



Does the life-history strategy determine the freezing resistance of flowers and leaves of alpine herbaceous species?

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Abstract

In high-mountain habitats, summer frost events can have negative consequences for plant fitness. Despite this, most studies have evaluated the consequences of frosts for vegetative structures of perennial plants, and neither for leaves nor for flowers of annual plants. We hypothesize that the degree of freezing resistance of flowers and leaves of a species depends on its life-history strategy (LHS), and is probably the consequence of a trade-off between growth/reproduction and the cost of the freezing resistance. Specifically, flowers and leaves of short-lived annual species should be less freezing resistant than those of perennial plant species. We compared the freezing resistance of flowers and leaves of 10 annual and 12 perennial plant species from the Andes of central Chile using the electrolyte leakage method. Temperature damage for 50% tissue (LT_{50}) of annual species was -9.6 °C in flowers and -11.9 °C in leaves. In perennial species, LT_{50} was similar in flowers (-12.3 °C) and leaves (-12.5 °C). Despite that, these differences were not significant (except the flowers of annual species), we found remarkable differences between LHS when freezing resistance was analyzed species by species. Like this, 58% and 83% of perennial species resist temperatures ≤ -10 °C in their flowers and leaves, respectively, compared with only 30% and 40% of annual species. Additionally, in most of the species, the freezing resistance of leaves was greater than that of flowers, with this proportion being greater in annual (58%) than in perennial species (43%). Thus, we concluded that the degree of freezing resistance depends on the LHS, such that annual species, which are less freezing resistant than perennial species, have an infrequent occurrence and a distribution restricted to low elevation in high-mountain habitats.

Keywords Alpine plants · Freezing damage · Growing season · Reproduction

Introduction

In high-mountain habitats, low temperatures are one of the main abiotic factors determining plant distribution (Woodward 1987). In these cold habitats, frost events occur throughout the year, but the negative consequences for plants

are greater in spring and summer (Körner 2003). During these periods, plants exhibit their lowest degree of freezing resistance, apparently caused by a compromise between resource allocation to tissue protection and growth/reproduction (Larcher 2003). Therefore, unpredictable frost events during the growing season can have important ecological consequences for high-mountain plants.

Growing season frosts can alter plant development and cause significant damage in vegetative and reproductive structures (Inouye 2000, 2008; Augspurger 2009; Gerdol et al. 2013; Lenz et al. 2013; Vitasse et al. 2014; CaraDonna and Bain 2016). In vegetative structures, an increase in frost exposure can reduce foliar expansion and growth (Gerdol et al. 2013), as well as cause damage to leaves and apical shoots (Rixen et al. 2012). This can cause a loss in stored carbon and nutrients as well as a reduction in the photosynthetic carbon gain capacity (Dumlao et al. 2012). In reproductive structures, frost exposure can cause tissue damage and reduce flower and fruit production (Bokhorst et al. 2008;

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Gerdol et al. 2013). Consequently, the loss of flowers and seeds due to frost may reduce plant fitness, decrease recruitment and alter the relative abundance of those plant species that depend upon sexual reproduction (Bokhorst et al. 2008; Gerdol et al. 2013).

Regardless of the high frost sensibility of some tissues in alpine species, studies have shown that a huge amount of plants that inhabit high mountains are freezing resistant during the growing season. Specifically, the freezing resistance of vegetative structures during growing season has been well studied (e.g. Squeo et al. 1996; Loik et al. 2004; Taschler and Neuner 2004; Bannister et al. 2005; Larcher et al. 2010; Sierra-Almeida et al. 2009, 2010, 2012, 2016; Sklenář et al. 2010; Venn et al. 2013; Pescador et al. 2016). These studies have demonstrated that leaves of alpine plants could resist freezing temperatures ranging from -4° to -10° C in temperate and high latitude mountains (Sakai and Otsuka 1970; Taschler and Neuner 2004; Körner and Alsos 2009). Actually, some alpine plants that inhabit in mountains in dry summers can even withstand temperatures close to -20° C (Squeo et al. 1996; Sierra-Almeida et al. 2009; Venn et al. 2013). Contrary to the well-studied vegetative structures, our knowledge about freezing resistance of reproductive structures in alpine plants is limited and have been focused mainly in alpine plants from the Austrian Alps and Rocky Mountains (Neuner et al. 2013; Ladinig et al. 2013; Caradonna and Bain 2016). In these studies, the reproductive structures were found to be more sensitive to freezing damage than vegetative structures. For example, Caradonna and Bain (2016) found in the eight studied perennial species that floral tissue was able to tolerate freezing temperatures between -1.9° and -10° C. Likewise, Ladinig et al (2013) reported that the mean LT_{50} was around -4° C during bolting and anthesis in seven perennial species. Despite these studies, there is still much to be learned about how freezing resistance may differ among plant organs and which could be the intrinsic and/or environmental factors that could determine the degree of freezing resistance, especially in reproductive structures.

Some authors assume an inverse relationship between the freezing resistance ability of a species and its growth rate during summer (Sakai and Larcher 1987; Neuner and Beikircher 2010; Wringer 2015; Bucher et al. 2019). For example, Bucher et al. (2019) reported a negative relationship between freezing resistance and specific leaf area (a proxy of growth rate), confirming this trade-off and, as stated by the authors, also in line with plant economics spectra (Wright et al. 2004; Reich 2014; Díaz et al. 2004). If the arguments raised by these authors are valid, then contrasting LHS (i.e., perennial vs. annual) should have differences in the ability to resist freezing temperatures in vegetative and reproductive structures in response to their inherent growth rates. Therefore, annual species with short lifespans

and high growth rates, which produce aboveground organs rapidly after snowmelt and die at the end of the growing season, are expected to be less freezing resistant than perennial species with a long lifespan and low growth rate, which invests resources in underground organs to persists beyond the growing season.

