resistance, both PGF and PFT classifications are potentially informative for predicting changes.

In the present study, we investigated growing season freezing sensitivity of a range of different plant species from a long-term *in situ* CO_2 enrichment and 2-year soil warming experiment at treeline in the Swiss Alps. In this experiment, we previously observed increased *in situ* leaf tissue damage under elevated CO_2 in *V. myrtillus* ($F_{1,8} = 5.54$, $P = 0.047$) but not in *Vaccinium gaultherioides* ($F_{1,8} = 0.01$, $P = 0.93$) after a natural freezing event in June 2005 (I. T. Handa *et al*., unpublished data). We also found a trend of increased damage under elevated CO_2 in *Larix decidua* after freezing conditions in late May 2007 ($F_{1,8} = 3.80$, $P = 0.087$; M. Martin, unpublished data). These observations prompted us to examine this effect further through an experimental freezing study including 10 prominent species from four PFTs spanning three PGFs. We aimed to determine how atmospheric CO_2 enrichment and soil warming affect the freezing sensitivity of different alpine treeline species, PFTs and PGFs. By additionally tracking phenology of the selected species, we determined if treatment effects on freezing resistance were associated with changes in phenology. Our primary hypotheses were that (1) CO_2 enrichment would negatively affect early growing season freezing resistance but that this effect is intrinsic to CO_2 and not associated with a phenological shift induced by the treatment and (2) soil warming would advance phenology, leading to more mature leaf tissue in the early growing season and, therefore, reduced freezing sensitivity of fully expanded leaves. We also predicted that (3) plants of different PFT and PGF groups would have distinct freezing damage thresholds under current conditions, and plants within the same group would respond similarly to the experimental treatments.

Materials and methods

Site description

The study site is located at Stillberg, Davos in the Central Alps, Switzerland (9°52′E, 46°46′N), where a free air CO_2 enrichment (FACE) experiment was set up in 2001 (Hättenschwiler *et al*., 2002). The FACE experiment is situated at or slightly above the natural climatic treeline (2180 m a.s.l.) on a NE-exposed 25–30° slope. The site is part of a long-term afforestation research area planted in 1975 by the Swiss Federal Institute for Forest, Snow and Landscape Research (WSL). For the FACE experiment, 40 circular 1.1 m^2 plots were established, 20 with a European larch (*L. decidua*) in the centre and 20 with a mountain pine (*Pinus mugo* ssp. *uncinata*) in the centre. Although these trees planted at treeline are ca. 35 years old, they are not taller than 3 m and have a stem basal diameter of <10 cm. The trees are widely spaced and do not form a closed canopy, thus allowing for a dense heath-like understorey layer in each experimental plot surrounding the tree base (see Hättenschwiler *et al*., 2002 for additional information about the site and experimental setup). CO_2 enrichment (multiple-year mean 566 ± 75 vs. ca. 370 ± 3 ppm ambient) has been supplied throughout each growing season since 2001; the technical setup and performance of the CO_2 enrichment facility has been described in detail previously (Hättenschwiler *et al*., 2002; Handa *et al*., 2005). In the summer of 2007, a soil warming treatment (growing season mean + 4 K) was introduced to the FACE experiment by laying heating cables on the ground underneath the dwarf shrub layer (details in Hagedorn *et al*., 2009).

The resulting experimental design was a completely randomized split–split–plot model: 10 whole plots, each consisting of four individual trees and their associated understorey layer, were randomly assigned to a CO_2 treatment, two split plots within each whole plot were randomly assigned a soil warming treatment, and finally one of the two tree species (larch or pine) was present at the split–split–plot level (hereafter simply referred to as 'plot'). For the freezing experiment, therefore, each tree species had up to five replicates of the four individual combinations of CO_2 and warming treatments. Three of the pines died before 2008, which

resulted in a replication of four in three of the treatment combinations. The maximum replication of five was also applicable to measurements made on understorey plants in the freezing experiment because we distinguished between larch or pine presence in the plots, resulting in a total of eight treatment combinations. Each understorey species was present in at least 30 of the 40 experimental plots to allow for sufficient replication of each treatment combination.

Air temperature data (height at 2 m above ground) was available from a climate station located on Stillberg at 2090 m a.s.l., approximately 100 m below the FACE site. Additionally, HOBO Pro v2 dataloggers (Onset Computer Corporation, Bourne, MA, USA, part U23-003) were installed in the canopy of eight trees within the experiment (four pine, four larch; height at 1–2 m above ground) to obtain a closer estimate of daily air temperature minima throughout the growing season.

Plant species and field sampling

We sampled a total of 10 plant species from the experimental plots, spanning four PFTs (classification after Chapin *et al*., 1996): (a) deciduous woody: tree *L. decidua* Mill. (European larch) and dwarf shrubs *V. myrtillus* L. (bilberry) and *V. gaultherioides* Bigelow (group *Vaccinium uliginosum* agg.; northern bilberry); (b) evergreen woody: tree *P. mugo* spp. *uncinata* (DC.) Domin (mountain pine) and dwarf shrub *Empetrum nigrum* spp. *hermaphroditum* (Hagerup) Böcher (crowberry); (c) forb: *Gentiana punctata* L., *Homogyne alpina* (L.) Cass., *Leontodon helveticus* Mérat, and *Melampyrum pratense* L.; and (d) graminoid: *Avenella flexuosa* (L.) Drejer. The selected species also represented three PGFs: tree, shrub, and herb. Names of the listed species are according to Lauber & Wagner (2007).

