RESEARCH ARTICLE

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Freezing resistance, safety margins, and survival vary among big sagebrush populations across the western United States

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PREMISE: Physiological responses to temperature extremes are considered strong drivers of species' demographic responses to climate variability. Plants are typically classified as either avoiders or tolerators in their freezing-resistance mechanism, but a gradient of physiological-threshold freezing responses may exist among individuals of a species. Moreover, adaptive significance of physiological freezing responses is poorly characterized, particularly under warming conditions that relax selection on cold hardiness.

METHODS: Freezing responses were measured in winter and again for new foliage in spring for 14 populations of *Artemisia tridentata* collected throughout its range and planted in a warm common garden. The relationships of the freezing responses to survival were evaluated in the warm garden and in two colder gardens.

RESULTS: Winter and spring freezing resistance were not correlated and appeared to be under differing selection regimes, as evident in correlations with different population climate of origin variables. All populations resisted considerably lower temperatures in winter than in spring, with populations from more continental climates showing narrower freezing safety margins (difference in temperatures at which ice-nucleation occurs and 50% reduction in chlorophyll fluorescence occurs) in spring. Populations with greater winter freezing resistance had lower survivorship in the warmest garden, while populations with greater spring freezing resistance had lower survivorship in a colder garden.

CONCLUSIONS: These survivorship patterns relative to physiological thresholds suggest excess freezing resistance may incur a survival cost that likely relates to a trade-off between carbon gain and freezing resistance during critical periods of moisture availability. This cost has implications for seed moved from cooler to warmer environments and for plants growing in warming environments.

KEY WORDS *Artemisia tridentata*; Asteraceae; cold hardiness; freezing avoidance; freezing resistance; freezing tolerance; freezing safety margin; ice nucleation temperature; LT_{50}; relaxed selection.

Range shifts of many species are related to changing climate (Parmesan and Yohe, 2003; Walther et al., 2005; Kelly and Goulden, 2008; Chen et al., 2011). Understanding the mechanisms of plant responses to minimum temperatures, which greatly influence the distribution of plant species (Iversen, 1944; Sakai and Weiser, 1973; Walther et al., 2005; Zimmermann et al., 2009), is important for predicting and understanding demographic responses of plant species to climate variation. Intraspecific variation in cold hardiness among populations is common in widespread species (e.g., Sakai and Weiser, 1973; Alexander et al., 1984; Morin et al., 2007; Corcuera et al., 2011) and may be attributed to local adaptation or to recognized subspecies, varieties, or ecotypes. Species-distribution modeling studies that have considered intraspecific variation found that it altered predictions of species' responses to climate change (e.g., Pearmen et al., 2010; Valladares et al., 2014; Maguire et al., 2018). However, intraspecific variation in physiological freezing responses has rarely been compared with survival, which makes it difficult to evaluate the adaptive significance of these responses (but see Darychuk, 2012). Such information is particularly lacking in warm or warming environments, where selection on cold hardiness traits may be relaxed.

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How cold hardiness is defined and measured differs among studies and botanical subdisciplines. Here, "freezing resistance" refers to the low tissue temperature at which 50% of function is lost (LT_{50}). We use the more general term "cold hardiness" to refer to any other measure of a plant's ability to withstand low temperatures (e.g., percentage survival or injury upon cooling to a particular temperature).

FREEZING RESISTANCE MECHANISMS: AVOIDANCE, TOLERANCE, OR A CONTINUUM?

Freezing resistance is achieved by mechanisms that fall into two categories, freezing avoidance and freezing tolerance (Levitt, 1980). Freezing avoidance mechanisms allow plant tissues to cool without the formation of ice in extracellular spaces. Freezing tolerance mechanisms enable a plant to tolerate the formation of extracellular ice and the accompanying dehydration that results from the large vapor pressure gradient that extracellular ice creates. Intracellular ice formation is lethal in nearly all cases (Burke et al., 1976). Avoidance of extracellular freezing is typically accomplished by freezing point depression or by supercooling, both of which allow liquid to cool to temperatures below freezing without ice formation. Production of solutes facilitates both of these processes (Burke et al., 1976). Supercooling capacity is also enhanced by anti-freeze proteins (Griffith et al., 2005) and structural properties (e.g., small cell size, little intercellular space, thick cuticles) that inhibit ice nucleation and propagation (Levitt, 1980; Wisniewski and Fuller, 1999). Tolerance of extracellular freezing is accomplished by changes to the lipid composition of the cell membrane and the production of solutes or proteins that function to reduce water loss (e.g., compatible osmolytes) or ameliorate its effects by stabilizing membranes and enzymes and repairing oxidative damage (e.g., dehydrins, cryoprotectants, anti-oxidants; Guy, 1990; Xin and Browse, 2000; Kalberer et al., 2006).