It is noteworthy that the freezing resistance of flowers and/or leaves in annual species, inhabiting high mountain ecosystems, has been rarely studied (e.g., Sierra-Almeida et al. 2010; Pescador et al. 2016). The lack of studies dealing with the freezing resistance of annual species may be due to their lower presence compared to perennial species (1–2% of the total flora in high mountain areas are annual species: Billings and Mooney 1968; Billings 1974), which is presumably due to the difficulty of completing their life cycle during a single, short, and cold growing season (Billings and Mooney 1968; Billings 1974). Additionally, one of the possible explanations of their low abundance and restrictive altitudinal distribution toward higher elevations could be their degree of freezing resistance, but this has not been previously studied. Given this distributional pattern, it is particularly interesting to study how much sensitive to freezing are flowers and leaves of annual species in comparison to herbaceous perennial that dominate alpine habitats.

In the central Chilean Andes, as occurs in other seasonal alpine habitats worldwide, plants' growth and reproduction are restricted to spring and summer, when the snow has melted and temperatures are warm (Arroyo et al. 1981; Cavieres et al. 2006, 2007). Moreover, this high elevation zone is influenced by a Mediterranean climate type, which imposes soil moisture limitations for plants, resulting in an even shorter period for growth and reproduction (Arroyo et al. 1981; Sierra-Almeida et al. 2016). Despite these abiotic constraints, a great proportion of the alpine plants in the Andes of central Chile depends highly on sexual reproduction (Arroyo et al. 1981). Even at higher elevations, plants have developed strategies to increase their sexual reproduction success, such as long flowering periods and slower floral development (Arroyo et al. 1981; Torres-Díaz et al. 2011; Pacheco et al. 2016). Unlike the global pattern, where annual plants are rare in high-mountain habitats (Billings and Mooney 1968; Billings 1974; Chabot and Billings 1972), in the central Chilean Andes, annual plants are particularly abundant (representing 10% of total plant cover at the Sub-Andean belt, Arroyo et al. 1981; Cavieres et al. 2000), turning this alpine ecosystem into an attractive scenario to compare the freezing resistance of annual and perennial species.

Thus, we investigated the summer freezing resistance of flowers and leaves of 10 annual and 12 perennial herb species inhabiting the Central Chilean Andes. We expected that freezing resistance depends on LHS, where annual herbs should have a lower freezing resistance than perennial herbs in flowers and leaves. Additionally, we explored and

discussed the differences in the freezing resistance of flowers and leaves and how plant traits such as flower and leaf morphology, phenology, and elevation distribution determine the freezing resistance.

Materials and methods

Study site and species

This study was carried out near Farellones (33°17' S 70°25' W), in the central Chilean Andes, 50 km east of Santiago. The climate in this area is alpine with a strong Mediterranean influence, which predominates at lower elevations, generating a dry growing season (Santibañez and Uribe 1990). This area is characterized by the dominance of shrubs and dwarf shrubs such as *Anarthrophyllum cumingii*, *Chusqueira oppositifolia* and *Tetraglochin alatum*, perennial herbs such as *Alstroemeria exerens* and *Stachys philippiana*, and annual species such as *Chaetanthera* spp, *Collomia biflora* and *Microsteris gracilis* (lower Andean vegetation belt, Teillier et al. 2011).

The growing season begins with the snowmelt in September–October and ends in April with the first snowfall (Sierra-Almeida and Cavieres 2012). During this period, the daily mean air temperature is 15.6 °C, with daily maximum and minimum temperatures of 32 and 5 °C, respectively (average temperature between 2200 and 2800 m above sea level; Table 1). Frost events are distributed throughout the growing season, though they are more frequent at the beginning and end of this season (October, November and March). There is a high inter-annual variation in the number and intensity of frost events, ranging from 2 to 59 frost events per growing season (2009–2010). The duration of these frost events is also highly variable, ranging from 1 to 14 h (2011–2012), and air temperatures can drop below –10 °C (Table 1, Fig. S1).

A total of 22 species were studied, including 10 annual and 12 perennial herbaceous species (Table 2). Plant material for each studied species was collected in the sub-Andean belt (2100–2500 m, Cavieres et al. 2000), with the exception of *Alstroemeria exerens*, *Draba gilliesii*, *Hordeum comosum*, *Ipheion sessile* and *Perezia carthamoides* which were collected at 2800 m above sea level, in the lower range of the lower-Andean belt (2600–3400 m, Cavieres et al. 2000). The studied species were primarily selected based on their observed abundance in the plant herbaceous community. Then, we considered the morphological characteristics of vegetative (leaves) and reproductive structures (flowers/inflorescences), which should be present simultaneously to determine their respective freezing resistances (Table 2). Plant material was collected between 17-Oct-2017 and 01-Jan-2018, coinciding with the flowering peak of each studied species (see Table 2 for collection date).

