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Frost sensitivity of leaves and flowers of subalpine plants is related to tissue type and phenology

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Summary

1. Harsh abiotic conditions – such as low temperatures that lead to spring and summer frost events in high-elevation and high-latitude ecosystems – can have strong negative consequences for plant growth, survival and reproduction.

2. Despite the predicted increase in episodic frost events under continued climate change in some ecosystems, our general understanding of the factors associated with frost sensitivity of reproductive and vegetative plant structures in natural plant communities is limited. The timing of growth and reproduction may be an important strategy by which plants can avoid frost.

3. In this study, we experimentally investigated the frost sensitivity of eight long-lived perennial herbaceous plant species from a subalpine ecosystem in the Colorado Rocky Mountains, USA. The study taxa represent four congeneric pairs from four flowering plant families; within each pair, there is a species with early and late growth and reproductive phenology. Thus, we control for evolutionary history – and therefore additional traits shared through common ancestry – to some degree, while examining the influence of phenology on frost sensitivity. Specifically, we compared frost sensitivity of vegetative and reproductive structures for each species and asked whether frost sensitivity was similar between species within congeneric pairs or, instead, was related to phenology (i.e. differences in the timing of growth and reproduction).

4. For most species (6 of 8), flowers were more sensitive to frost than leaves. Within most congeneric pairs (3 of 4), the leaves of species with later phenology were significantly more sensitive to frost than the leaves of species with earlier phenology. For flowers, the later flowering species were more sensitive in two of the four congeneric pairs.

5. *Synthesis.* This study contributes to our general understanding of factors related to interspecific differences in plant sensitivity to episodic frost events of naturally occurring species. The increased frost sensitivity of reproductive structures compared to vegetative structures may be a widespread pattern for long-lived perennial plants. Furthermore, we find evidence for a trade-off between phenology and frost sensitivity, whereby species with later phenology exhibit higher frost sensitivity compared to species with earlier phenology. These results have implications for plant populations, species interactions and ecological communities.

Key-words: climate change, freezing, frost resistance, phenology, phylogeny, plant–climate interactions, Rocky Mountain Biological Laboratory

Introduction

> 'Low temperature represents, together with drought and salt stress, one of the most important environmental constraints limiting the productivity and distribution of plants on Earth'. –Sakai & Larcher (1987)

In many ecosystems, the potential for plants to be exposed to unfavourable abiotic conditions that can have negative consequences for their survival, growth and reproduction is high (Sakai & Larcher 1987; Inouye 2000). This is particularly the case in high-elevation and high-latitude ecosystems with the occurrence of episodic spring and summer frost events. Historically, many studies have investigated frost damage and

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freezing sensitivity of economically important plant species, with an emphasis on understanding freezing tolerance during winter (e.g. Sakai & Larcher 1987). Only more recently have studies explicitly focused on episodic frost events that occur during the growing season for plants in natural communities (e.g. Inouye 2008; Augspurger 2009; Lenz *et al.* 2013; Vitasse *et al.* 2014). These episodic frost events – despite their infrequent nature – have considerably different ecological and evolutionary implications than freezing winter temperatures (Inouye 2000). For example, a single frost event during the growing season can have strong detrimental effects on plants, including the destruction of vegetative and reproductive structures (Inouye 2000; Augspurger 2009; Lenz *et al.* 2013; Vitasse *et al.* 2014), with implications for reproduction (Forrest & Thomson 2010), demography (Inouye 2008) and species interactions (Nixon & McClain 1969). Under recent climate change, the probability of plant exposure to extreme episodic frost events has increased in many high-elevation and high-latitude ecosystems, including our own study site in Colorado (e.g. Hänninen 1991; Inouye 2008; Augspurger 2012; Wheeler *et al.* 2014; but see Scheifinger *et al.* 2003 and Bennie *et al.* 2010 for counter examples), making it increasingly important to understand the ecological consequences of frost. This seemingly paradoxical phenomenon occurs when late winter and early spring conditions are particularly warm, or spring snowmelt is early, which advances the timing of plant growth and reproduction; the probability of frost damage is high under these conditions because plants are growing earlier in the season at a time when night-time temperatures can dip well below freezing (Inouye 2000, 2008; Augspurger 2012). For example, in our study system in Colorado, substantial frost damage to plants is associated with early snowmelt dates (Inouye 2008). In particular, there is still much to be learnt about how frost sensitivity may differ among vegetative and reproductive structures, interspecific differences in frost sensitivity within natural communities, and the potential population and community-level consequences of episodic frost events.

It is generally assumed that reproductive structures are more sensitive to frost than vegetative structures (Sakai & Larcher 1987). However, available evidence from observations in natural plant communities is limited and relatively ambiguous: some studies find evidence for higher sensitivity in flowers compared to leaves (e.g. Inouye 2000; Inouye, Morales & Dodge 2002), others find no difference (e.g. Nixon & McClain 1969; Augspurger 2009), and yet another finds the opposite pattern (Augspurger 2011). It is possible that episodic frost events occurring during the growing season represent such an extreme abiotic condition that both vegetative and reproductive structures are damaged equally. Still, it remains unclear how vegetative and reproductive structures differ in response to episodic frost events occurring throughout the growing season.

When the negative consequences of episodic frost events are severe, plants may invest in a phenologically mediated frost avoidance strategy over a tolerance strategy (Sakai & Larcher 1987; Cannell 1997; Lenz *et al.* 2013). It has been proposed that optimal timing of life-history events (e.g. growth and reproduction) in temperate ecosystems should be a balance between avoiding the negative effects of abiotic extremes – which are more likely early in a growing season – and biotic competition, which should increase the later a species becomes active (Iwasa & Levin 1995; Post 2013). Under this logic, plant traits, as well as evolutionary history, will constrain this optimum: plants with the greatest ability to withstand harsh abiotic conditions should have earlier phenological events that relieve the effects of biotic competition to some degree and provide a longer growing season. However, if evolutionarily conserved physiological limitations constrain frost sensitivity (*sensu* Zanne *et al.* 2014), then related taxa may show no difference in frost sensitivities despite differences in phenology.