In addition to these mechanisms, which are general to many tissue types, leaves that overwinter in freezing climates must also protect their photosynthetic apparatus from photoinhibition, the light-dependent reduction of photosynthesis that occurs year-round but is exacerbated by greater cold inhibition of carbon-fixing than light-harvesting systems. This imbalance generates a risk of photochemical overexcitation and oxidative damage (Huner et al., 1993; Long et al., 1994). Photoprotective responses vary from rapidly reversible changes in the composition of xanthophyll pigments to longer-term pigment changes and deformation or deconstruction of key photosystem II proteins (Demmig-Adams and Adams, 2006; Demmig-Adams et al., 2012).

Freezing resistance varies seasonally as plants undergo acclimation and deacclimation (also referred to as hardening and dehardening) to freezing temperatures in response to seasonal changes in temperature and day length. Cold acclimation is a slow process (weeks to months), while deacclimation typically occurs over a shorter timescale (days to weeks) and can be reversible (Kalberer et al., 2006). Seasonal variation in freezing resistance necessitates evaluation of freezing resistance at multiple times of year. The capacity to supercool (and therefore avoid freezing) is less energetically demanding and more quickly reversible than the structural, biochemical, and physiological changes required for tolerance but also less effective against very low temperatures or over longer periods (Sakai and Larcher, 1987; Wisniewski et al., 2018).

Operationally, plant species are often grouped into two categories by their physiological freezing resistance mechanism, which is determined by the relationship between the temperature at which extracellular water freezes (ice-nucleation temperature [NT]) and the temperature leading to a 50% loss of function or lethal response (LT_{50}; Bravo et al., 2001; Sierra-Almeida et al., 2009), though it is recognized that a species can employ different mechanisms at different times (e.g., Sierra-Almeida et al., 2009; Sklenář, 2017). Freezing avoiders are damaged by freezing at approximately the same temperature that extracellular ice forms in their tissues (i.e., $NT - LT_{50} = 0$). Freezing tolerators incur freezing damage only at temperatures significantly lower than their ice nucleation temperatures (i.e., $NT - LT_{50} > 0$; Bravo et al., 2001; Sierra-Almeida et al., 2009).

This binary avoider or tolerator classification approach to freezing-resistance mechanisms can group together plants that are damaged only a few degrees below their NT with plants that are not damaged until tissues cool well below their NT, possibly obscuring important clinal variation in freezing response. Consider that when extracellular ice forms (at the NT), a large vapor pressure gradient is created between the relatively wet cell and dry extracellular space, creating osmotic stress (Hansen and Beck, 1988). The larger the difference between NT and LT_{50}, the greater the amount of intracellular ice and corresponding osmotic stress that can be tolerated before a catastrophic response to freezing occurs (i.e., freezing injury). Thus, rather than viewing freezing-resistance mechanisms in a binary fashion, greater insight on adaptive variation in freezing-resistance may be gained by considering that freezing resistance mechanism may vary in a gradient-like fashion among or within species. This gradient ranges from avoidance, in which there is little difference between NT and LT_{50} and thus a small "freezing-safety margin" (equivalent to the "thermal difference" in Sierra-Almeida et al., 2009), to tolerance, in which relatively more negative LT_{50} compared to NT results in larger safety margins. When this margin is narrow, the plant can tolerate little additional ice-related stress within its tissues, and any additional freezing poses a hazard of damage (is unsafe). This freezing–safety margin continuum concept is somewhat analogous to the commonly used hydraulic safety margin (the difference between midday xylem pressure potential and the critical pressure triggering catastrophic embolism, Alder et al., 1996). Isohydric species (which close stomata during drought to maintain higher midday water potential) have wider hydraulic safety margins, while anisohydric species (which leave stomata open during drought and thus reach lower midday water potentials) have narrower hydraulic safety margins (McDowell et al., 2008). Isohydry and anisohydry were previously treated as mutually exclusive behaviors with species falling in one category or the other (Tardieu and Simonneau, 1998). They are now considered end points of a continuum along which all species operate (Klein, 2014; Fu and Meinzer, 2018), and the influence of environmental conditions on the determination of where a species operates in the continuum is now also recognized (Hochberg et al., 2018).

Just as the concept of a dichotomy has been replaced with that of a continuum in water relations, consideration of the size of the freezing safety margins along with absolute values of NT or LT_{50} should provide an elegant and ecologically meaningful estimate of threshold temperature responses, provided that the variation relates to ecological patterns or differences in survival. This continuum of variation in freezing responses is of particular importance for evergreen species, which must utilize physiological avoidance and

tolerance mechanisms. In contrast, deciduous species can use temporal avoidance mechanisms (e.g., delayed phenology) to maintain a "thermal safety margin", which Lenz et al. (2013) defined as the difference between minimum seasonal air temperature and LT_{50}, that is relatively constant across species and elevations.

SEASONAL TEMPERATURE AND MOISTURE DYNAMICS

In many continental, often semi-arid ecosystems such as the cold desert of the Great Basin, winters are wet and freezing and summers are hot and dry, with only a brief spring period when daytime temperatures and soil moisture are optimal for growth (Caldwell, 1985). Plants in these ecosystems must not only be able to withstand low winter temperatures, they must also be able to capitalize on the short period of optimal moisture and temperature for growth in spring, when exposure to nighttime frosts can be appreciable (Brabec et al., 2017). Genetic variation in the timing and depth of winter hardening and spring dehardening could therefore strongly influence growth and survival in these environments. Climate affecting these processes can be reflected coarsely by monthly minimum, mean, or maximum temperature, by thermal accumulation measured with degree days, and by continentality measured as the difference between average winter minimum and summer maximum temperatures.