We completed six sampling events during the early growing season of 2008 (days 169–209), with up to three plant species tested on a single date (Fig. 1). Each species was sampled only once, as limited plant material available for destructive sampling and space constraints in the freezing units prevented multiple tests. The one exception was *Larix*, for which both short shoots (leaf fascicles on last year wood; day of year 169) and long shoots (expanding new-wood growth; day 188) were used. Newly formed leaf and shoot tissue was sampled upon full leaf expansion. As a result, each species was tested at a similar point relative to leaf phenology, but calendar date and number of days since snow melt varied.

Springtime phenology was quantified for each species present in a given plot by estimating the percentage of plant cover that had experienced (a) leaf bud break and (b) at least 50% of full leaf expansion (Hartley *et al*., 1999). We inspected individual ramets from all regions of each plot and averaged out any small-scale variation due to microsite differences for a percentage estimate at the plot level for a given species. Observations were made weekly from snow melt to early July (days of year 154, 161, 168, and 175).

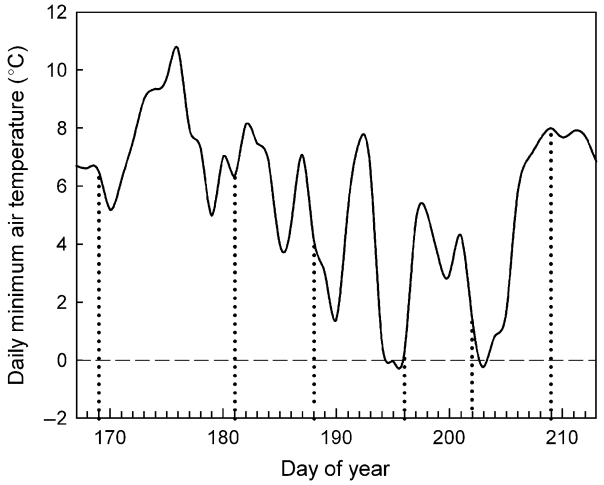

Fig. 1 Period of plant sampling during the summer of 2008. The six freezing trials (dotted vertical lines) included the species: day 169, *Larix decidua* (short shoots); 181, *Gentiana punctata*; 188, *L. decidua* (long shoots); 196, *Homogyne alpina*, *Leontodon helveticus*, and *Melampyrum pratense*; 202, *Empetrum hermaphroditum*, *Vaccinium gaultherioides*, and *Vaccinium myrtillus*; 209, *Avenella flexuosa* and *Pinus uncinata*.

Freezing experiments

Freezing resistance was determined using detached tissue collected between 07:00 and 09:00 hours in the morning. For *V. myrtillus*, *V. gaultherioides*, and *Melampyrum*, one or two (depending on availability) entire individual leaves per experimental plot were used for each freezing treatment. For forbs with large leaves (*Leontodon*, *Gentiana*, and *Homogyne*), two 5 mm leaf discs were used instead (Gurvich *et al*., 2002; Bannister, 2007). Whole leaf fascicles were used for *Pinus* and short shoots of *Larix* (first sampling), and the newly grown shoot increment (with leaves attached) was used for *Empetrum* and long shoots of *Larix* (second sampling). For *Larix* and *Pinus*, material was sampled from the top half of the tree on the downslope (NE) side. Grass blades (*Avenella*) were cut into approximately 1 cm sections to standardize the size of sample material. All samples were rinsed with deionized water to remove surface impurities, blotted dry, and subsequently wrapped in aluminum foil to (a) prevent desiccation during freezing and (b) ensure a homogenous thermal

environment during freezing cycles (Obrist *et al*., 2001). Samples were kept in darkness at 5 °C until freezing, which occurred within 12 h of collection.

Freezing conditions were implemented overnight and different freezing temperature treatments were applied concurrently, rather than sequentially as is common practice, to most closely simulate naturally occurring summer freezing events. One sample per species per experimental plot was inserted into each of six polystyrene-insulated cylindrical aluminum freezing chambers (radius 4 cm, depth 7 cm), as in Obrist *et al*. (2001). Each of the six freezing chambers was used for a different final target temperature in order to create a temperature gradient spanning the species-specific critical range. Samples from each tested species were arranged on small racks within the freezing chambers; this arrangement was consistent for all freezers with respect to experimental plots to account for any temperature gradient effect within the chambers. Temperature inside each chamber was regulated separately by a computer-controlled Peltier cooling element and constantly monitored by thermocouples attached to extra leaf samples. After initial reduction to 0 °C, temperature within each freezer was linearly decreased 1 K every 20 min ($3\,K\,h^{-1}$) and then held at a target value. The precise duration at each specific target value was shorter with each increment to a colder target temperature (20 min shorter per 1 K), in accordance with typical natural freezing events at the site. This slow cooling process also helped reduce the possibility of either excessive supercooling or failure to nucleate (Sakai & Larcher, 1987), artefacts that have been observed for small, excised samples (Neuner *et al*., 1997). The span of target temperatures for the different freezers varied for each freezing cycle, based on preliminary tests for each species, ranging from −3 to −14 °C. After being held at the target temperature for about 5 h, freezing chambers were switched off to gradually return samples to room temperature, not exceeding $6\,K\,h^{-1}$. Samples were then kept in darkness at 5 °C. In addition to the six custom-made freezing chambers, we included a control (5 °C) and a maximum damage (−30 °C) treatment using a standard household commercial unit, providing a total of eight treatment units.