To explore the roles of phenology (timing of growth and reproduction) and shared evolutionary history as they relate to the ability of plants to cope with episodic frost events, we experimentally investigated frost sensitivity of leaves and flowers of eight long-lived perennial herbs from a subalpine ecosystem in the Colorado Rocky Mountains, USA. These eight taxa comprise four congeneric pairs from four flowering plant families; each of these pairs includes a species with early and late growth and reproductive phenology within the season (Table 1). This sampling of taxa controls for evolutionary his-

Table 1. Taxonomic information, habitat preference, mean flowering date (1974–2014), peak flowering date for the 2014 study season and frost trial number for the eight subalpine plant species used in this study. For each species, mean date of flowering is the average of peak flowering date across the 41-year study period (1974–2014); standard error (SE) in all cases is standard error of the mean. Mean flowering date is significantly different between each early and late pair ($P \ll 0.05$). Frost trial number corresponds to the date range for each experimental frost trial (see Table 2 for specific dates)

Species	Family	Habitat	Mean flowering date ± SE	Peak flowering date, 2014	Frost trial
Mertensia fusiformis	Boraginaceae	Wet and dry meadows	06 June ± 1.8	09 June	1
Mertensia ciliata	Boraginaceae	Wet meadows	09 July ± 1.3	13 July	2
Hydrophyllum capitatum	Hydrophyllaceae	Wet and dry meadows	11 June ± 1.8	13 June	1
Hydrophyllum fendleri	Hydrophyllaceae	Wet meadows, aspen forest	01 July ± 1.8	27 June	2
Delphinium nuttallianum	Ranunculaceae	Wet and dry meadows	18 June ± 1.5	19 June	1
Delphinium barbeyi	Ranunculaceae	Wet meadows	23 July ± 1.6	29 July	3
Erigeron flagellaris	Asteraceae	Dry meadows	17 July ± 4.1	12 July	2
Erigeron speciosus	Asteraceae	Wet and dry meadows	03 Aug ± 1.7	06 Aug	3

tory – and therefore other traits shared through common ancestry – to some degree, while investigating how phenology relates to frost sensitivity. Specifically, we examined the temperatures at which vegetative (leaves) and reproductive (flowers) structures are sensitive to frost and asked two primary questions: (i) Are frost sensitivities similar among vegetative and reproductive structures within species, and (ii) are frost sensitivities similar among closely related species (congeneric pairs), or do closely related species with early vs. late phenology exhibit differences in frost sensitivity? We discuss the implications of our findings from the perspectives of population dynamics, species interactions and community ecology.

Materials and methods

To determine temperatures at which species exhibit signs of frost damage and to relate interspecific variation in frost sensitivity to ecological and evolutionary patterns and processes, we selected eight subalpine plant species that varied in phenology (timing of growth and reproduction) and evolutionary relationships (Table 1). Our experimental procedure for examining the frost sensitivity of these taxa had three stages: (i) plant tissue collection in the field, (ii) exposure of plant tissue to experimental temperature treatments in the laboratory and (iii) assessment of frost damage following each treatment.

STUDY SITE AND STUDY SPECIES

All plant specimens used in this study were collected at the Rocky Mountain Biological Laboratory (RMBL) in Gothic, Colorado, USA (38°57.5′N, 106°59.3′W, 2900 m above sea level). The area surrounding the RMBL is a subalpine ecosystem consisting of a mosaic of wet and dry meadows, and aspen and spruce forest. This ecosystem is snow-dominated for much of the year; thus, the growing season is relatively brief, lasting approximately 3–5 months (CaraDonna, Iler & Inouye 2014). Snowmelt date in this ecosystem is highly variable from year to year, ranging from mid-April to mid-June, although the date of snowmelt is getting earlier in accordance with climate change (Anderson *et al.* 2012; Iler *et al.* 2013a). Freezing temperatures during springtime are common in this plant community: from 1973 to 2005, minimum temperatures in June ranged from −1.7 to −8.3 °C (mean = −4.3 °C; Inouye 2008). The frequency of freezing temperatures has not changed over the course of this time period (1973–2005; Miller-Rushing & Inouye 2009); instead, there is an ecologically relevant interaction between earlier snowmelt dates, plant phenology and frost damage. When snowmelt is early, flowering phenology is advanced (Iler *et al.* 2013b), and as a result, plants are exposed to a greater occurrence of freezing temperatures during growth and reproduction (Inouye 2008; Miller-Rushing & Inouye 2009). For example, at our study site in Colorado, Inouye (2008) found that when snowmelt date is early, a substantial proportion of flowers of three herbaceous species experience severe frost damage; such frost damage does not occur when snowmelt date is later. As snowmelt dates are predicted to become earlier and earlier in this ecosystem, the severity and frequency of frost damage to plants are also likely to increase (Inouye 2008).

Our eight taxa represent four congeneric pairs from four flowering plant families: *Erigeron flagellaris* and *E. speciosus* (Asteraceae); *Mertensia fusiformis* and *M. ciliata* (Boraginaceae); *Hydrophyllum capitatum* and *H. fendleri* (Hydrophyllaceae); and *Delphinium nuttallianum* and *D. barbeyi* (Ranunculaceae). Each of these congeneric pairs includes a species with early and late-flowering phenology (Table 1), which allows us to explore the relationship between phenology and frost sensitivity while controlling for evolutionary relationships to some degree. Each early and late pair has significantly different flowering dates (*t*-test, *Mertensia* spp. $t = -14.85$, $P < 0.001$; *Hydrophyllum* spp. $t = -7.9$, $P < 0.001$; *Delphinium* spp. $t = -16.17$, $P < 0.001$; *Erigeron* spp. $t = -4.10$, $P < 0.001$; Table 3, Fig. 2). All eight taxa are native and widely distributed across western North America in subalpine and montane ecosystems. Furthermore, all taxa are long-lived perennial herbs, which is the dominant life-history strategy of the plant taxa in this subalpine community. For these species, at the end of each growing season, the above-ground biomass senesces. All species overwinter below-ground and upon snowmelt at the start of each new growing in the spring, the growth of vegetative and reproductive structures occurs once again.