SELECTION AND RELAXED SELECTION ON COLD HARDINESS

Identification of genetic variation within species across landscape gradients, sometimes referred to as genecological patterning (Turesson, 1923), is basic evidence for the adaptive value of ecophysiological traits and, furthermore, is of practical value (e.g., for parameterizing seed-transfer guidelines; Campbell, 1986; St. Clair et al., 2013). Genecological variation that occurs in a common garden provides evidence that natural selection has acted upon the trait in question (Heslop-Harrison, 1964; Endler, 1986), such as greater cold hardiness in populations originating from colder environments (e.g., Zhen and Ungerer, 2008). Cold hardiness is selected in relatively cold environments, but in relatively warmer places or times adaptations to freezing may undergo "relaxed selection", that is, a weakening or loss of a previously important source of selection (Lahti et al., 2009). Under conditions of relaxed selection, cold-hardiness traits may be selectively neutral (have no fitness consequences) or excess cold hardiness may incur a cost and thus be selected against (Oakley et al., 2014).

Few studies have investigated a potential survival cost of excess cold hardiness in warm environments, though many studies have shown a trade-off between plant size or growth rate and cold hardiness, which implies a growth cost of cold hardiness (e.g., Loehle et al., 1998; Darychuk et al., 2012; Prada et al., 2016). However, smaller size does not necessarily translate to lower fitness or survival (e.g., Grime, 1974) and thus does not imply a trade-off between cold hardiness and fitness or survival in warm environments, though it may incur a competitive disadvantage (e.g., Rehfeldt et al., 2018). The question of whether plant cold hardiness incurs a fitness or survival cost in warm environments has, to date, been addressed chiefly in the model species *Arabidopsis thaliana,* an herbaceous annual. Conclusions vary among studies, with some showing a fitness or survival cost (Jackson et al., 2004; Oakley et al., 2014) and others selective neutrality (Jackson et al., 2004; Zhen et al., 2011). Whether freezing-response traits are selectively neutral or incur a fitness or survival cost under conditions of relaxed selection has important implications for understanding plant responses to climate change and for selection of seed for restoration.

BIG SAGEBRUSH AS A STUDY SYSTEM FOR INTRASPECIFIC VARIATION IN COLD HARDINESS

Here we evaluate freezing responses in big sagebrush (*Artemisia tridentata* Nutt.; Asteraceae), a widespread perennial shrub that is a foundational species affecting biodiversity across much of western North America (Prevey et al., 2010). This species is a suitable study model because of its high genetic diversity that is thought to have resulted in its ability to occupy a broad geographic range in cold deserts (Mahalovich and McArthur, 2004). Big sagebrush is threatened by the combination of exotic-species invasions and altered wildfire over millions of hectares (Balch et al., 2013), and large-scale seed transfers from wildland populations to restoration-seeding sites have become routine, albeit with mixed success (Knutson et al., 2014). Most studies on understanding intraspecific variation in big sagebrush have focused on differences in adaptation between the three dominant subspecies, which differ in their morphology and leaf chemical composition (Rosentreter, 2005; Jaeger et al., 2016) and occupy different landscapes within the broader species distribution: basin big sagebrush (*A. tridentata* subsp. *tridentata*) of deep basin soils, Wyoming big sagebrush (*A. tridentata* subsp. *wyomingensis*) of low-elevation plains or ridges, and mountain big sagebrush (*A. tridentata* subsp. *vaseyana*) of high elevations, or their hybrids (McArthur, 1992).

Our objective was to determine whether populations of big sagebrush varied physiologically in freezing response and whether the variation corresponded to the subspecies identity or climate of origin of the population. We hypothesized that populations from colder climates would have greater freezing resistance and that freezing resistance would vary by season and reflect dynamic freezing safety margins. We predicted there would be no relationship between freezing resistance and survival (i.e., selective neutrality on freezing response traits) in a relatively warm location but that plants with greater freezing resistance would have higher survivorship in colder locations. These questions were evaluated for 14 populations collected from various locations in the western United States and grown together in three common gardens that spanned a climate gradient.

MATERIALS AND METHODS

We measured several physiological responses to freezing in late winter (before shoot elongation) and again in early spring (during shoot elongation) for 4–6 populations from each of the three major subspecies of big sagebrush, grown together in a relatively warm common garden. We investigated season and subspecies differences in freezing responses, and we compared freezing responses to population climate of origin and to survival in this and two colder common gardens.