Damage assessment

Leaf tissue damage was determined both by the electrolyte leakage (EL) method (Bernier-Cardou & Bigras, 2001) and by visual scoring (e.g. Obrist *et al*., 2001; Taschler & Neuner, 2004). After freezing, samples were unwrapped and transferred to polypropylene vials with 10 mL of deionized water, shaken, and stored in darkness at 8 °C for 24 h to allow ions to leach across damaged cell membranes. Conifer needle samples were cut into 5 mm segments beforehand, as this step has been shown to improve freezing damage detection with the EL method (Burr *et al*., 2001). Electrical conductivity of the solution was measured once, and sealed vials were then heated at 100 °C using an oven for 1 h to completely disrupt all cell membranes. Another 24 h lapsed (conditions as above) followed by a second electrical conductivity measurement, thus allowing us to express EL after freezing as a percent of maximum EL [relative electrolyte leakage (REL)]. REL values are not influenced by the amount of material sampled, by routine mechanical damage caused by the sample preparation method, e.g. leaf disc punching, or by the inherent membrane permeability of individual species.

Freezing resistance of plant tissue was expressed as lethal temperature 50 (LT50), the estimated temperature at which half of the samples were killed (Sakai & Larcher, 1987). There is generally only a narrow range of temperatures between no damage (low REL) and complete damage (high REL), which allows a relatively precise calculation of this value. LT50 was determined by fitting a sigmoid curve (Boltzmann equation, ORIGIN v.8 software, OriginLab Corporation, Northampton, MA, USA) through the eight individual REL data points for each species from an individual plot and calculating the inflection point (Burr *et al*., 1990; Taschler *et al*., 2004). In cases where the sigmoid curve did not have a clear inflection point, the corresponding LT50 value was omitted from statistical analysis (10% of the total number of LT50 value calculations). Damage induced by freezing was also assessed visually and quantified as the proportion of plant tissue with altered colouration and/or loss of turgor (Obrist *et al*., 2001). Although not feasible for all species, this basic yet reliable technique (Ritschie, 1991) helped to verify that high REL values actually corresponded to freezing damaged tissue. Pooled across the species for which visible assessment was possible (*V. myrtillus*, *V. gaultherioides*, and *Homogyne*), REL measurements and visual damage score showed a strong correlation ($R^2 = 0.845$). This comparison rendered the EL method a reliable proxy for freezing damage; hence, only REL results were used for further data analysis.

Statistical analysis

LT50 values were analyzed according to our completely randomized split–split–plot design. Type I analysis of variance was performed by fitting a linear mixed model, using the restricted maximum likelihood method to account for cases where species were not present in all plots. The model for all tested plants together included plant species, CO_2 and temperature treatments, and all

two- and three-way interactions as fixed effects. As tree type (larch or pine presence in a plot) was not applicable to the two tree species in the model, this factor could not be included as a fixed effect. For the complete model, we explored different ways of including LT50 values of *Larix* short and long shoots: only short shoots, only long shoots, both shoot types, and mean values from the two types. Different approaches yielded nearly identical results, and mean values were ultimately used in the complete model. Based on the results from this overall analysis, we also tested for treatment effects on LT50 for each species individually. For larch and pine, 'tree' was omitted from the data structure (split–plot model). Short and long shoots of *Larix* were tested separately at this more detailed level of analysis to investigate potential differences in the response of short and long shoots.

The effect of PGF (tree, shrub, or herb) and its interaction with the experimental treatments were tested with a statistical model analogous to that for all plant species together. The effect of PFT was tested in the same way as PGF. *A. flexuosa* was excluded from the PFT analysis because it was the only grass species, leaving a total of three PFTs: deciduous woody (three species), evergreen woody (two species), and forb (four species). We used a Tukey's multiple comparisons test to compare the LT50 values of individual PFTs (Hothorn *et al*., 2008). Although *post hoc* tests can give inaccurate results when applied to structurally complex statistical models (Quinn & Keough, 2002), the lack of significant interactions between main effects in the overall model justified such an analysis in this case.

For statistical analysis of phenology, we considered only *Larix*, *Pinus*, *V. myrtillus*, *V. gaultherioides*, and *Empetrum* because limited presence and unclear phenological stages prevented accurate analysis of the forb and grass species. Statistical tests were completed for each species and observation date separately, using the same fixed and random effects as in the model for LT50 values of individual species described above. The percentages were arcsine(sqrt) transformed in the model to satisfy the requirement of linearity. Percent budburst and/or percent expanded leaves were only tested statistically on dates where variation was observed among the plots for a given species (not all 0% or 100%). Leaf budburst (all four observation dates) and leaf expansion (last three dates) were tested for *V. myrtillus*, whereas only budburst was tested for *V. gaultherioides* (all four dates). Expansion of short shoot leaves on all four dates was analyzed for *Larix*. Budburst on the last observation date (175) was the only data used for both *Empetrum* and *Pinus*.

For all statistical analyses, assumptions of linearity and constant variance were checked visually using diagnostic plots. Results with P values ≤ 0.05 were considered significant. Replication was limited to five in this study due to the pre-existing design of the long-term FACE experiment, and consequently results with lower statistical significance might have had ecological significance. To acknowledge this possibility, P values >0.05 but ≤ 0.10 were considered marginally significant. All analyses were performed using R version 2.7.2 (R Development Core Team, 2008).

Results

Snow melt date and air temperature

Snow was completely melted from each of the experimental plots between days 145 and 154 of 2008. Daily minimum air temperature in the canopy of FACE trees ranged from 0 to 10 °C during the sampling period between day 169 and day 209 (Fig. 1).