PLANT TISSUE COLLECTION

Vegetative (leaves) and reproductive (flowers) plant tissues were collected from approximately 100–120 randomly chosen plant individuals around the RMBL. The total number of samples collected for each plant species varied based on the availability of plant tissue during each collection period (see Tables S1 & S2 in Supporting Information); for one plant species, we had difficulty collecting this amount of plant tissue, and the sample size is reduced (*Hydrophyllum capitatum*: $n = 62$). For all species, plant tissue was collected at the same developmental stage. Specifically, for leaves, only mature, fully expanded leaf tissue was collected, and for flowers, only newly opened floral tissue was collected. From each individual plant, a single undamaged leaf and a single undamaged flower were collected and then immediately placed inside a plastic bag with a damp paper towel and placed inside a cooler. For consistency, and so we did not damage leaf tissue or floral tissue, all leaves were cut near the base of the petiole, below the leaf blade, and flowers were cut near the base of the pedicel, below the receptacle. Within 2 h of collection, all detached leaf and flower tissues were transferred from the field cooler to a laboratory refrigerator and stored at 5 °C until prepared for an experimental temperature treatment. The treatments were applied within 24 h of field collection. We collected fresh plant tissue each day prior to each experimental treatment to control for plant tissue senescence that may occur after collection from the field. In addition, the temperatures experienced by herbaceous plants during the growing season may influence their frost sensitivity (e.g. Sakai & Larcher 1987; Rapacz & Janowiak 1998). In our subalpine ecosystem, temperatures fluctuate between warm daytime (i.e. > 15 °C) and cool night-time temperatures (i.e. < 5 °C) for the vast majority of the growing season (see Fig S1). Thus, all of our study taxa experienced relatively similar temperature regimes, and it is therefore unlikely that temperatures experienced prior to field collection would strongly bias our results. Air temperatures at the RMBL before and during plant sampling and frost trials are reported in Figure S1.

FROST EXPERIMENT

We used a Sigma M26-C4 programmable temperature chamber (Sigma Systems Corporation, Mansfield, MA, USA) to expose plant tissue to five independent temperature treatments: 0, −2.5, −5.0, −7.5 and −10 °C. Our selection of these temperature treatments encompasses the range of night-time freezing temperatures that occur at our study site in Colorado during early spring after snowmelt (minimum temperature range from 1973 to 2005: −1.7 to −8.3 °C;

Inouye 2008); our lowest temperature treatment was meant to represent an extreme condition that lies just beyond what is observed in our ecosystem. The actual target temperatures reached during each treatment are reported in Table 2 and are the temperatures used in all subsequent analyses. For each complete experimental frost trial – that is, all five temperature treatments – approximately 100 samples of flowers and leaves were used for each species; approximately 20 samples were used for each individual temperature treatment (i.e. 20 samples at five temperature treatments = 100 samples per species). Across the growing season, three complete experimental frost trials were conducted that coincided with the availability of vegetative and reproductive tissue for our focal taxa at our field site (Table 1). Because we had only one temperature chamber, individual temperature treatments within a complete frost trial occurred on separate days (or at night) during this time period, assignment of each treatment was random within each complete frost trial to prevent any biases that may arise as a result of experimental treatments being conducted on separate days. Because liquid CO_2 is the cooling agent for the temperature chamber, all plant tissues were wrapped in aluminium foil to prevent CO_2 poisoning and to prevent excess water loss and desiccation during the experiment (Sakai & Larcher 1987; Wheeler *et al.* 2014). Each leaf and flower sample was wrapped individually in its own foil packet.

Temperature treatments were programmed to model the rate and duration of night-time frost events at our field site, based on temperature data from a weather station at the study site (see Fig. S2). All frost simulations began at 5 °C. Plant tissue was placed inside the temperature chamber, and over the course of 6 h, the temperature ramped down from 5 °C to one of the five target temperatures (0, −2.5, −5.0, −7.5 or −10 °C; Table 2). Once the target temperature was reached, it was held for 2 h and then brought back up to 5 °C in 1 h. The total time for each temperature treatment was 9 h. Thus, we controlled for time, rather than cooling rate, because this better matches what occurs in our ecosystem during a frost event (Fig. S2).

ASSESSMENT OF FROST DAMAGE

Once each temperature treatment was complete, individual plant specimens were removed from the temperature chamber and visually examined for frost damage. Frost damage to leaves and flowers is generally obvious due to discoloration and wilting associated with oxidation of polyphenols, or a characteristic odour caused by the decompartmentalization and autolysis of the protoplast (Sakai & Larcher 1987). For consistency, assessment of frost damage was always conducted by the same three researchers (P.J.C., J.A.B. and R.M. Brennan). In addition, all experimentally manipulated plant tissue was compared to freshly collected plant tissue for reference. Frost damage was scored on a binary scale where 1 = frost damage, and 0 = no frost damage. For flowers, we examined frost damage to the entire flower (i.e. all floral parts were assessed simultaneously without distinction among specific floral parts). We used a binary damage scale over a continuous damage scale, because in the vast majority of cases, if frost damage occurred, it was either complete or extensive throughout the plant tissue (e.g. Fig. 1). Finally, visual inspection of frost damage has been shown to correspond with electrolyte leakage methods (Obrist 2001; Vitasse *et al.* 2014).