TABLE 1. Common garden location and climate information. Freezing responses were measured for sagebrush collected at the Orchard common garden, while survival was measured in all three gardens. Climate of origin temperature and precipitation variables for each population were extracted from a spline model of climate normals for the western United States (Rehfeldt, 2006). MAT = mean annual temperature, MAP = mean annual precipitation, MTCM = mean temperatures of the coldest month, MTWM = mean temperature of the warmest month, TD = temperature difference (continentality, MTWM − MTCM), D100 = Julian date at which the sum of degree days >5°C reaches 100. Degree days are calculated as the difference between the daily mean temperature and a threshold temperature (in this case, 5°C, see Rehfeldt, 2006).

Garden	Latitude	Longitude	Elevation (m a.s.l.)	MAT (°C)	MAP (mm)	MTCM (°C)	MTWM (°C)	TD (°C)	D100 (Julian date)
Ephraim, Utah	39.369	−111.578	1690	8.6	301	−4.5	21.8	26.3	109
Majors Flats, Utah	39.339	−111.52	2105	6.1	412	−5.4	18.9	24.3	128
Orchard, Idaho	43.322	−115.998	974	10.2	330	−1.8	23.2	25	97

Sagebrush common gardens

Seeds from wild populations of big sagebrush collected from throughout its range were used to propagate seedlings, which were then planted in three common gardens in locations with climates typical of the three major subspecies (Table 1, Fig. 1; Appendix S1; Chaney et al., 2017): Orchard, Idaho on a warm-plains site with climate typical of subsp. *wyomingensis*); Ephraim, Utah on a cold-basin site with climate typical of subsp. *tridentata*; and Majors Flat, Utah on a mesic mountain site with climate typical of subsp. *vaseyana*). Plants were raised from seed in a greenhouse for 3 months, hardened outside for 2 weeks, and planted in late April (Ephraim, Orchard) or early June (Majors Flat) 2010 in a lightly tilled plot surrounded by an above- and belowground hardware cloth fence to exclude herbivores. Plant spacing was 1 m within rows and 1.5 m between rows with a border row planted around the perimeter to minimize edge effects. Plants were watered periodically during the first growing season to ensure establishment.

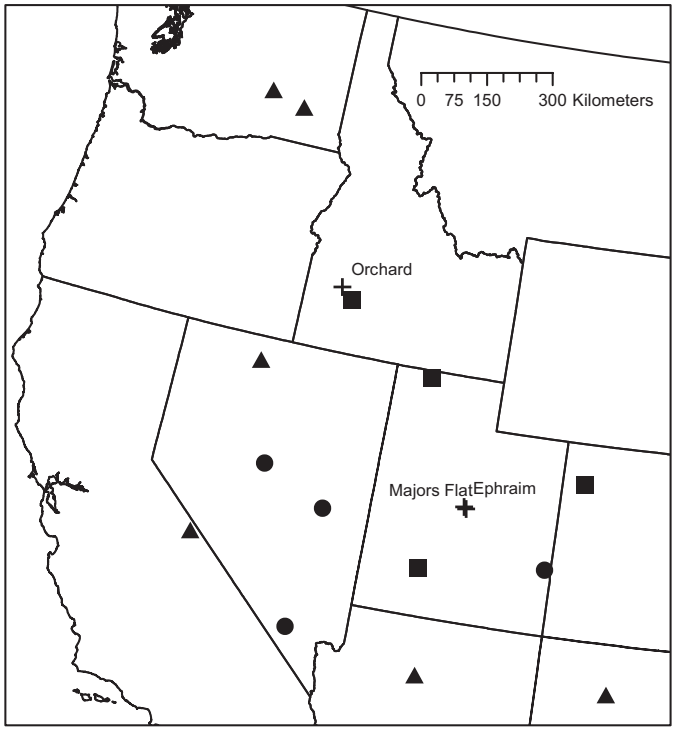

FIGURE 1. Map of common garden (crosses) and population origin locations. Triangles = basin big sagebrush (subspecies *tridentata*); circles = mountain big sagebrush (subspecies *vaseyana*); squares = Wyoming big sagebrush (subspecies *wyomingensis*). Freezing responses were measured for sagebrush collected at the Orchard common garden, while survival was measured in all three gardens.

The Orchard site is relatively warm, the Ephraim site is relatively continental (large difference between mean temperature in the coldest month [MTCM] and mean temperature in the warmest month [MTWM]), and the Majors Flat site is relatively wet compared to the range of climate for big sagebrush and the climates of origin of many of the planted populations (Table 1; Appendix S1).

Tissue sample collection at Orchard garden

Tissue samples were collected at the Orchard common garden in late winter before the initiation of new growth (11 March 2015) and again in early spring after shoot elongation was underway (28 April 2015). Logistical and feasibility constraints limited tissue sampling and physiological measurements to this garden. Plants were nearly 5 years old and were reproductively mature and adult-sized at the time of collection. We collected two subsample terminal branches (10 cm long) from two individuals from each of 4–6 populations of each subspecies, 28 plants total from 14 populations (Fig. 1; Appendix S1). The late winter collection was overwintering foliage, while the early spring collection was new foliage. In either case, we collected the foliar age class that was the most current, most abundant, and arguably the most important for carbon gain at the time of collection. During the week before collection and including collection date, the lowest minimum and highest maximum air temperatures at the site were −5.9°C and 22.1°C for late winter and −0.7°C and 24.0°C for early spring (NWCC 2017). Average minimum air temperatures for March (late winter) and April (early spring) for 2005–2015 at Orchard are −0.3°C and 1.5°C, respectively, with average maximum air temperatures 13.1°C and 16.6°C, respectively (NWCC, 2017).