Plant leaf phenology

Overall, we observed only few significant treatment effects on plant phenology, and none of the interactions between the main factors CO_2, warming, and tree species identity in the plot (if applicable) were significant. Of the five species for which we could analyze leaf phenology, only *Empetrum* showed an effect of CO_2 enrichment (marginally significantly lower percent budburst in elevated CO_2 plots on day 175, $F_{1,8} = 3.61$, $P = 0.094$). *V. myrtillus* was the only species that showed some effect of soil warming on phenology (Fig. 2). Leaves expanded earlier in warmed plots, with a higher percent of leaves >50% expanded on days 161 ($F_{1,8} = 4.36$, $P = 0.070$, marginally significant) and 168 ($F_{1,8} = 5.48$, $P = 0.047$, significant) but not on days 154 or 175 (Fig. 2). There was no significant warming effect on leaf budburst. Neither of the experimental treatments significantly affected leaf phenology of *Larix* or *Pinus*.

Leaf phenology of the two deciduous dwarf shrub species was influenced by the identity of the tree species in the plot, irrespective of the other treatments. *V. gaultherioides* growing under pine showed earlier budburst than when growing under larch, with significant differences on days 161 ($F_{1,16} = 8.00$, $P = 0.012$) and 168 ($F_{1,16} = 12.12$, $P = 0.003$), and a nonsignificant trend in the same direction on the two other observation dates (day 154, $F_{1,16} = 2.84$, $P = 0.111$ and day 175, $F_{1,16} = 2.28$, $P = 0.151$; Fig. 3). The opposite pattern was observed in *V. myrtillus*, for which plants growing under pine showed marginally significantly lower percent budburst on day 161 only ($F_{1,16} = 3.95$, $P = 0.064$) and

marginally significantly lower percent expanded leaves on day 168 only ($F_{1,16} = 3.95$, $P = 0.064$; Fig. 2).

Freezing sensitivity of co-occurring plant species, functional types, and growth forms

The freezing damage threshold (LT50) of the 10 treeline species under ambient CO_2 and temperature conditions spanned from $-6.7 \pm 0.3\,^{\circ}C$ (*Larix*) to $-9.9 \pm 0.6\,^{\circ}C$ (*V. gaultherioides*), and plant species varied significantly in their LT50 in our complete model ($F_{9,241} = 27.69$, $P < 0.0001$; Fig. 4). PFT explained a significant amount of the observed interspecific variation ($F_{2,230} = 7.18$, $P = 0.001$): the deciduous woody group was significantly more resistant than the forbs ($z = 2.52$, $P = 0.031$), while the evergreen woody group had intermediate resistance and did not differ significantly from the other two groups. PGF did not significantly influence freezing resistance ($F_{2,269} = 0.48$, $P = 0.621$). Within the tree growth form, deciduous *Larix* was the most sensitive to freezing conditions out of all tested species, whereas new foliage of evergreen *Pinus* was one of the hardiest. The two deciduous dwarf shrub species varied widely in their freezing resistance, with *V. gaultherioides* showing the most resistant leaves of all species tested. Both deciduous dwarf shrubs tolerated colder temperatures than the evergreen species *Empetrum*, the opposite pattern as that observed in the trees. Among the herbaceous species, *Homogyne*, *Leontodon*, and *Melampyrum* had LT50 values within 0.3 K of each other, but *Gentiana* and the alpine grass species *Avenella* showed greater resistance to freezing.

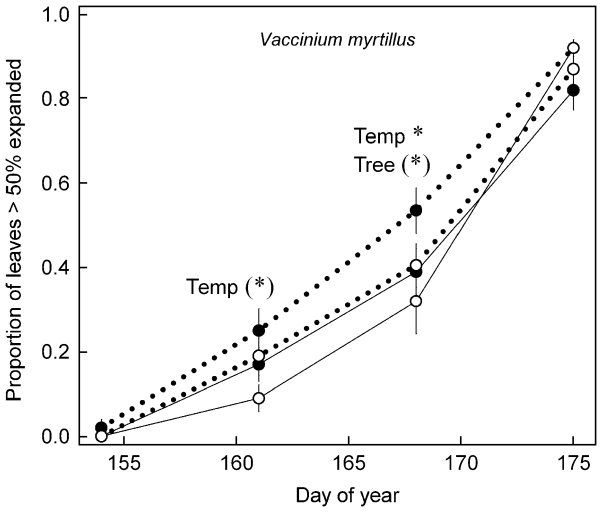

Fig. 2 Effects of soil warming (solid line, control temperature; dotted line, warmed) and identity of the tree species (larch or pine; filled circle, growing under larch; open circle, growing under pine) in the experimental plot on leaf phenology of *Vaccinium myrtillus*. The proportion of leaves at least 50% expanded are shown. Mean values across all plots per treatment combination $\pm$ 1 standard error are presented. Treatment effects are marked as marginally significant: (*)$P \leq 0.10$; and significant: *$P \leq 0.05$. For each of the four treatment combinations shown, $n = 10$ plots (averaged across CO_2 treatments).