Table 2. Target and actual temperatures reached during each of the three frost trials. During each treatment, the temperature in the chamber began at 5 °C and then over the course of 6 h was brought down to the each target temperature, held for 2 h and then brought back up to 5 °C in 1 h; with this design, we controlled for time, rather than cooling rate, because this more closely resembles what occurs at our study site in the Colorado Rocky Mountains, USA (Fig S2). 'Frost trial 3' occurred over fewer days compared to the other frost trials because some of the individual temperature treatments were conducted at night

Frost trial	Treatment temperature (˚C)					Frost trial date range
Target	0.0	−2.5	−5.0	−7.5	−10.0	NA
1	0.0	−2.5	−4.9	−7.1	−9.8	19–26 June 2014
2	0.0	−2.3	−4.8	−6.9	−7.4	02–09 July 2014
3	0.0	−2.5	−4.8	−7.4	−9.4	28–31 July 2014

ASSESSMENT OF PLANT PHENOLOGY

Data on plant phenology for our study species come from a long-term study of flowering phenology at the RMBL (1974–2014). In this long-term study, the number of open flowers per stalk was counted every other day from 1974 to 2014 for all flowering plant species that occur in 30 2 × 2 m plots. In our study of frost sensitivity, we define phenology for each species as the mean date of peak flowering averaged across the 41-year study period (Table 1) (Inouye 2008; Iler *et al.* 2013b; CaraDonna, Iler & Inouye 2014). Specific peak flowering dates for each species during the 2014 study year are reported in Table 1. Peak flowering date is the day on which 50% of the flowers were counted as open for each species. We do not have species-level data on vegetative phenology; thus, we use flowering phenology as an approximate measure for overall vegetative and reproductive phenology for each species. Reproductive and vegetative traits are often highly correlated (Primack 1987),

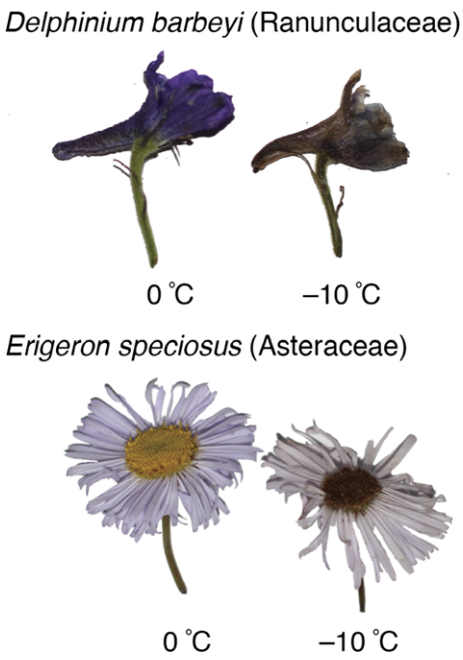

Fig. 1. The flowers of *Delphinium barbeyi* and *Erigeron speciosus* after the warmest (0 °C) and the coolest (−10 °C) experimental temperature treatments. Plant tissue was collected from a subalpine ecosystem near the Rocky Mountain Biological Laboratory in the Colorado Rocky Mountains, USA (2900 m above sea level).

and indeed, flowering and leaf-out times appear to be highly correlated in many species (*sensu* Schwartz & Reiter 2000; Post *et al.* 2008 Polgar, Gallinat & Primack 2014; Zohner & Renner 2014). Based on our (P.J.C. and J.A.B.) personal observations for our study taxa, plants that flower early tend to be plants that begin vegetative growth earlier in the season; additionally, because the growing season is brief, the duration between emergence of vegetative structures and the timing of reproduction is relatively short. Therefore, in this study, we are assuming that flowering phenology and vegetative phenology are correlated to some degree.

DATA ANALYSIS

We analysed the relationship between experimental temperature and frost damage to leaves and flowers using standard logistic regression models (implemented in R using the glm function; R Development Core Team 2014). The lethal temperature at which 10%, 50% and 90% of the individuals across temperature treatments experienced frost damage (LT_{10}, LT_{50}, LT_{90}) and the standard error (standard error of inverse prediction) were calculated from each logistic regression model. In one case (leaf frost sensitivity analyses of *Hydrophyllum fendleri*), a standard logistic regression model could not be fit due to perfect separation of data; here, we used a Bayesian logistic regression model that accounts for this standard logistic model violation (implemented in R using the ARM package).

We analysed differences in frost sensitivity between leaves and flowers within species, and between species within congeneric pairs, by comparing the intercepts of each logistic regression model fit via a Wald test. Specifically, different logistic regression intercepts indicate different relationships between temperature and the probability of frost damage (which is conceptually analogous to comparing slopes in a linear regression); in other words, a significantly different intercept means that plant tissue types, or species within congeneric pairs, differ in their frost sensitivity. In the few cases where one species in a comparison did not exhibit any frost damage within the experimental treatments (i.e. frost sensitivity occurred below −10 °C), we used a one-sided *t*-test to examine whether LT_{50} of the damaged species was significantly less than −10 °C. Furthermore, we limit our analyses of frost sensitivity to within species comparisons (leaf vs. flower) or within congeneric pair analyses (early- vs. late-flowering species) because our selection of taxa is necessarily a biased sample from the community. Thus, we did not analyse data in a continuous framework using a linear regression model because our selection of species does not represent independent samples.