Plant tissue collection

Flowers and leaves were collected from individual plants selected randomly ($n=7$ per species). Plant tissue of all of the studied species was collected at the same developmental stage. For reproductive structures, flowers and inflorescence were collected during anthesis (corolla open and flower sexually functional; Neuner et al. 2013). For vegetative structures, only healthy, mature and fully expanded leaves were collected. Seven flowers and seven leaves were collected from each individual plant; each one was covered with a moist paper towel and immediately placed inside a plastic sealed bag (total = 49 flowers and 49 leaves per species). In cases where some individuals did not have enough flowers and leaves, the plant samples were completed with tissue obtained from the closest individual. Plant samples were stored inside a cooler to avoid changes in the tissue water status. Within 2 h of collection, all plant samples were transferred to a refrigerator at 4 °C until freezing treatments

Table 1 General characteristics of the frost events that have occurred during the growing season in the central Chilean Andes

Elevation	Growing season	Daily mean temperature (°C) ± SE	Daily mean minimum temperature (°C) ± SE	Daily mean maximum temperature (°C) ± SE	Number of frosts events	Frost intensity (°C) ± SE	Frost duration (h below 0 °C) ± SE	Absolute minimum temperature (°C)	Date of min. temperature (day/month/year)
2200 m	2017–2018	17.7 ± 0.2	6.4 ± 0.3	35.6 ± 0.9	2	–0.4 ± 0.4	1	–0.9	21-11-17
	2009–2010	5.9 ± 0.4	2.6 ± 0.5	9.7 ± 0.4	59	–1.7 ± 0.2	11.9 ± 2.0	–12.1	05-09-09
	2011–2012	7.9 ± 0.3	4.7 ± 0.3	11.3 ± 0.3	26	–1.4 ± 0.3	14.2 ± 4.4	–10.7	06-09-11
2800 m	2013–2014	8.3 ± 0.3	5.8 ± 0.3	12.0 ± 0.3	13	–0.9 ± 0.2	6.2 ± 1.9	–4.6	10-11-13
	2014–2015	9.6 ± 0.3	6.6 ± 0.3	12.8 ± 0.3	7	–1.2 ± 0.4	13 ± 5.9	–5.3	29-11-14
	2017–2018	13.5 ± 0.2	3.4 ± 0.2	28.7 ± 0.7	27	–1.5 ± 0.1	4.4 ± 0.6	–5.3	16-11-17

*Air temperature data were recorded every 1 h at weather stations (HOBO micro station, Onset Computer Corporation) located 1.5 m above the ground. For the growing season, 2017–2018 data were recorded with a Temperature/Relative Humidity Data Logger (HOBO Pro v2.6 Ext., Onset Computer Corporation) located 50 cm above the ground. Standard error (SE) in all cases is standard error of the mean

Table 2 Taxonomic, morphological and geographical information for 22 herbaceous species from the central Chilean Andes included in the freezing resistance determinations

Species	Family	Growth form	Leaf type	Flower type	Date of collection	Altitudinal distribution in the area (masl)
Annual species						
<i>Chaetanthera flabellata</i>	Asteraceae	Rosette	Simple lanceolate	Inflorescence capitulum	Dec.17	2200–3500
<i>Chaetanthera linearis</i>	Asteraceae	Forb	Simple linear	Inflorescence capitulum	Dec.28	NA–2200
<i>Collomia biflora</i>	Polemoniaceae	Forb	Simple ovate	Terminal inflorescence	Nov.17	2100–2400
<i>Lupinus microcarpus</i>	Fabaceae	Forb	Compound	Solitary flower (a group of 6)	Nov.17	2100–2500
<i>Madia sativa</i>	Asteraceae	Forb	Simple lanceolate	Inflorescence capitulum	Dec.28	2300–2500
<i>Microsteris gracilis</i>	Polemoniaceae	Forb	Simple lanceolate	Solitary flower	Oct.17	2100–3100
<i>Phacelia brachyantha</i>	Hydrophyllaceae	Forb	Simple elliptical	Terminal inflorescence	Nov.17	1800–2200
<i>Quinchamalium parviflorum</i>	Schoepfiaceae	Forb	Simple linear-fleshy	Spherical inflorescence	Dec.28	2300–2800
<i>Schizanthus hookeri</i>	Solanaceae	Forb	Simple pinnatisecta	Inflorescence cyme	Dec.28	1800–2300
<i>Scyphanthus elegans</i>	Loasaceae	Forb	Simple pinnatisecta	Inflorescence cyme	Dec.28	1800–2300
Perennial species						
<i>Acaena splendens</i>	Rosaceae	Forb	Compound	Inflorescence like-spikes	Nov.17	2100–2400
<i>Alstroemeria exerens</i>	Alstroemeriaceae	Forb	Simple ovate-elliptic	Small umbels	Jan.29	2300–2700
<i>Barneoudia chilensis</i>	Ranunculaceae	Rosette	Simple obovate	Solitary flower	Oct.17	2320–2700
<i>Draba gilliesii</i>	Brassicaceae	Forb	Simple oblong	Inflorescence corymbs	Nov.17	2700–3550
<i>Hordeum comosum</i>	Poaceae	Grass	Simple linear	Spike inflorescence	Jan.29	1900–3800
<i>Ipheion sessile</i>	Amaryllidaceae	Forb	Simple linear	Umbel inflorescence	Nov.17	NA-2700
<i>Olsynium junceum</i>	Iridaceae	Forb	Simple cylindrical	Solitary flower	Oct.17	2500–3500
<i>Oxalis cinerea</i>	Oxalidaceae	Forb	Compound	Terminal inflorescence	Oct.17	2100–2900
<i>Perezia carthamoides</i>	Asteraceae	Forb	Simple linear	Inflorescence capitulum	Jan.29	1800–2100
<i>Poa holciformis</i>	Poaceae	Grass	Simple linear	Spike inflorescence	Oct.17	2300–3300
<i>Sanicula graveolens</i>	Apiaceae	Forb	Compound	Umbel inflorescence	Nov.17	2300–2900
<i>Stachys philippiana</i>	Lamiaceae	Forb	Simple oblong	Solitary flower	Nov.17	1800–2300

*Altitudinal distribution and morphological traits according to Teillier et al. (2011); Cavieres et al. (2000); Hoffmann et al. (1998) and personal observations. Plant nomenclature follows Rodríguez et al. (2018)

started. To avoid senescence and dehydration of the plant samples, freezing resistance determinations were carried out in a field laboratory within 24 h after sample collection.