Effects of elevated CO_2 and soil warming on freezing sensitivity

Plants at the Stillberg treeline site experienced increased susceptibility to early growing season freezing events when grown under elevated atmospheric CO_2 (LT50 across all species: $F_{1,8} = 16.38$, $P = 0.004$). When plant species were investigated individually, the negative CO_2 effect on freezing resistance was significant for three, and marginally significant for two, of the 10

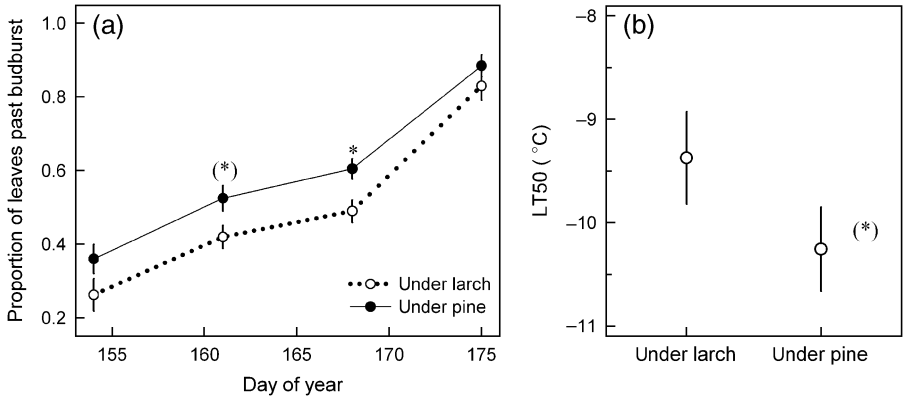

Fig. 3 Phenology (a) and freezing resistance (b) of *Vaccinium gaultherioides* as affected by the tree species (larch or pine) under which it was growing. (a) Time series of the proportion of leaves past budburst under the two different tree species ($n = 20$ plots, mean across CO_2 and warming treatments); (b) effect of tree species on freezing resistance ($n = 20$ plots, mean across CO_2 and warming treatments). Mean values across all plots per treatment combination $\pm$ 1 standard error are presented. Treatment effects are marked as marginally significant: (*)$P \leq 0.10$; and significant: *$P \leq 0.05$.

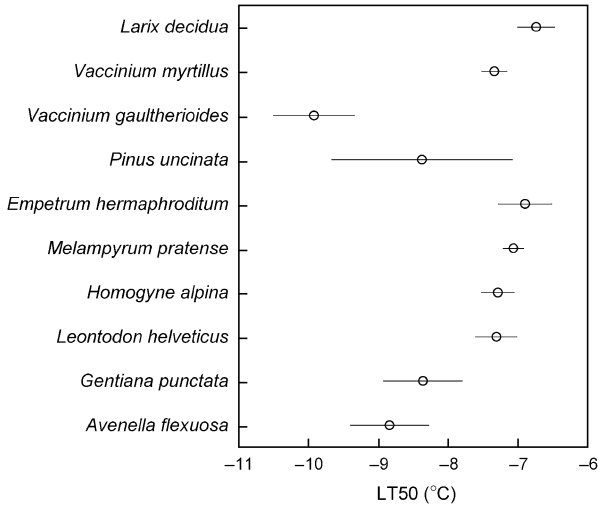

Fig. 4 Freezing sensitivity of individual treeline plant species. Only plants that experienced ambient CO_2 and no soil warming treatments are shown. Mean LT50 values across all plots per treatment combination ± 1 standard error are presented. Sample size in each treatment group was determined by species presence in the plots and missing samples. $n = 10$ for *Larix decidua*, mean across short and long shoots; $n = 4$ for *Pinus uncinata*; $n = 10$ for *Vaccinium myrtillus*, *Vaccinium gaultherioides*, *Homogyne alpina*, and *Leontodon helveticus*; $n = 9$ for *Melampyrum pratense*; $n = 5$ for *Empetrum hermaphroditum*, and *Gentiana punctata*. For all understorey species, mean across larch and pine tree identity are shown.

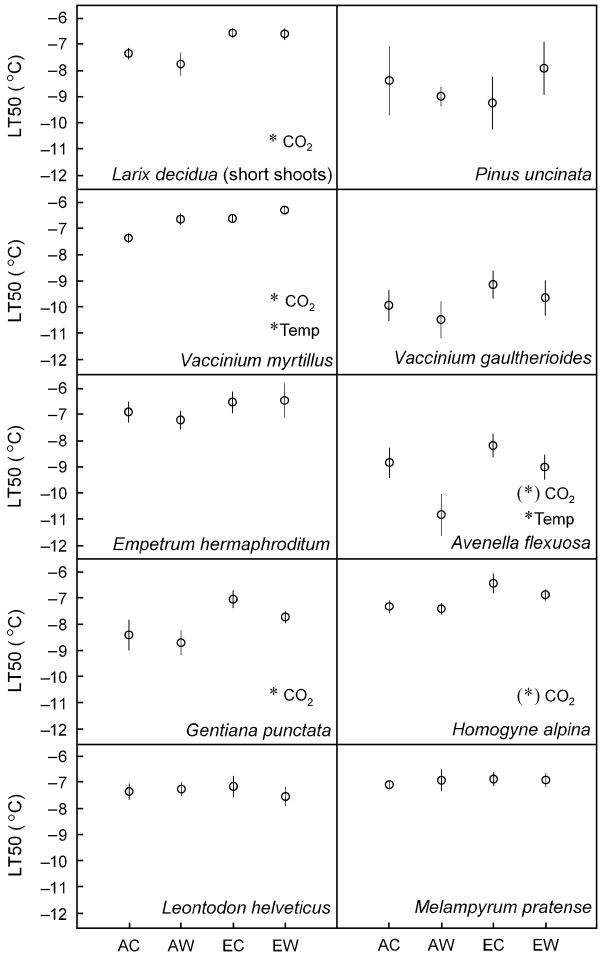

Fig. 5 CO_2 (elevated, E; ambient, A) and soil warming (warmed, W; control, C) treatment effects on the freezing resistance (LT50) of treeline plant species at Stillberg. Mean LT50 values across all plots per treatment combination ± 1 standard error are presented. Treatment effects are marked as marginally significant: (*)$P \leq 0.10$; and significant: *$P \leq 0.05$. Sample size in each treatment group was determined by species presence in the plots and missing samples. For trees, $n = 3$–5; for understorey plants, $n = 5$–10 and means across larch and tree identity are shown.