Results

Frost sensitivity varied considerably among the eight study species. For leaves, frost damage ranged from −5.1 °C to below −10.0 °C, and for flowers, frost damage ranged from −1.9 °C to below −10.0 °C (Figs 2 and 3; Table 3). (A complete list of logistic regression model fit coefficients is reported in Tables S1 & S2). For the majority of species, leaf and flower frost sensitivity (LT_{10}, LT_{50} and LT_{90}) occurred within the experimental temperature range. However, in four cases, frost damage did not occur within the experimental temperature range (leaves and flowers of *Erigeron flagellaris*,

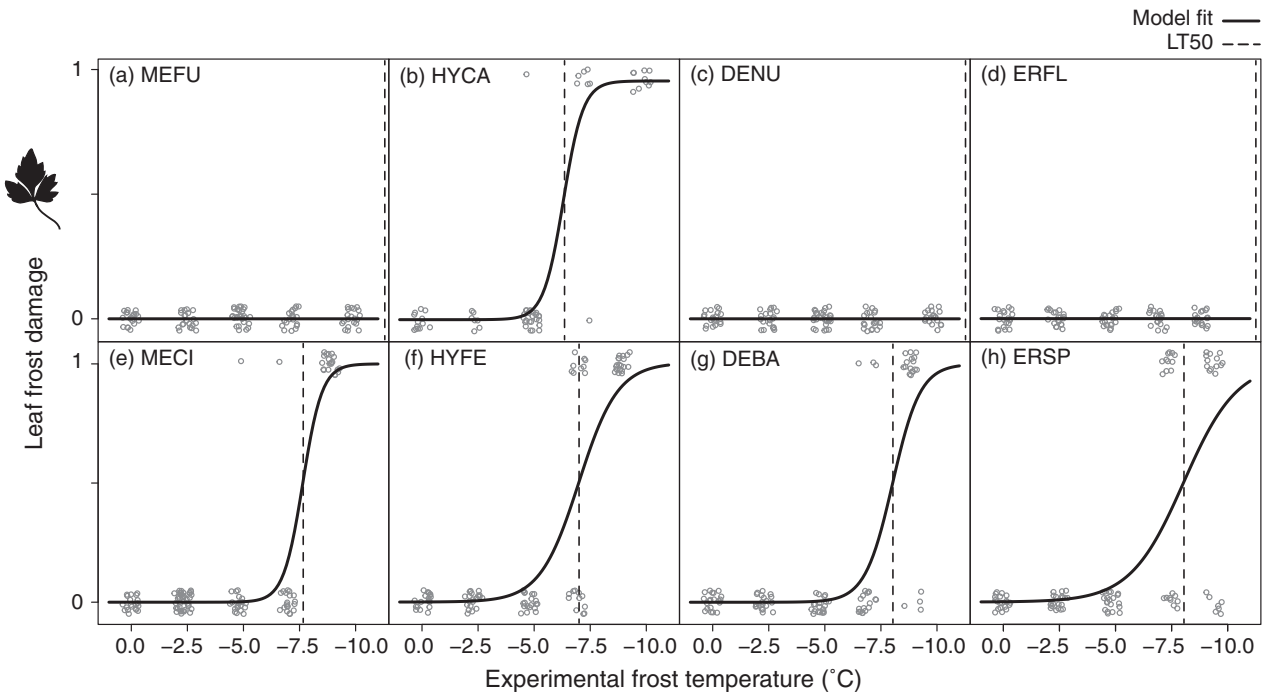

Fig. 2. Leaf frost sensitivities of subalpine plant species from the Colorado Rocky Mountains, USA. (a–h) Logistic regression model estimates showing the relationship between leaf frost damage and experimental temperatures. Black lines represent logistic regression model fits; points represent actual data points, which are jittered for clarity and visualization purposes; dashed lines indicate LT_{50} (dashed lines are shown at the end of the *x*-axis in cases where LT_{50} did not occur prior to −10 °C). Species are ordered by relative phenology (Table 1), and congeneric pairs are stacked on top of each other. Four letter species abbreviation codes are as followed: MEFU = *Mertensia fusiformis*, HYCA = *Hydrophyllum capitatum*, DENU = *Delphinium nuttallianum*, ERFL = *Erigeron flagellaris*, MECI = *Mertensia ciliata*, HYFE = *Hydrophyllum fendleri*, DEBA = *Delphinium barbeyi* and ERSP = *Erigeron speciosus*. Note that the *x*-axis has been reversed so that an increase in freezing temperature is shown from left to right.