Ice nucleation temperature

Fresh individual leaves (1 per plant) were placed in plastic bags secured around copper-constantan thermocouples (Type-T, 30 gauge, Cole Parmer, Vernon Hills, IL, USA). Leaves were cooled at a rate of ≤4°C/h to −18°C in a custom lab freezer and their temperatures recorded at 5-s intervals (CR7 data logger, Campbell Scientific, Logan, Utah, USA). Exotherms were identified as rapid increases in temperature caused by freezing of extracellular water, and ice nucleation temperature (NT) was recorded as the lowest temperature reached before the onset of an exotherm.

Initial F_v/F_m and LT_{50}

Dark-adapted F_v/F_m, the ratio of variable to maximum chlorophyll fluorescence, is a measure of the potential quantum efficiency of photosystem II. This unitless ratio is generally close to 0.83 for unstressed leaf tissue, and values decrease upon exposure to various stresses (Maxwell and Johnson, 2000). As such, F_v/F_m is often used in freezing experiments to determine LT_{50} of photosynthetic tissue (e.g., Loik et al., 2004; Sierra-Almeida et al., 2009).

We measured dark-adapted F_v/F_m after at least several hours of dark adaptation with a chlorophyll fluorometer (model LCF, LI-6400, LiCor, Lincoln, NE, USA) before ("Initial F_v/F_m") and after cooling a set of detached leaf samples from each individual plant to a set of temperatures (−5, −15, −25, −35, −45, and −55°C in late winter; −7, −10, −12.5, −15, −17.5, and −20°C in early spring). Control groups were maintained at 3°C for each time period. All cooling occurred in a −80°C freezer with groups of samples in individual plastic bags placed at the center of a 25 kg box of sand, which provided thermal mass for a steady cooling rate of 4.3–5.6°C/h, monitored by thermocouples. Upon reaching their target temperatures, plants were removed from the freezer and allowed to thaw in the dark at 3°C. F_v/F_m was measured the day after freezing and then every second day, until F_v/F_m values no longer changed (7–9 days for lowest temperatures in late winter, 3–5 days for lowest temperatures in early spring, see time series in Appendix S2). Measurements of control (non-frozen) and frozen leaves occurred at the same time. All measurements that were taken after F_v/F_m no longer changed with time were averaged to obtain a final measurement used in LT_{50} calculations. LT_{50} was calculated as the inflection point of a 4-parameter sigmoid-logistic response of F_v/F_m to temperature, thus identifying the temperature at which the value of F_v/F_m was half way between lower and upper asymptote values (JMP 12.1.0; SAS Institute, Cary, NC, USA). One population (WAT1) had very low initial F_v/F_m in spring, and we were unable to determine spring LT_{50} for this population because its F_v/F_m responses did not clearly show asymptotes.

Population climate of origin and survivorship

Long-term temperature and precipitation for each population's origin (seed collection site) were extracted from a spline model of climate normals for the western United States (Rehfeldt, 2006). We tested a wide range of climate metrics related to the amount and seasonality of precipitation, temperature (mean, minimum, and maximum), thermal accumulation over time (i.e., degree days), and continentality (difference between summer and winter temperatures, Appendix S3). Survivorship was calculated for each population in each garden as the number of individuals alive divided by the number of individuals originally planted (usually 10), as measured in the spring of 2015.

Statistical analyses

Linear mixed models—We tested the significance of seasonal and subspecies differences in freezing responses for plants collected at the Orchard common garden using linear mixed models (JMP 12.1.0) with initial F_v/F_m, NT, LT_{50}, or NT − LT_{50} as response variables. Fixed effects were collection date (late winter or early spring), subspecies identity (T, V, W) and collection date × subspecies. Random effects were population and individual. We treated populations as random because the 4–6 populations of each subspecies represented a random subset of all populations rather than a fixed set of specific populations we wished to characterize. We treated individuals as random because we had multiple measurements (i.e., winter and spring values) per individual. Tukey–Kramer analyses were used to compare values among subspecies. For homogenizing variance, NT was square-root transformed and LT_{50} was log transformed.

Pearson correlations—To determine the strength of genecological relationships, we evaluated correlations between freezing responses and climate of origin at the population level (i.e., population average used as modeling variable). Where multiple significant correlations existed (as was generally the case because climate variables are related), we report the climate variable with the best fit (highest $|r|$, lowest AIC) for any given freezing response but detail all results in a supplementary table (Appendix S4).