Frost experiments

A cryostat (F34-ME, Julabo Labortechnik GmbH, Germany) was used to expose the flowers and leaves to six independent freezing treatments: – 5, – 7.5, – 10, – 12.5, – 15 and

– 20 °C. The selection of the freezing treatments was based on earlier frost records during the growing season (Table 1, Fig. S1) and previous data on the freezing resistance of leaves for species in the study area (Sierra Almeida et al. 2009, 2010, 2016). For each of the six freezing treatments and the control, seven single flowers or capitula (Table 2; *C. flabellata*, *C. Linearis*, *M. sativa* and *P. carthamoides*) and seven leaves from different individuals were used for each species. All the flowers were cut near the base of the pedicel,

below the receptacle and the leaves were cut near the base of the petiole. Detached flowers and leaves were placed inside hermetically sealed Falcon tubes (15 or 50 ml depending on the size of the sample) and put inside the cryostat. For the studied species, flower and leaf size is a much-conserved trait. Therefore, the amount of tissue placed inside Falcon tubes was standardized by size, using flowers and leaves of similar size.

Frost simulations started at 0 °C and at a cooling rate of -5 K h^{-1} every target temperature was reached within 1–4 h. After this, the samples were kept for 2 h at the target temperature and were then returned to 0 °C at a thawing rate of 5 K h^{-1} inside the cryostat. The cooling rate was selected according to the maximum cooling rates during natural summer freezing events in the Central Chilean Andes, obtained from previous weather station recordings located 1.5 m above the ground. Likewise, the time of exposition of the samples to the target temperature was selected to conform to summer frost characteristics in the study area. In natural frosts, when the minimum temperature is reached, it is maintained for approximately 2 h, then the temperature starts to rise again (Fig. S1). It should be noted that the highest target temperature (-5 °C) keeps the plants for 4 h at temperatures below zero, while the lowest target temperature (-20 °C) keeps them at temperatures below zero for 10 h, which almost entirely cover the variations in the frost duration for the study area (Table 1).

Assessment of freezing damage

The temperature that caused 50% damage (LT_{50}) in flowers and leaves was estimated by using the membrane electrolyte leakage method. This method is based on the increase in electrolyte leakage (mainly K^+) induced by the loss of integrity and stability of the cell membranes (Wilner 1960; Lipp et al. 1994). This method was chosen over others (e.g., photoinactivation, visual inspection), because it allows assessment and comparison of the freezing damage of both plant structures: flowers and leaves. LT_{50} in flowers was estimated in the whole functional structure, as fertile and infertile tissues play important roles in promoting/protecting sexual reproduction (e.g., corolla has a role in the attraction of pollinators and herbivory defense) and seed production (i.e., stamens and pistils).

Once each temperature treatment was finished, the tubes were removed from the cryostat and 10 ml of deionized water was then added to each tube. The tubes were kept at ambient temperature in a shaker for 6 h (18 °C approximately). Afterward, the electrical conductivity (EC) of the solution was measured with a conductance/resistance meter (Hanna

HI 8733, Hanna Instruments, Woonsocket, RI, USA). After measuring the EC, the tubes were enclosed in a boiling water bath for 30 min to obtain the maximum ion leakage by freezing-induced damage. Boiling plant tissue to achieve complete electrolyte leakage is a conventional procedure for determining tissue freezing resistance with the membrane leakage method (e.g., Wilner 1960; Arias et al. 2015; Zhang et al. 2016).

The relative electrical conductivity (REC), an indicator of membrane electrolyte leakage, was calculated for each sample as a percentage:

$$\text{REC} = (\text{EC after freezing treatment} / \text{EC after boiling}) \times 100.$$

In addition, given that the samples' manipulation and the incubation in deionized water can alter the REC, a control treatment was carried out for each species. Falcon tubes with tissue samples (7 replicates per tissue incubated separately in 10 ml of deionized water) were incubated at ambient temperature for 10 h, the time needed to achieve the longest freezing treatment (-20 °C). After that, the same procedure used to measure the relative EC after freezing treatments was used to obtain the control treatment's EC. Then, a corrected relative EC was calculated as follows:

$$\begin{aligned} \text{Corrected REC} &= \text{REC of the freezing treatment} \\ &\quad - \text{REC of the control treatment.} \end{aligned}$$

The temperature that produced 50% damage (LT_{50}) was determined by linear interpolation using the temperature that caused the highest percentage of electrolyte leakage (corrected REC) of $< 50\%$ and the temperature that caused the lowest corrected REC of $> 50\%$.

Data analysis

The differences in freezing resistance (LT_{50}) of flowers/leaves between annual and perennial herbs species were evaluated using the non-parametric Kruskal–Wallis because LT_{50} data did not comply with the required assumptions of parametric models (bimodal distribution). Given that 22 species with different morphological and phenological characteristics were included in this study, a linear model (LM) was used to determine the extent of the relationship between the flower/leaf freezing resistance and the independent variables: life-history strategy (LHS), flower and leaf type, altitudinal distribution, elevation and date of measurement (Tables S1 and S2). We assumed a Gaussian distribution and checked the normal distribution of model residuals to confirm goodness of fit. We also checked the plotted residuals of the model to ensure homoscedasticity. All the statistical analysis was performed in RStudio (Version 1.1.463—© 2009–2018 RStudio, Inc.).