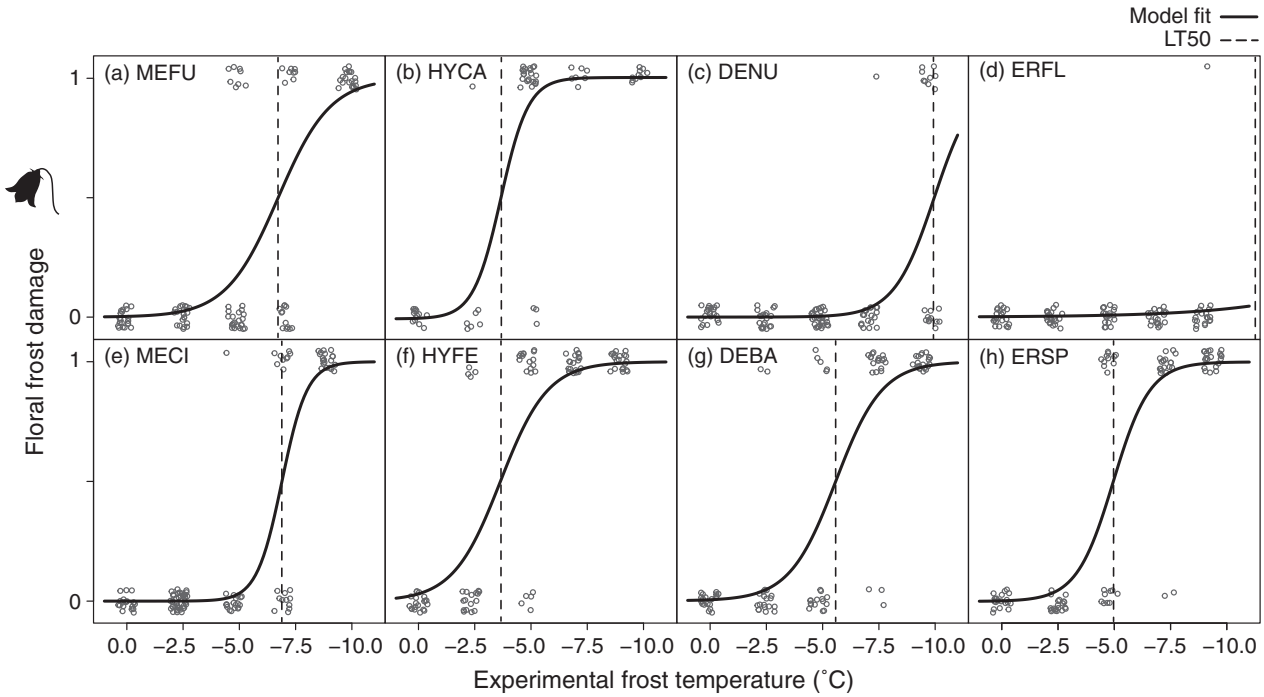

Fig. 3. Floral frost sensitivities of subalpine plant species from the Colorado Rocky Mountains, USA. (a–h) Logistic regression model estimates showing the relationship between floral frost damage and experimental temperatures. Black lines represent logistic regression model fits; points represent actual data points, which are jittered for clarity and visualization purposes; dashed lines indicate LT_{50} (dashed lines are shown at the end of the *x*-axis in cases where LT_{50} did not occur prior to −10 °C). Four letter species abbreviation codes are the same as in Fig. 2. Note that the *x*-axis has been reversed so that an increase in freezing temperature is shown from left to right.

Table 3. Leaf and flower frost sensitivities of eight subalpine species from the Colorado Rocky Mountains. Standard error (SE) in all cases is standard error of the mean. Temperature values for species that did not experience leaf or flower frost damage within the experimental treatments are reported as occurring below −10 °C (see Materials and Methods for details). For *Delphinium nuttallianum,* LT_{90} for flowers fells outside of the experimental treatments and is thus an extrapolated model estimate

	Leaf frost sensitivity (˚C)			Floral frost sensitivity (˚C)		
Species	$LT_{10} \pm SE$	$LT_{50} \pm SE$	$LT_{90} \pm SE$	$LT_{10} \pm SE$	$LT_{50} \pm SE$	$LT_{90} \pm SE$
Mertensia fusiformis	< −10.0	< −10.0	< −10.0	−5.7 ± 0.5	−6.9 ± 0.4	−8.1 ± 0.7
Mertensia ciliata	−6.5 ± 0.3	−7.7 ± 0.2	−8.8 ± 0.4	−4.2 ± 0.4	−6.7 ± 0.2	−9.3 ± 0.2
Hydrophyllum capitatum	−5.1 ± 0.7	−6.3 ± 0.4	−7.6 ± 0.4	−2.3 ± 0.7	−3.7 ± 0.4	−5.0 ± 0.4
Hydrophyllum fendleri	−5.8 ± 0.5	−7.0 ± 0.3	−8.12 ± 0.5	−1.9 ± 0.5	−3.7 ± 0.3	−5.5 ± 0.5
Delphinium nuttallianum	< −10.0	< −10.0	< −10.0	−8.0 ± 0.6	−10.0 ± 0.4	−11.9 ± 0.9
Delphinium barbeyi	−6.4 ± 0.6	−8.0 ± 0.4	−9.7 ± 0.6	−3.3 ± 0.6	−5.6 ± 0.4	−7.8 ± 0.6
Erigeron flagellaris	< −10.0	< −10.0	< −10.0	< −10.0	< −10.0	< −10.0
Erigeron speciosus	−5.4 ± 0.7	−8.0 ± 0.4	−10.5 ± 0.8	−3.2 ± 0.5	−5.0 ± 0.3	−6.7 ± 0.5

and leaves of *Mertensia fusiformis* and *Delphinium nuttallianum*), indicating frost resistance even at the most extreme treatments; in these cases, frost sensitivity values are reported as less than −10.0 °C (Table 3). In one case (flowers of *Delphinium nuttallianum*), LT_{90} only did not occur within the experimental temperature range, and the reported LT_{90} value is based on extrapolated logistic regression model estimates (Table 3).

FLORAL & LEAF FROST SENSITIVITY AMONG SPECIES

Floral tissue was significantly more sensitive to frost than leaf tissue in six of the eight study species (Table 4, Figs 2 and 3). For the remaining two species (*Delphinium nuttallianum* and *Erigeron flagellaris*), little or no frost damage occurred within the experimental treatments, making it unclear in these cases whether leaves and flowers differ in frost sensitivity.

COMPARISON OF LEAF AND FLOWER FROST SENSITIVITY WITHIN CONGENERIC PAIRS

There was a significant difference in leaf frost sensitivity between species within three out of the four congeneric pairs (Fig. 2). In these three cases, the later flowering species was more sensitive to frost (one-sided *t*-test, *Mertensia* spp. $t = 114$, $P < 0.0001$; *Delphinium* spp. $t = 49.5$, $P < 0.0001$;

Table 4. Logistic regression analyses results comparing frost sensitivity between leaf tissue and floral tissue for each species. For all cases where there is a significant effect, floral tissue is more sensitive to frost than leaf tissue (Table 3, Figs 2 and 3). Significant comparisons are indicated in bold. We did not compare leaf and flower frost sensitivity for *Delphinium nuttallianum* and *Erigeron flagellaris* because LT_{50} occurred outside of the experimental temperature range (See Materials and methods for details)