To evaluate whether individuals maintained the same relative ranking in freezing response in winter and spring (e.g., whether the plants with the greatest freezing resistance in winter also had the greatest freezing resistance in spring), we evaluated correlations between freezing responses measured in late winter (initial F_v/F_m, NT, LT_{50}, and NT − LT_{50}) and the same responses for the same individuals measured in early spring.

To determine whether variation in freezing safety margins was driven by NT or LT_{50} or jointly by both variables, we evaluated correlations between NT and LT_{50} and between freezing safety margin and NT and LT_{50} in both late winter and early spring. We used individual rather than population as the unit of replication because measurements were made at the individual level, and, unlike other analyses described here, comparisons were not being made with population-level variables such as climate of origin or survivorship.

To link freezing responses and survivorship, we evaluated correlations between each of the freezing responses (measured at Orchard) and survivorship (as of spring 2015) at each of the three gardens. We report all significant correlations in the manuscript and list all correlations in Appendix S5. Comparing Orchard freezing responses with survivorship for the same populations at Ephraim and Majors Flat assumed that the expression of the genetic variation and any possible within-population selection (during mortality in years 1–5 after planting) did not differ among gardens.

RESULTS

Ice nucleation temperature

Ice nucleation temperature (NT) decreased significantly from −6.89 ± 0.17°C (ranging −5.61 to −8.96°C) in late winter to −7.97 ± 0.16°C (from −6.69 to −10.08°C), in early spring, showing no significant differences among subspecies (Table 2). There were no significant correlations between NT and population climate of origin in winter or spring ($P > 0.07$; Appendix S4).

Initial F_v/F_m

The initial F_v/F_m of plant tissue measured upon collection in the field (before freezing in the laboratory) was lower in early spring (0.756 ± 0.013) than late winter (0.812 ± 0.005; Table 2, Fig. 2), despite colder temperatures in late winter. Initial F_v/F_m did not vary

among subspecies in late winter but was greatest in subspecies *vaseyana* and least in subspecies *tridentata* in spring (significant date × subspecies interaction; Table 2, Fig. 2). There were no significant correlations between initial F_v/F_m and population climate of origin in late winter ($P > 0.17$; Appendix S4). In early spring, initial F_v/F_m was greater in populations that originated in colder, wetter places (e.g., F_v/F_m was correlated with D100, the date by which sum of degree days reaches 100; Fig. 3, but see also Appendix S4).

LT_{50}

LT_{50} increased significantly from −31.1 ± 1.1°C (ranging −19.2 to −46.9°C) in late winter to −13.1 ± 0.3°C (ranging −10.30 to −17.0°C) in early spring, indicating a loss in freezing resistance, with no differences among subspecies (Table 2). Mean F_v/F_m during chilling trials decreased to nearly 0 upon cooling to −20°C in spring, compared to decreasing to only 0.4 at −40°C or colder in winter (Fig. 4). Blackening, an unambiguous indicator of leaf mortality, was observed for the lowest temperatures immediately upon thawing for leaves collected in spring but not winter (not shown).

LT_{50} varied considerably among populations in both winter and spring (Fig. 5A, B). LT_{50} in winter was lower (and thus freezing resistance greater) for populations that originated from colder places that experienced delayed onset of spring warmth (e.g., LT_{50} was negatively correlated with D100, the date by which the sum of degree days reaches 100, Fig. 5A, but see also Appendix S4). LT_{50}

TABLE 2. R^2 values for model fits; *P* values for fixed effects (season, subspecies and subspecies × season) in mixed models for freezing responses measured at the Orchard common garden (NT = nucleation temperature); and percentage of variance explained by the random effects of individual or population. *P* values < 0.05 are in bold.

Statistic	NT	Initial F_v/F_m	LT_{50}	NT − LT_{50}
R^2	0.33	0.81	0.86	0.9
P values				
Season	<0.0001	<0.0001	<0.0001	<0.0001
Subspecies	0.735	0.051	0.165	0.333
Season × subspecies	0.087	**0.010**	0.376	0.191
Percentage variance explained				
Population	0.00	6.69	15.74	0.00
Individual	1.14	31.08	0.00	1.41

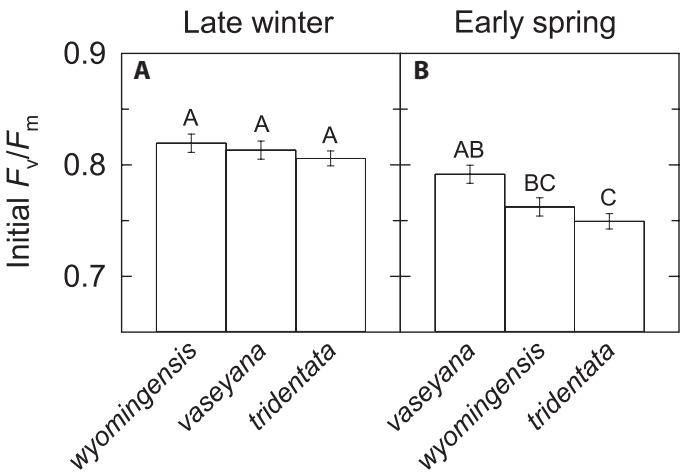

FIGURE 2. Means and standard errors of F_v/F_m before freezing by subspecies for (A) late winter (overwintering foliage) and (B) early spring (new foliage) measured at the Orchard common garden. Letters denote Tukey–Kramer comparisons among subspecies for both time periods. Subspecies not labeled with the same letter are significantly different.