Results

Effect of LHS on the freezing resistance of flowers and leaves

Linear model analysis indicated that LHS significantly affects the freezing resistance of flowers and leaves ($p < 0.0001$) (Tables S1, S2). When all species were taken together, the mean LT_{50} of annual species was -9.6 ± 0.6 °C and -11.9 ± 0.8 °C, in flowers and leaves, respectively. In perennial species, the mean LT_{50} for flowers was -12.3 ± 0.3 °C, while for leaves it was -12.5 ± 0.5 °C (Fig. 1). LT_{50} of flowers differed significantly between annual and perennial species ($p < 0.0001$). LT_{50} of flowers was on average 2.7 K lower (more negative) in perennial than in annual species (Fig. 1). However, there were no significant differences in the LT_{50} of leaves between annual and perennial plants.

It is important to note that freezing resistance among species in both LHS differed significantly (Table 3; Fig. 2). In annual species, LT_{50} of flowers ranged from -5.5 ° (*P. brachyantha*) to -20 °C (*C. linearis*), while LT_{50} of leaves ranged from -6.6 ° (*C. biflora*) to -20 °C (*C. flabellata*, *C. linearis* and *L. microcarpus*). In perennial species, LT_{50} of flowers ranged from -6.2 ° (*A. exerens*) to -19 °C (*D. gilliesi*), while LT_{50} of leaves ranged from -7.3 ° (*P. holciformis*) to -20 °C (*A. splendens*). Notwithstanding that the LT_{50} of flowers and leaves had similar values in annual and perennial species, when species were analyzed case by case, meaningful patterns came to light. Specifically, perennial species had a greater safety margin for frosts than

annual species, both in their flowers and leaves. 58% and 83% of perennial species resisted temperatures ≤ -10 °C in their flowers and leaves, respectively (Fig. 2). In annual species, only 30% and 40% of the studied species resisted temperatures ≤ -10 °C in flowers and leaves, respectively (Fig. 2).

Freezing resistance differences between flowers and leaves

Interestingly, we observed significant differences in the mean LT_{50} between flowers and leaves (Fig. 3; Table 3). LT_{50} of leaves was 1.2 K lower (more negative) than LT_{50} of flowers ($p < 0.05$) when all species were evaluated together. This general result coincided with that observed in the majority of the studied species, wherein 56% of annual and 42% of perennial studied species, leaves were, on average, 5.6 K more freezing resistant than flowers (LT_{50} difference ranged from 2.1 K in *S. philippiana* to 10.1 K in *L. microcarpus*). Moreover, in 22% of annuals and 25% of perennial studied species, flowers and leaves resisted similar freezing temperatures (Table 3). Interestingly, in 22% of annuals and 33% of perennial studied species, flowers were on average 5.2 K more freezing resistant than leaves, with a LT_{50} difference of 2.5 K in *C. biflora* to 9.1 K in *M. gracilis* (Table 3).

Linear model analysis indicated that the five predictors (i.e., LHS, flower and leaf type, altitudinal distribution, elevation, and date of measurement) explained 66% and 75% of the variance of flowers' and leaves' freezing resistance, respectively ($p < 0.0001$). In flowers, the date of measurement ($R^2 = 0.29$, $F_{(3,111)} = 32.4$, $p < 0.0001$) and the altitudinal distribution ($R^2 = 0.14$, $F_{(1,111)} = 47.5$,

Fig. 1 Summer freezing resistance (LT_{50}) measured in the flowers and leaves of 10 annual and 12 perennial herbs species. Boxes indicate the median (= second quartile; line inside the box) and extend from the first to the third quartile. The whiskers show at maximum the 1.5-fold interquartile range. Black circles represent mean values. Different superscripts indicate significant differences in LT_{50} between organs ($p < 0.05$)

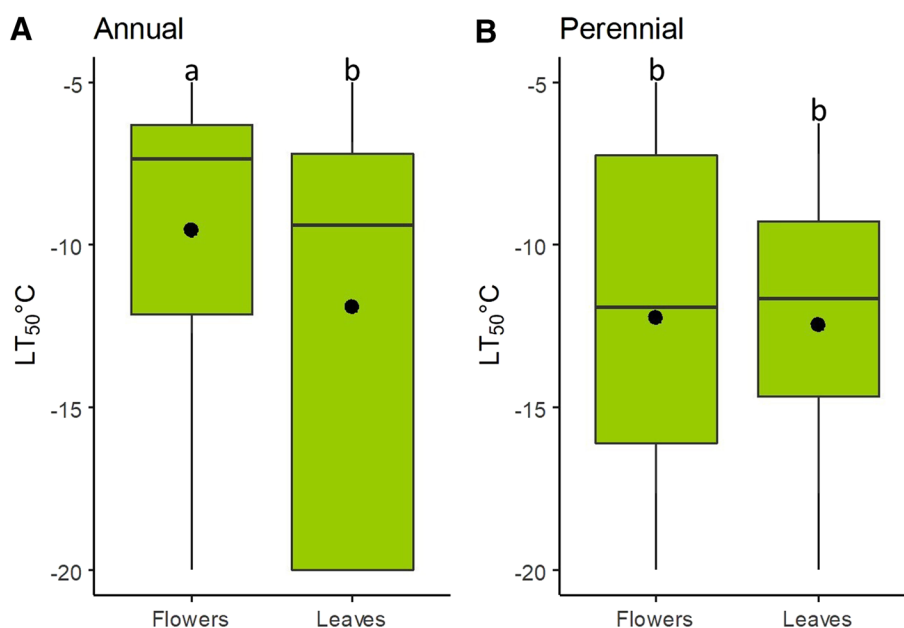


Table 3 Freezing resistance of leaves and flowers measured during the growing season in 10 annual and 12 perennial herb species from the Andes of central Chile