studied species. LT50 was reached at significantly higher temperatures in *Larix* short shoots (+ 1.0 ± 0.3 K; $F_{1,8} = 8.93$, $P = 0.017$), in *V. myrtillus* (+ 0.5 ± 0.2 K; $F_{1,8} = 9.91$, $P = 0.014$), and in *Gentiana* (+ 1.2 ± 0.4 K; $F_{1,7} = 9.21$, $P = 0.019$) growing under elevated CO_2 compared with ambient CO_2 (Fig. 5). The negative effect of CO_2 on freezing resistance was marginally significant in *Homogyne* (+ 0.7 ± 0.3 K; $F_{1,8} = 3.92$, $P = 0.083$) and *Avenella* (+ 1.2 ± 0.6 K; $F_{1,8} = 3.91$, $P = 0.083$; Fig. 5). At the given resolution of our experiment, no CO_2-induced effect on freezing resistance was observed in *Larix* long shoots, *Pinus*, *Empetrum*, *V. gaultherioides*, *Leontodon*, and *Melampyrum* (Fig. 5).

Soil warming did not have a consistent effect on freezing sensitivity across the studied species (LT50 across all species: $F_{1,8} = 2.14$, $P = 0.182$). However, species differed in how their freezing sensitivity was affected by warming, and we found a marginally significant species by warming interaction in the analysis of all species together ($F_{9,241} = 1.69$, $P = 0.092$). Soil warming significantly increased freezing sensitivity in *V. myrtillus* (+ 0.5 ± 0.2 K; $F_{1,8} = 9.25$, $P = 0.016$), but the opposite was found for *Avenella* (−1.4 ± 0.6 K; $F_{1,8} = 5.37$, $P = 0.049$; Fig. 5). None of the remaining eight species showed a significant warming effect on freezing sensitivity (Fig. 5).

The identity of the tree species in the experimental plot (larch or pine) had only a marginally significant effect on freezing sensitivity in two of the understorey plants. Under pine, freezing damage was detected at a warmer temperature for *Empetrum* (+ 1.1 ± 0.5 K; $F_{1,2} = 9.57$, $P = 0.091$) but at a lower temperature for *V. gaultherioides* (−0.9 ± 0.6 K; $F_{1,12} = 3.34$, $P = 0.092$; Fig. 3). None of the two- or three-way interactions involving elevated CO_2, soil warming and tree species identity were significant. Responses to the experimental

treatments were not consistent within the three PFTs or within the three PGFs (Fig. 5).

Discussion

Distinct freezing damage threshold for co-occurring plant species

Our freezing study was novel for including several species, from three PGFs and four functional types, growing in a natural treeline setting. During the early growing season, all plant species sampled had a mean freezing damage threshold (LT50) between −6.7 and −9.9 °C when grown under ambient conditions. The narrow span of this range, despite high diversity of growth form and life strategy among species, can be explained by the common local temperature regime to which the sampled plants are adapted and acclimated (Gurvich *et al*., 2002; Körner, 2003). Similar peak growing season LT50 values from −5.5 to −9.5 °C have been reported for alpine forbs and grasses at the Furka Pass (2470 m a.s.l.) in the Swiss Alps (Körner, 2003). While the definition of freezing damage, e.g. initial vs. complete damage, varies among studies, a similar range of critical temperatures has been observed for nondormant trees, dwarf shrubs, and herbaceous plants growing in other high-elevation (Sakai & Larcher, 1987; Taschler & Neuner, 2004) and high-latitude (Beerling *et al*., 2001) locations.

We predicted that plants of different functional types and growth forms growing at the treeline would have distinct critical freezing temperatures under current conditions. Across all treatments, classification into three PFTs (grass PFT excluded) based on deciduousness and woodiness explained some of the variation among species. The forb group was significantly more sensitive to freezing than the deciduous, but not the evergreen, woody group. No difference was observed between deciduous and evergreen woody species, likely because the effect of deciduousness on dwarf shrubs was in the opposite direction as that on trees. This surprising result could reflect other characteristics of the individual species available for testing, such as microclimate preference and changes in freezing resistance throughout the early growing season. Given the high variability observed among the 10 species sampled, a clear understanding of the importance of deciduousness or woodiness in the freezing resistance of plants growing near treeline would require sampling many more species than were available in our experimental plots.

Regarding PGF, we predicted that trees would be more sensitive to freezing than low stature dwarf shrubs and herbaceous plants because they experience greater conductive heat exchange (Squeo *et al*., 1991). However, PGF did not help explain differences among species in LT50 and there was high variability in freezing resistance within PGFs. It is possible that a survey including many species would reveal patterns regarding PGFs, although a previous study including 33 treeline and alpine species also showed no correlation between PGF and freezing resistance (Taschler & Neuner, 2004). Within the tree growth form, deciduous *Larix* was the species most sensitive to freezing conditions during the early growing season, whereas new foliage of evergreen *Pinus* tolerated lower temperatures. In contrast, the average growing season LT50 value of *L. decidua* long shoots, sampled from June through August at an Austrian treeline site, was found to be more freezing resistant than other woody species (Taschler *et al*., 2004).