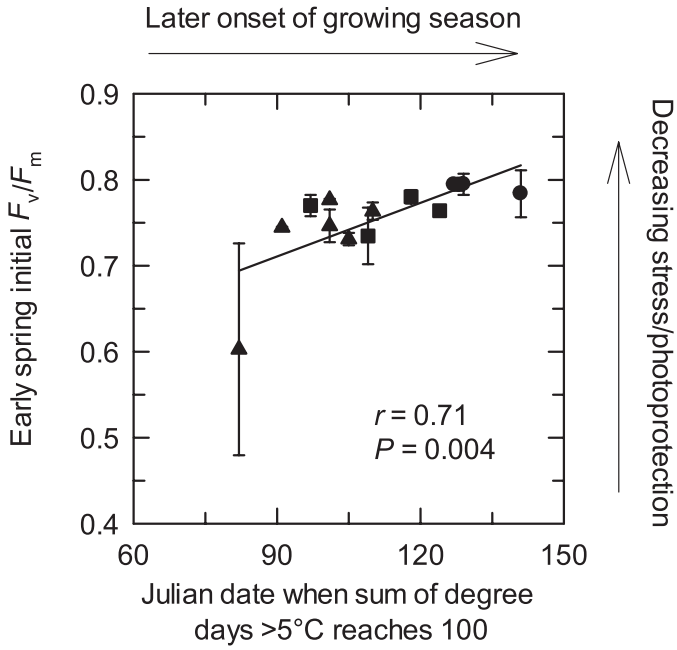

FIGURE 3. Correlation between early spring initial F_v/F_m of each population (±SE, measured at the Orchard common garden) and the Julian date when the sum of degree days that are >5°C reaches 100 at the location of population origin. Triangles = basin big sagebrush (subspecies *tridentata*); circles = mountain big sagebrush (subspecies *vaseyana*); squares = Wyoming big sagebrush (subspecies *wyomingensis*). The climate variable with the best fit (highest |*r*|, lowest Akaike information criterion) for this freezing response is plotted here. All climate correlations for this freezing response are listed in Appendix S4. Degree days are calculated as the difference between the daily mean temperature and a threshold temperature (here 5°C; see Rehfeldt, 2006).

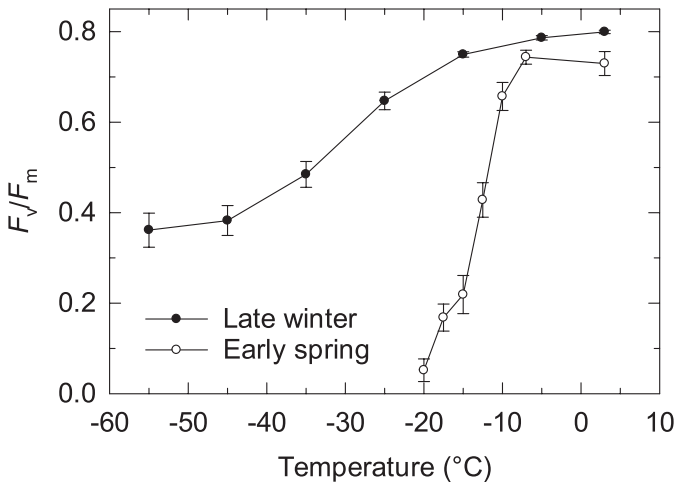

FIGURE 4. Mean (±SE) dark-adapted F_v/F_m values for all 28 big sagebrush individuals collected at the Orchard common garden and frozen to a series of temperatures (shown on *x*-axis) in late winter (overwintering foliage, filled circles) or early spring (new foliage, open circles).