Species	Leaf freezing resistance	Flower freezing resistance	Thermal difference	Trend
	LT ₅₀ (°C)	LT ₅₀ (°C)		
Annuals herbs				
<i>Chaetanthera flabellata</i>	-20.0±0.0	-12.9±0.2	7.1**	L>F
<i>Chaetanthera linearis</i>	-20.0±0.0	-20.0±0.0	0.0	ND
<i>Collomia biflora</i>	-6.6±0.5	-9.1±0.9	2.5*	L<F
<i>Lupinus microcarpus</i>	-20.0±0.0	-9.9±0.9	10.1**	L>F
<i>Madia sativa</i>	-9.3±1.1	-6.4±0.4	2.9*	L>F
<i>Microsteris gracilis</i>	-8.2±0.6	-17.9±0.3	9.8**	L<F
<i>Phacelia brachyantha</i>	-8.1±0.4	-5.5±0.4	2.6***	L>F
<i>Quinchamalium parviflorum</i>	-11.8±0.4	-7.4±0.6	4.4**	L>F
<i>Schizanthus hookeri</i>	-7.5±0.8	-7.5±0.6	0.1 ^{ns}	L=F
<i>Scyphanthus elegans</i>	-7.0±0.6	-6.5±0.2	0.5 ^{ns}	L=F
Perennial herbs				
<i>Acaena splendens</i>	-20.0±0.0	-14.4±1.1	5.6**	L>F
<i>Alstroemeria exerens</i>	-10.9±1.0	-6.2±0.4	4.6**	L>F
<i>Barneoudia chilensis</i>	-7.8±0.4	-11.3±1.9	3.4 ^{ns}	L=F
<i>Draba gilliesii</i>	-12.7±0.8	-19.0±0.9	6.3*	L<F
<i>Hordeum comosum</i>	-11.5±0.8	-14.3±0.1	2.9*	L<F
<i>Ipheion sessile</i>	-12.7±1.1	-11.8±0.9	0.9 ^{ns}	L=F
<i>Olsynium junceum</i>	-15.5±0.9	-19.9±0.1	4.4**	L<F
<i>Oxalis cinerea</i>	-16.2±1.6	-9.0±0.8	7.2**	L>F
<i>Perezia carthamoides</i>	-12.5±0.8	-17.7±1.3	5.2**	L<F
<i>Poa holciformis</i>	-7.3±0.3	-8.9±0.8	1.6 ^{ns}	L=F
<i>Sanicula graveolens</i>	-16.3±1.2	-6.8±0.3	9.5***	L>F
<i>Stachys philippiana</i>	-10.5±0.4	-8.5±0.8	2.1***	L>F

LT₅₀ are shown as mean ± standard error. Thermal differences are shown as level of significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and ^{ns} not significant. Trend: L>F: leaves (L) had greater freezing resistance than flowers (F), L=F: there were no differences and L<F: flowers had greater freezing resistance than leaves

$p < 0.0001$) explained most of the variance. In leaves, the leaf type ($R^2 = 0.55$, $F(5, 93) = 40.9$, $p < 0.0001$) and the date of measurement ($R^2 = 0.09$, $F(3, 93) = 11.3$, $p < 0.0001$) explained most of the variance (Tables S1, S2).

Discussion

In this study, we evaluated the importance of life-history strategy (LHS) on the freezing resistance of flowers and leaves in a subalpine community of the central Chilean Andes. In particular, our results showed that perennial species were more freezing resistant than annual species, considering reproductive and vegetative structures. Besides, in most of the studied species, leaves were more freezing resistant than flowers, indicating that the higher freezing resistance of leaves compared to flowers is independent of the LHS, and could be a common feature in alpine plants. To the best of our knowledge, this study represents the first report that simultaneously determines the freezing resistance

of reproductive and vegetative structures in herbaceous species, including the highest number of annual alpine species reported to date.

Perennial species are more freezing resistant than annual species

As we expected, LHS has a significant effect on the freezing resistance, where perennial species were more freezing resistant, in flowers and leaves, than annual species. As we stated in our hypothesis argumentation, this pattern could be explained by trade-off between growth rates and freezing resistance (Sakai and Larcher 1987; Neuner and Beikircher 2010; Wrangler 2015; Bucher et al. 2019). By definition, annual plant reproduction is sexual and occurs once (Bazzaz and Morse 1991). To assure reproductive success, annual plants have a high growth rate in the pre-reproductive phase and invest most of their energetic and mineral resources into reproduction (Bazzaz et al. 1987; Hancock and Pritts 1987; Garnier 1992). Resource availability is finite; consequently,