	Wald test coefficients		
Species	χ^2	d.f.	*P*
Mertensia fusiformis	23.50	1	**< 0.0001**
Mertensia ciliata	5.40	1	**0.02**
Hydrophyllum capitatum	22.20	1	**< 0.0001**
Hydrophyllum fendleri	25.70	1	**< 0.0001**
Delphinium nuttallianum	NA	NA	NA
Delphinium barbeyi	18.50	1	**< 0.0001**
Erigeron flagellaris	NA	NA	NA
Erigeron speciosus	21.10	1	**< 0.0001**

Erigeron spp. $t = 50$, $P < 0.0001$; Table 3, Fig. 2), suggesting a relationship between plant phenology and frost sensitivity independent of evolutionary history. However, there was no statistical difference in sensitivity of leaves to frost between the two *Hydrophyllum* species (Wald test, $\chi^2 = 2.5$, d.f. = 1, $P = 0.11$; Table 3, Fig. 2), suggesting that at least some closely related species exhibit similar leaf frost sensitivity.

For two sets of congeneric pairs, flowers of the later flowering species were more sensitive to frost than the earlier flowering species (Wald test, *Delphinium* spp.: $\chi^2 = 30.00$, d.f. = 1, $P < 0.0001$; *Erigeron* spp. $\chi^2 = 27.60$, d.f. = 1, $P < 0.0001$; Table 3, Fig. 3). In contrast, for the remaining two congeneric pairs, there was no difference in floral frost sensitivity between species (Wald test, *Mertensia* spp. $\chi^2 = 0.40$, d.f. = 1, $P = 0.53$; *Hydrophyllum* spp. $\chi^2 = 0.85$, d.f. = 1, $P = 0.77$; Table 3, Fig. 3).

Discussion

In this study, we experimentally investigated the frost sensitivity of vegetative and reproductive structures for a series of eight long-lived perennial herbs from a subalpine ecosystem in the Colorado Rocky Mountains, USA. Specifically, we explored two primary questions: (i) whether frost sensitivity differed between leaves and flowers within species, and (ii) whether phenology (timing of growth and reproduction) or evolutionary history was related to interspecific variation in frost sensitivity. Overall, frost sensitivity varied considerably among species and plant structures. Within species, flowers were consistently more sensitive to frost than leaves (Tables 3 and 4, Figs 2 and 3). Within congeneric pairs, the leaves of species with later-season phenological events were generally more sensitive to frost than the species with early-season phenological events. For flowers, the relationship between phenology and frost sensitivity was less clear: in two of the congeneric pairs, the species with later-season phenological events were more sensitive to frost than the species with early-season phenological events, as predicted; for the remaining two congeneric pairs, frost sensitivity was virtually identical despite differences in the timing of phenological events. Thus, our results provide evidence in support of a phenology-frost sensitivity trade-off, but also show that, in some cases, more closely related taxa may exhibit physiological constraints related to frost sensitivity.

Our assessment of frost sensitivity was within controlled experimental conditions and was conducted on detached plant tissues. Detachment of plant tissues may prevent recovery after minor frost damage, especially if physiological processes involved in recovery are inhibited. However, in this study, plant tissue was most often severely damaged (e.g. complete discoloration and wilting) or unaffected (Fig. 1); in these cases, recovery of destroyed plant tissue is unlikely. Frost sensitivities of plants under naturally occurring conditions are likely to vary with cooling and warming rates, microclimate and other habitat heterogeneities (e.g. slope, aspect and humidity) that may buffer or further expose plants to freezing temperatures (Inouye 2000). Thus, our findings from our controlled experimental temperatures are not directly applicable to natural settings but still have important implications for natural plant populations and communities. Furthermore, in a few cases, we were unable to determine specific frost sensitivity values for some species because frost damage did not occur within our experimental temperature treatments (Table 3). However, this limitation does not influence the interpretation of our results because we can infer that frost sensitivity for these species is below −10.0 °C, a freezing temperature that is beyond what is observed in our ecosystem during spring (Inouye 2008).

FLOWERS ARE MORE SENSITIVE TO FROST THAN LEAVES

Reproductive structures are assumed to be more sensitive than vegetative structures, in part because rapidly differentiating and actively growing plant tissues tend to be most sensitive to frost events (Sakai & Larcher 1987). However, the limited evidence for plants from natural communities does not consistently support this assumption (e.g. Nixon & McClain 1969; Inouye 2000; Augspurger 2009, 2011). In our experimental study, we found that flowers were generally more sensitive to frost than leaves (Table 3, Figs 2 and 3). We propose that our finding of greater frost sensitivity in flowers compared to leaves is consistent with demographic life-history trade-offs for long-lived perennial plant species (Franco & Silvertown 2004). All eight of the study species are long-lived iteroparous herbs. Such plants should invest in strategies that ensure survival and growth over short-term reproductive success in order to increase overall population growth (λ). This is because survival and growth characteristically contribute more strongly to population growth for long-lived perennial herbs compared to reproduction (Franco &

Silvertown 2004). In other words, there is less incentive to invest in protection of ephemeral reproductive structures compared to vegetative structures because when vegetative structures survive, another reproductive cycle is possible in the following year.