Among shrubs, the two closely related deciduous *V. myrtillus* and *V. gaultherioides* diverged widely in their freezing resistance. *V. gaultherioides* extends >3000 m in the Alps and, as to be expected, was more freezing tolerant than *V. myrtillus*, which has a montane centre of distribution and has previously been found to have low freezing tolerance when active (Taschler & Neuner, 2004). *V. gaultherioides* occupies microhabitats with a shorter snow duration than *V. myrtillus* (Körner, 2003), which likely also contributed to its greater freezing resistance (Bannister *et al*., 2005). Similarly, the evergreen dwarf shrub *Empetrum* is known for its obligatory snow cover requirement in winter (Tybirk *et al*., 2000) and reached LT50 at a higher temperature than the other two dwarf shrubs. Acute freezing desiccation upon snow removal during winter has been reported for *Rhododendron ferrugineum*, another species requiring snow cover for protection against winter freezing conditions (Larcher & Siegwolf, 1985). However, new shoots of *Empetrum* were tested approximately 3 weeks closer to budburst than the deciduous dwarf shrubs, and this difference in timing could have also played a role in its lower freezing resistance.

Within the herbaceous PGF, three of the four forb species had similar thresholds of freezing damage and were close to the −7 °C threshold commonly observed for herbaceous alpine and arctic plants (Körner, 2003; Körner & Alsos, 2008).

The grass *Avenella* was more freezing resistant, consistent with previous evidence that graminoids tend to resist lower temperatures than broad-leaved species (Gurvich *et al*., 2002; Körner, 2003; Taschler & Neuner, 2004; Hacker & Neuner, 2008).

Comparisons of LT50 values among plant species, functional types and growth forms are unavoidably influenced by sampling date. In our study, we chose

to sample each species as soon as possible after full leaf expansion to minimize differences in LT50 among species due to differences in leaf developmental stage and also to standardize across species the stage tested for CO_2 and warming treatment effects. This decision, along with spatial constraints in the freezing chambers, led us to sample individual species on different dates over a 40-day period. Therefore, our comparison of LT50 values among species might reflect variation in the recent temperature history before sampling. However, relatively consistent air temperature, specifically no freezing conditions, for the duration of the study likely reduced this timing effect (Fig. 1). The alternative approach, though not feasible in our study, of sampling all species on the same date would have permitted a more realistic comparison of relative freezing resistance on one specific date but would have been more influenced by differences in developmental stage of the compared species.

For comparisons of LT50 values between species, it is also important to note that the freezing process potentially initiated with different degrees of supercooling for different species. In particular, small, cut samples have a tendency to supercool to lower temperatures before ice nucleation occurs compared with intact leaves and stems (Neuner *et al*., 1997). Constraints on the availability of plant material and space in the freezing chambers necessitated segmentation of *Avenella* leaf blades and the use of leaf discs for *Gentiana*, *Homogyne*, and *Leontodon*, and consequently these species might have been more likely to experience delayed ice nucleation. However, our protocol using a slow, natural cooling rate followed by several hours at the target freezing temperature reduced the possibility of excessive supercooling (Sakai & Larcher, 1987). We observed a range of LT50 values that corresponded closely to values documented previously for alpine plants (Körner, 2003), which suggests that the freezing process was indeed realistic. Therefore, while it is important to recognize the potential effect of excessive supercooling on the observed LT50 values, we are confident that any delayed ice nucleation did not strongly affect our comparison among species. Important for the main study objectives, we also expect that any supercooling effects in a given species were systematic across all experimental treatments. As freezing occurred at higher temperatures under elevated CO_2 than in ambient conditions, the same nucleation delay in both elevated and ambient CO_2 treatments would have tended to reduce the CO_2 signal by freezing at lower temperatures than with undelayed nucleation. Hence, our analysis and conclusions regarding the effect of elevated CO_2 are conservative.

Effects of simulated atmospheric and climate change on plant freezing resistance

In our study, multiple treeline plant species grown in a CO_2-enriched atmosphere showed increased sensitivity to early growing season freezing events. This result supports the well-established pattern of reduced freezing resistance in plants growing under elevated CO_2 (for a review see Woldendorp *et al*., 2008) and provides the first evidence of this effect for treeline plants *in situ*. However, the three dwarf shrub species we studied showed different responses than their European subarctic counterparts. We found that freezing resistance of *V. myrtillus* decreased in response to elevated CO_2 at our alpine treeline site, whereas no response was detected in low-elevation *in situ* studies of the same species in a subarctic heathland community (Abisko Scientific Research Station; Beerling *et al*., 2001). Conversely, *V. gaultherioides* and *E. hermaphroditum* were not affected by CO_2 enrichment in our study but both experienced a slight (<1 K) but significant increase in ice nucleation temperature under elevated CO_2 during the early growing season at the subarctic Abisko site (Beerling *et al*., 2001). These inconsistencies could be due to the potential difference in physiological characteristics between populations in northern Scandinavia and in the Alps. In particular, *Empetrum* species and *V. gaultherioides* are two highly heterogeneous species complexes with, at present, unclear systematic positions (Bell & Tallis, 1973; Jacquemart, 1996). The freezing resistance response of an individual species or species complex to CO_2 enrichment can apparently vary depending on general environmental conditions or plant genotype, making habitat-specific field studies important for predicting future plant responses.

It is also possible that our experimental design, using 1 K steps near the expected critical temperature, was not sensitive enough to capture the differences detected by determining ice nucleation temperatures at a resolution of 0.1 K (Beerling *et al*., 2001).