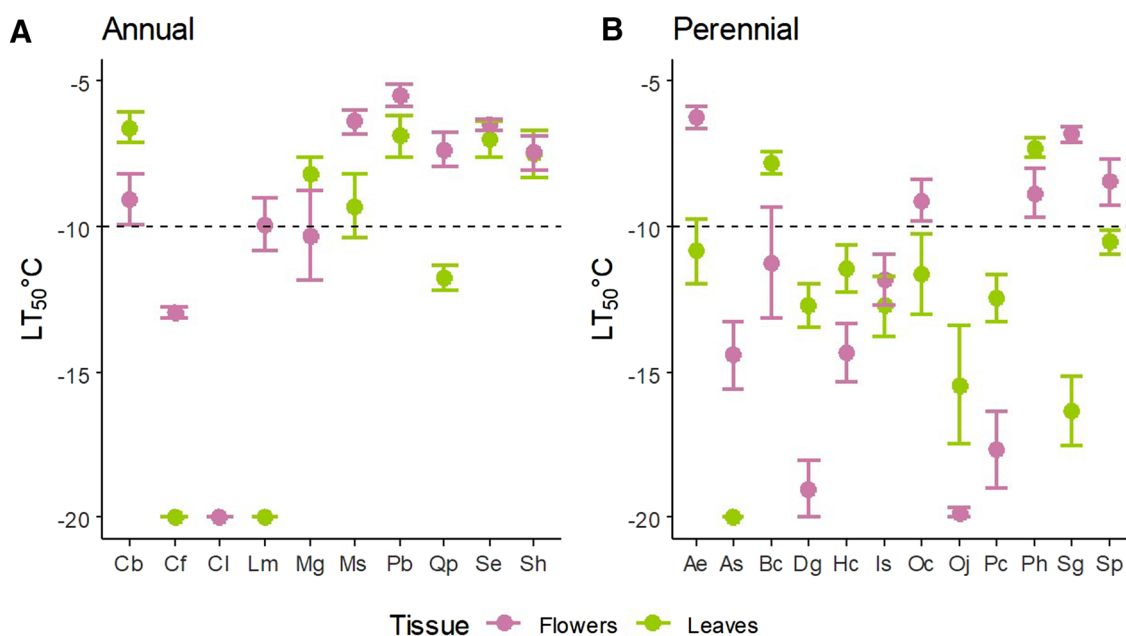


Fig. 2 Summer freezing resistance (LT_{50}) measured in the flowers (pink) and leaves (green) of 10 annual and 12 perennial herbs species. Black circles represent mean values and the whiskers show the standard error

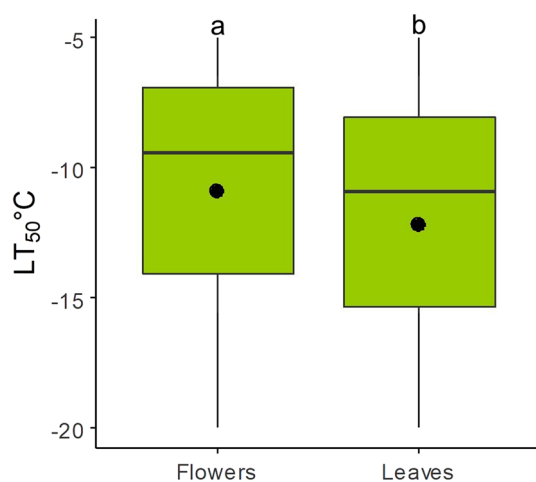


Fig. 3 Summer freezing resistance (LT_{50}) measured in the flowers and leaves of 22 alpine species. Boxes indicate the median (=second quartile; line inside the box) and extend from the first to the third quartile. The whiskers show at maximum the 1.5-fold interquartile range. Black circles represent mean values. Different superscripts indicate significant differences in LT_{50} between organs ($p < 0.05$)

the high-resource demands for rapid growth and reproduction could produce a trade-off with other functions, in this case, with freezing resistance. The resource allocation trade-offs between growth and freezing resistance have been described in leaves (Neuner and Beikircher 2010; Wringer 2015; Bucher et al. 2019). For example, Neuner and Beikircher (2010) found that when *Picea abies*' leaves were

elongating, the onset of the mesophyll cell wall thickening coincided with the lowest freezing resistance, suggesting that the ongoing metabolic and cytological changes leave little leeway for frost hardening in cells during elongation growth. Although this trade-off has not yet been assessed in reproductive structures, this suggests that it could also be occurring and could be even more severe given the high costs of production and maintenance of flowers, and their low contribution to the energetic balance at the whole plant level, which would explain its lower freezing resistance both in perennial species but especially in annual species. On the other hand, perennial plants allocate more resources to vegetative tissues and reserve organs. They have a longer growth period than annual plants (Bazzaz et al. 1987; Arroyo et al. 1981). For example, a perennial's transition development phase is longer than that of annual plants, due to a lower relative growth rate (Garnier 1992). This implies that during their lifespan, perennial herbs perform more photosynthesis and are highly productive; it also implies that they use their resources conservatively. This conservative use of resources allows them to store non-structural carbohydrates for perennial growth, as well as allow them to respond to resource imbalances provoked by environmental stresses (Bazzaz and Morse 1991). Given the above, perennial species avoid or diminished the trade-off between growth and freezing resistance, which could be the main explanation for the higher freezing resistance in flowers and leaves compared to those of annual plants.

On one hand, these results suggest that perennial species invest in highly effective freezing protection mechanisms to cope with summer frosts. On the other hand, it reflects the fact that, since the tissues of annual species tend to be more sensitive to freezing temperatures (especially in flowers), the avoidance mechanisms that hinder and/or prevent the formation of ice should have a more significant role in the protection of these tissues against frost damage. Whether the freezing resistance is a response to the type of the underlying mechanism or is related to temporal or spatial escape/avoidance strategies is still uncertain. Therefore, it should be considered for future research.

Leaves were more freezing resistant than flowers regardless of LHS

Overall, our results showed that leaves were more freezing resistant than flowers (56% of annual and 42% of perennial studied species, respectively), coinciding with previous reports for Austrian alpine species (Neuner et al. 2013; Ladinig et al. 2013) and subalpine species from the Rocky Mountains (CaraDonna and Bain 2016). Arguments explaining a lower frost resistance in flowers compared to leaves seem to be related to the resource-use efficiency of plants. Provided that the lifespan of most plants' reproductive structures is shorter than that of their vegetative structures, their time exposure to potential stressors during the growing season is therefore also shorter. For example, the flower longevity of herbaceous species from the Rocky Mountains is expected not to exceed 21 days (Ashman and Schoen 1994). In the central Chilean Andes, flower longevity is even shorter, varying from 4 to 9 days at 2320 m and 3550 m, respectively (Arroyo et al. 1981, 2013; Pacheco et al. 2016). In contrast, leaf lifespan in herbs from the tropical Andes at elevations between 4000 and 4600 m was on average 193 days (Diemer 1998), at least 90% more than flowers' lifespan. At the same time, the beginning of flowering is a highly regulated process, where environmental signals for flowering (temperature and photoperiod, Amasino and Michaels 2010) allow plants to avoid conditions that are detrimental to reproduction.