VARIATION IN FROST SENSITIVITY WITHIN CONGENERIC PAIRS: EVIDENCE FOR THE IMPORTANCE OF PHENOLOGY

Recent work has highlighted a trade-off between plant phenology and frost sensitivity (e.g. Lenz *et al.* 2013; Vitasse, Lenz & Körner 2014; Vitasse *et al.* 2014). Plants that are sensitive to freezing temperatures should be selected to have later phenological events (e.g. leaf-out time and flowering time) that decrease the probability of experiencing a detrimental frost event (Lenz *et al.* 2013). We hypothesize that selection should be more extreme for vegetative structures compared to reproductive structures because the destruction of vegetative structures via frost damage may partially or completely retard growth, which can subsequently lead to partial or complete reproductive failure. Furthermore, this hypothesized relationship between plant phenology and frost sensitivity is consistent with theoretical expectations for optimal timing in seasonal environments. Plant species with earlier phenological events should receive a fitness benefit because they can withstand unfavourable abiotic conditions, and as a result, can avoid more intense biotic competition (Iwasa & Levin 1995; Post 2013) and simultaneously take advantage of a longer growing season (Lockhart 1983). However, this general hypothesis does not account for the possibility that related taxa may have evolutionarily conserved physiological constraints that prevent them from coping with harsh abiotic conditions despite flexibility in their phenological events. In this study, we found strong evidence for a relationship between plant phenology and frost sensitivity, and only limited evidence that, in some cases, closely related taxa may exhibit physiological constraints related to frost sensitivity (Tables 1 and 3, Figs 2 and 3). These results suggest that the related taxa in our study may not have strong physiological constraints related to frost sensitivity, or the benefits of earlier growth and reproduction are strong enough to override any physiological constraints shared among related taxa. Additional research on species representing broader taxonomic coverage and from different ecosystems will be important to further evaluate these findings. Nonetheless, our results from four congeneric pairs suggest that within many clades, taxa may show divergence in phenological traits that mediate frost sensitivity, supporting the phenology–frost sensitivity relationship, whereas in some cases in other clades, related taxa may exhibit no difference in frost sensitivity, despite variation in the timing of phenological events, which may be related to evolutionarily conserved physiological constraints. Our study adds to a growing body of evidence that highlights the relationship between ecological timing and harsh abiotic events (e.g. Inouye 2000, 2008; Augspurger 2009; Forrest & Thomson 2010; Lenz *et al.* 2013; Vitasse, Lenz & Körner 2014; Vitasse *et al.* 2014).

IMPLICATIONS FOR SPECIES INTERACTIONS AND COMMUNITY DYNAMICS

The differences in frost sensitivities between vegetative and reproductive structures we report here have implications for species interactions and community dynamics. Our findings suggest that a single episodic frost event may not be detrimental for plant growth and survival, but can severely reduce floral production via frost damage and reduce reproduction in these subalpine plant species (e.g. Forrest & Thomson 2010; Thomson 2010). Frost sensitivity of flowers among our eight species was highly variable, with some species experiencing frost damage at relatively mild freezing temperatures (−1.9 °C), while others remained undamaged by freezing temperatures exceeding −10.0 °C (Table 3). This means that a frost event at any given time during the growing season is likely to have variable effects on species, depending on their specific frost sensitivity and phenology, as well as evolutionary history. Assuming that plants under natural conditions exhibit a similar range of frost sensitivity to those observed in our experiment, this variation in frost sensitivity has the potential to alter flowering plant community composition and hence the floral resource landscape available to pollinators following an episodic frost event. Changes in flowering plant community composition can affect pollinator visitation patterns (Waser 1978; Ghazoul 2006), as well as plant–plant competition for abiotic resources (Veresoglou & Fitter 1984), both of which can influence plant reproductive output. Importantly, changes in floral resource composition and abundance are also likely to have consequences for pollinator population dynamics (e.g. Roulston & Goodell 2011; Burkle, Marlin & Knight 2013). Indeed, episodic frost events have been linked to subsequent population declines in squirrels due to reductions in food resources (Nixon & McClain 1969), and a similar scenario is possible for pollinator populations. In this subalpine plant community, coflowering patterns have been shown to be reshuffled under climate change conditions (Forrest, Inouye & Thomson 2010; CaraDonna, Iler & Inouye 2014). As the climate continues to warm in this subalpine ecosystem, and snowmelt and plant phenology continue to become earlier, detrimental frost events are likely to increase, especially for plants commencing growth early in the season (Inouye 2008; Miller-Rushing & Inouye 2009; Forrest & Thomson 2010). The findings presented here are consistent with the interpretation that at least some of the reshuffling observed within this flowering plant community may be attributed to the effects of frost damage.

Conclusions

Harsh abiotic conditions, such as freezing temperatures, are a key challenge limiting the distribution and abundance of plants (Sakai & Larcher 1987; Zanne *et al.* 2014). As climate

change continues to alter the frequency and magnitude of extreme abiotic events, it is increasingly important to understand interspecific variation in species' responses to these events, and ultimately, how these responses may influence both population and community patterns. This study contributes to our general understanding of factors related to interspecific differences in plant sensitivity to episodic frost events of naturally occurring species. The increased sensitivity of reproductive structures to frost compared to vegetative structures may be a widespread pattern for long-lived perennial plants, as is evidenced here, but this hypothesis needs to be experimentally examined in more species and in more ecosystems. As we hypothesized, interspecific variation in plant frost sensitivity may represent different strategies employed by different clades. Our results suggest that the timing of important life-history events (e.g. growth and reproduction), more so than evolutionary history, is likely to influence how plant species respond to harsh abiotic conditions.

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Data accessibility

Frost sensitivity data used in this study are archived at the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad/v4cv6 (CaraDonna & Bain 2015). Long-term flowering phenology data and metadata are archived at www.rmbl.org and at the Digital Repository at the University of Maryland: http://drum.lib.umd.edu/.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Snowmelt date, daily maximum and daily minimum air temperatures before and during plant sampling and temperature manipulation.

Figure S2. Examples of night-time cooling rates that occur during the spring after snowmelt at the Rocky Mountain Biological Laboratory, Gothic, Colorado, USA.

Table S1. Logistic regression model coefficients for the relationship between experimental temperature and leaf frost damage for the eight plant taxa in this study.

Table S2. Logistic regression model coefficients for the relationship between experimental temperature and floral frost damage for the eight plant taxa in this study.