There were minimal shifts in springtime phenology of the observed species when growing in a CO_2-enriched atmosphere, which follows findings from previous field studies of subarctic heathland dwarf shrubs (Abisko site; Gwynn-Jones *et al*., 1997), mature subarctic *P. abies* (Slaney *et al*., 2007), and young field-grown maple trees (Norby *et al*., 2003). Leaf elongation in *L. decidua* trees was advanced after one growing season of CO_2 enrichment (spring 2002) at the Stillberg site, but there were no significant phenological shifts under elevated CO_2 in subsequent years (Handa *et al*., 2005). Overall, the combined result of increased freezing sensitivity of plants growing under elevated CO_2 without a shift in phenology suggests a physiological, rather than

phenological, mechanism behind the CO_2 effect. To our knowledge, specific physiological changes caused by CO_2 enrichment that reduce the ability of plant cells to tolerate freezing conditions have yet to be determined. Proposed mechanisms include altered biomembrane lipid composition and reduced availability of calcium-binding sites in cell walls and membranes (Beerling *et al*., 2001) and a reduction in membrane stabilizing or osmotically active antifreeze compounds (Obrist *et al*., 2001).

Given the observed earlier start of key phenological events, such as bud break and leaf expansion, of photoperiod-insensitive taxa in response to climate warming (Keller & Körner, 2003), we hypothesized that any such soil warming effects at the alpine treeline would lead to a temporal shift of the period when plants are most sensitive to freezing conditions. However, the warming treatment had little, if any, impact on phenology of the studied plant species. *V. myrtillus* was the only species with a warming effect on phenology, showing earlier leaf expansion in response to warming. Surprisingly, we observed an increased freezing sensitivity in *V. myrtillus* experiencing soil warming. Because warming accelerated phenology, we expected the opposite response of higher resistance in the more mature leaf tissue on a given sampling date (Taschler *et al*., 2004). With our experimental protocol, we cannot interpret the apparent 0.5 K greater freezing sensitivity in more mature leaf tissue of *V. myrtillus*; a higher temporal resolution of freezing resistance dynamics in expanding young foliage would address this question. It seems that the state of freezing resistance is not simply a linear function of leaf aging, but may depend on various physiological processes with different dynamics. As elevated CO_2 also increased freezing sensitivity in *V. myrtillus*, this species, one of the most dominant species at our study site, may be particularly sensitive to freezing events during the early growing season under future atmospheric and climate change. Conversely, *Avenella* exposed to soil warming showed reduced freezing sensitivity, as would be expected with accelerated phenological development. However, phenological observations of this species were not possible.

We acknowledge the limitation of one sampling period per species, for the sake of a larger sampling across many different species, for detecting and understanding warming effects on freezing sensitivity. Also, for some of the less abundant species, plant material was too limited in the CO_2-treated area for several consecutive samplings to achieve better coverage of bud break and leaf expansion dynamics. In interpreting the effects of soil warming, we also note that this treatment was only initiated after all plots were snow-free to avoid the confounding effect of differences in snow cover in our experiment. Earlier snow melt in a warmer climate, resulting in increased plant exposure and advanced phenology in photoperiod-insensitive taxa, can lead to increased damage from springtime freezing events (see Inouye & Wielgolaski, 2003) but could not be tested in the present experiment. On the other hand, there are predictions of enhanced late winter snow falls under climate change and thus the possibility of greater snow pack at elevations >2000 m a.s.l. (IPCC, 2007). Thus, the conditions simulated in our experiment are likely to occur during at least some years in the future, and there is even a realistic possibility of prolonged snow cover duration at very high altitudes.

A totally unexpected but rather interesting finding was related to tree species identity in the experimental plots. Irrespective of CO_2 or warming treatments, *V. gaultherioides* growing under pine had advanced budburst and subsequently had greater freezing resistance than those in plots containing larch. This result suggests that earlier budburst under pine, possibly due to different light conditions or nutrient composition of the tree leaf litter (M. Martin, unpublished data), caused leaves to be more fully mature and therefore hardier when exposed to freezing conditions.

PFT classification as a predictor for freezing resistance in a future climate

Replicate species within deciduous woody (three), evergreen woody (two), and forb (four) PFTs and within tree (two), shrub (three), and herb (five) PGFs permitted us to test the hypothesis that plants within the same classification group would have a similar freezing resistance response to treatments simulating atmospheric and climate change (Dorrepaal *et al*., 2005). Contrary to our prediction, effects of elevated CO_2 and soil warming on freezing sensitivity were not consistent within the three PGFs or within the three testable PFTs. PFT and growth form groupings were clearly less meaningful than species-specific environmental preferences, such as altitudinal distribution and snow cover demand (Gurvich *et al*., 2002; Körner, 2003), for predicting responses to the treatments. Our result suggests that future shifts in the treeline plant community composition due to freezing events are likely to occur at the individual species level.

Conclusions

Our results are the first to demonstrate a CO_2-enrichment-induced decrease in freezing resistance of European treeline plants. Elevated atmospheric CO_2 concentration acted directly on freezing sensitivity and not via altered phenology. Soil warming showed

little to no influence on the phenology and freezing resistance of the sampled species. Advanced phenology occurred in only one of the 10 species tested, while LT50 was shifted in only two species and in opposite directions. Warmer temperatures could, however, have a greater impact if earlier snow melt dates affect species with poor photoperiod control of phenology. Plant responses to the experimental treatments were largely species specific, and grouping into PGFs or PFTs did not contribute to a more general prediction of expected freezing sensitivity under future atmospheric and climate change. In summary, our results suggest that leaf tissue damage caused by episodic early season freezing events will increase in frequency for some species in the coming decades. The resulting shifts in relative freezing resistance among co-occurring species could, in turn, alter competitive interactions among species.

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