Likewise, it is important to consider that a flower's maintenance is expensive as a sink organ (e.g., carbon metabolism, transpiration, pigment, and nectar production; Ashman and Schoen 1997; Stead et al. 2008) compared with leaves, which also act as a source of resources. In this sense, a trade-off involving resource allocation to flower building/maintenance and flower protection against freezing stress could be assumed. Thus, allocating resources to develop physiological mechanisms to protect flower structures from freezing damage becomes very expensive in a system where resources are limited and time to complete reproduction is short. This does not mean that reproductive structures are

not able to cope with freezing temperatures. Indeed, the mean freezing resistance for high-Andean species' flowers was c. $-11\text{ }^{\circ}\text{C}$ (Fig. 3), which is below the magnitude of frost events that frequently occur in the central Chilean Andes during the growing season (Sierra-Almeida et al. 2009, 2016; Sierra-Almeida and Cavieres 2012), offering a margin of safety for most of the studied species. These species most likely cope with summer frost events in their reproductive structures through freezing avoidance/escape strategies, related to spatial and/or temporal exclusion (e.g., plant architecture, structure and anatomy; Larcher 2003).

In this regard, in our study, the type of inflorescence significantly affects the degree of freezing resistance of the flowers ($p < 0.0001$; Table S2), where flowers arranged in a capitulum showed a greater degree of freezing resistance (except for *Madia sativa*) compared to solitary or erect inflorescences. Great freezing resistance of capitula could be the result of the temporal and/or spatial exclusion of ice formation through the arrangement of flowers, where small and numerous flowers are spatially distributed in a disk-like flowerhead. A compressed arrangement of flowers can buffer the daily temperature fluctuation, maintaining steady temperatures inside the inflorescence, and consequently retarding flower frost damage (LV Morales, UDEC, Concepción, Chile, unpubl. res). Capitulum inflorescence is present in plants of the *Chaetanthera* genus (Asteraceae, annual plants), where the freezing resistance of inflorescences ranged from -13° (*C. flabellata*) to below $-20\text{ }^{\circ}\text{C}$ (*C. linearis*). For the perennial herb, *Perezia carthamoides*, its capitulum was the most freezing resistant ($-17.7 \pm 1.3\text{ }^{\circ}\text{C}$; Table 3).

It is important to highlight that the timing of flowering is also one of the factors that determines freezing resistance of flowers ($R^2: 0.29$, $F_{(3,111)} = 32.4$, $p < 0.0001$, Table S3). For example, *Barneoudia chilensis* and *Olsynium junceum*, the first species to bloom at 2300 m, showed high values of flower freezing resistance ($-11.3\text{ }^{\circ}\text{C}$ and $-19.9\text{ }^{\circ}\text{C}$, respectively), similar to *Draba gilliesii* and *Ipheion sessile* ($-19.0\text{ }^{\circ}\text{C}$ and $-11.8\text{ }^{\circ}\text{C}$, respectively), the first species to bloom at 2800 m. High values of freezing resistance in early flowering plants have also been reported by CaraDonna and Bain (2016) and could be a strategy to decrease the probability of being damaged by a frost event (Lenz et al. 2013), which occur more frequently at the beginning of the growing season (Inouye 2008). The high degree of freezing resistance in flowers may be a response to the temperature that flowers have experienced during their development, since cold conditions may decrease flower vulnerability to frost (Proebsting and Mills 1978; Anderson and Seeley 1993; Lu and Rieger 1993; Rodrigo 2000).

Interestingly, 22% and 33% of the annual and perennial studies species' flowers proved to be more resistant to freezing temperatures than their leaves, and in 22%

and 24% of the annual and perennial species flowers and leaves resisted similar freezing temperatures (Table 3). The presence of alternative patterns of freezing resistance differences between reproductive and vegetative structures suggests a complex and integrated combination of life-history traits that could be determining the ability of these alpine species to resist freezing temperatures. In this respect, further studies should include additional life-history traits at the whole plant level that represent the available internal resource pool. Additionally, the timing of the biological process and microhabitat preferences of each species should be considered further, to try to understand how often the different tissues face freezing temperatures and, thus, how important it is for them to have freezing resistance.

In summary, we found a close relationship between LHS and the freezing resistance, where flowers and leaves of perennial species were more freezing resistant than those of annual species. This finding suggests that the freezing resistance could be an important functional trait to explain the altitudinal distribution of herbaceous species, since annual species that were found to be more frost sensitive tended to have a more limited altitudinal distribution, inhabiting and being more abundant at low elevations. Additionally, and confirming previous reports, we found that the most common pattern was that flowers were more sensitive to freezing damage than leaves (particularly in annuals, where 58% of the species shows this pattern). In this sense, annual plants in high-mountain habitats can be particularly sensitive to changes in frost regimes as a consequence of climate change. Therefore, it is crucial to develop monitoring plans in the central Chilean Andes, to protect this unique habitat that houses a particularly large number of annual species that survive and are successful in an environment dominated by perennial plants.

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Author contributions LVM and AS-A conceived and designed the research; LVM, CAC and CS carried out the collection and data analysis. LVM interpreted the results and wrote the manuscript with support from AS-A and AS.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study does not involve research on human participants or animals.

Informed consent Informed consent was obtained from all individual participants included in the study.

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