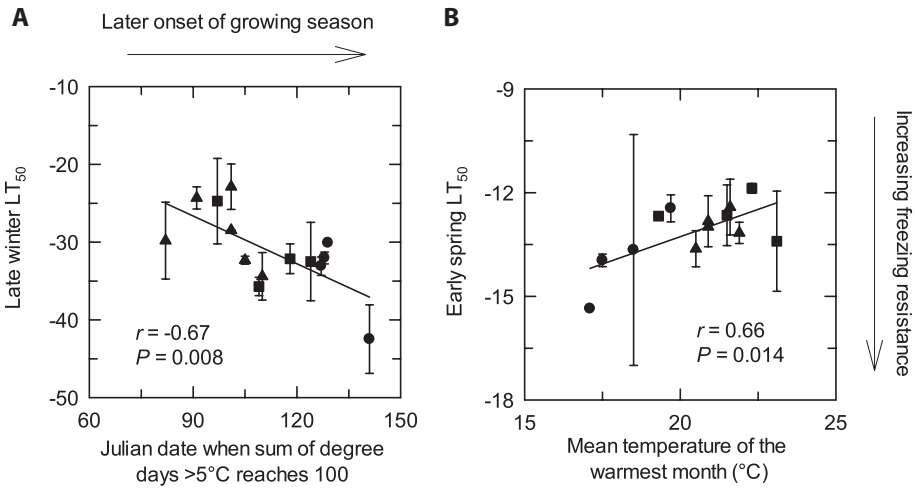

FIGURE 5. Correlations between winter or spring LT_{50} (±SE, measured at the Orchard common garden) and climate of origin by population. Triangles = basin big sagebrush (subspecies *tridentata*); circles = mountain big sagebrush (subspecies *vaseyana*); squares = Wyoming big sagebrush (subspecies *wyomingensis*). The climate variable with the best fit (highest $|r|$, lowest Akaike information criterion) for each freezing response is plotted here. All climate correlations for these freezing responses are listed in Appendix S4. Degree days are calculated as the difference between the daily mean temperature and a threshold temperature (here 5°C; see Rehfeldt, 2006).

in spring was lower for populations that originated in places with cooler summers (e.g., lower mean temperature of the warmest month, Fig. 5B, but see also Appendix S4).

Freezing safety margin (NT – LT_{50})

The freezing safety margin (NT – LT_{50}) decreased significantly from 24.2 ± 1.1°C in late winter (from 13.0 to 40.1°C) to 5.04 ± 0.30°C in early spring (from 2.54 to 9.73°C) and did not differ among subspecies (Table 2). The low LT_{50} relative to NT for all individuals in late winter (Fig. 6A) indicated a general freezing-tolerance mechanism, with the freezing safety margin driven entirely by variation in LT_{50} (Fig. 6C, E). We therefore did not further investigate correlations between late winter NT – LT_{50} and either population climate of origin or survivorship. In spring, variation in both NT (though marginal) and LT_{50} contributed to variation in the freezing safety margin (Fig. 6D, F), though all individuals tolerated at least some ice formation in their tissues (LT_{50} lower than NT, Fig. 6B). Populations that originated in more continental climates had smaller freezing-safety margins in spring (Fig. 7).

Winter vs. spring freezing responses

We observed a decoupling between winter and spring freezing responses, with no significant relationships between winter and spring NT, LT_{50}, or NT – LT_{50} at the plant level (P = 0.57–0.97, not shown). Only a marginal correlation between winter and spring was evident in initial F_v/F_m (r = 0.33, P = 0.086, not shown).

Correlations between Orchard freezing responses and survivorship

Populations with greater freezing resistance (lower LT_{50}) at Orchard in late winter had lower survivorship at Orchard (Fig. 8A). Survivorship at Ephraim was not correlated with any freezing response measured at Orchard (P > 0.18, Appendix S5). Populations that resisted lower temperatures at Orchard in early spring (lower LT_{50}) had lower survivorship at Majors Flat (Fig. 8B).

DISCUSSION

Freezing stress can have particularly strong impacts on cold-desert species due to the tendency for freezing nighttime temperatures to prevail during the brief spring periods when moisture is most available for growth (Caldwell, 1985). We found that the greater impacts of freezing in spring compared to winter in big sagebrush (as shown for seedlings, Brabec et al., 2017) are due to (1) reductions in freezing resistance and (2) a corresponding shift from a safer tolerance toward a riskier avoidance freezing-response mechanism from winter to spring. Freezing responses generally varied more among populations than among the subspecies associated with the populations, suggesting that adaptation to freezing occurs at the population and not subspecies level. The lack of variation in freezing responses among subspecies in spite of high variability among populations may be related to the generally greater range of values in low temperature (MTCM) and continentality of population origin among populations within compared to among subspecies averages (Appendix S1). Similar patterns of stronger population compared to subspecies variation have been observed for timing of germination (Meyer et al., 1990) and flowering (Richardson et al., 2017) in big sagebrush, though subspecies differed in drought adaptation (Kolb and Sperry, 1999a).

While all populations would conventionally be categorized as having a freezing tolerance mechanism, freezing safety margins (FSMs) became much narrower in spring, with populations from more continental origins having narrower FSMs. Relationships between climate of origin and freezing resistance among populations were generally as anticipated: populations from places with cooler/later springs had greater winter freezing resistance, and populations from places with cooler summers had greater spring freezing resistance. Relationships between freezing resistance and survival were unexpected: populations with greater winter freezing resistance had lower survival in the warmest garden, while populations with greater spring freezing resistance had lower survival in a colder and more continental garden. Below, we explain how these results suggest a cost to excess freezing resistance under warmer conditions and how the cost may be characterized by reduced photosynthesis during the wet but cool seasons due to retention of freezing resistance.

Winter cold hardiness ≠ spring cold hardiness

Winter and spring freezing resistance appeared to differ in their underlying physiology in big sagebrush, as evidenced by their different patterns of F_v/F_m loss upon cooling (Fig. 4). Although acclimated to freezing and not actively growing, the big sagebrush we measured

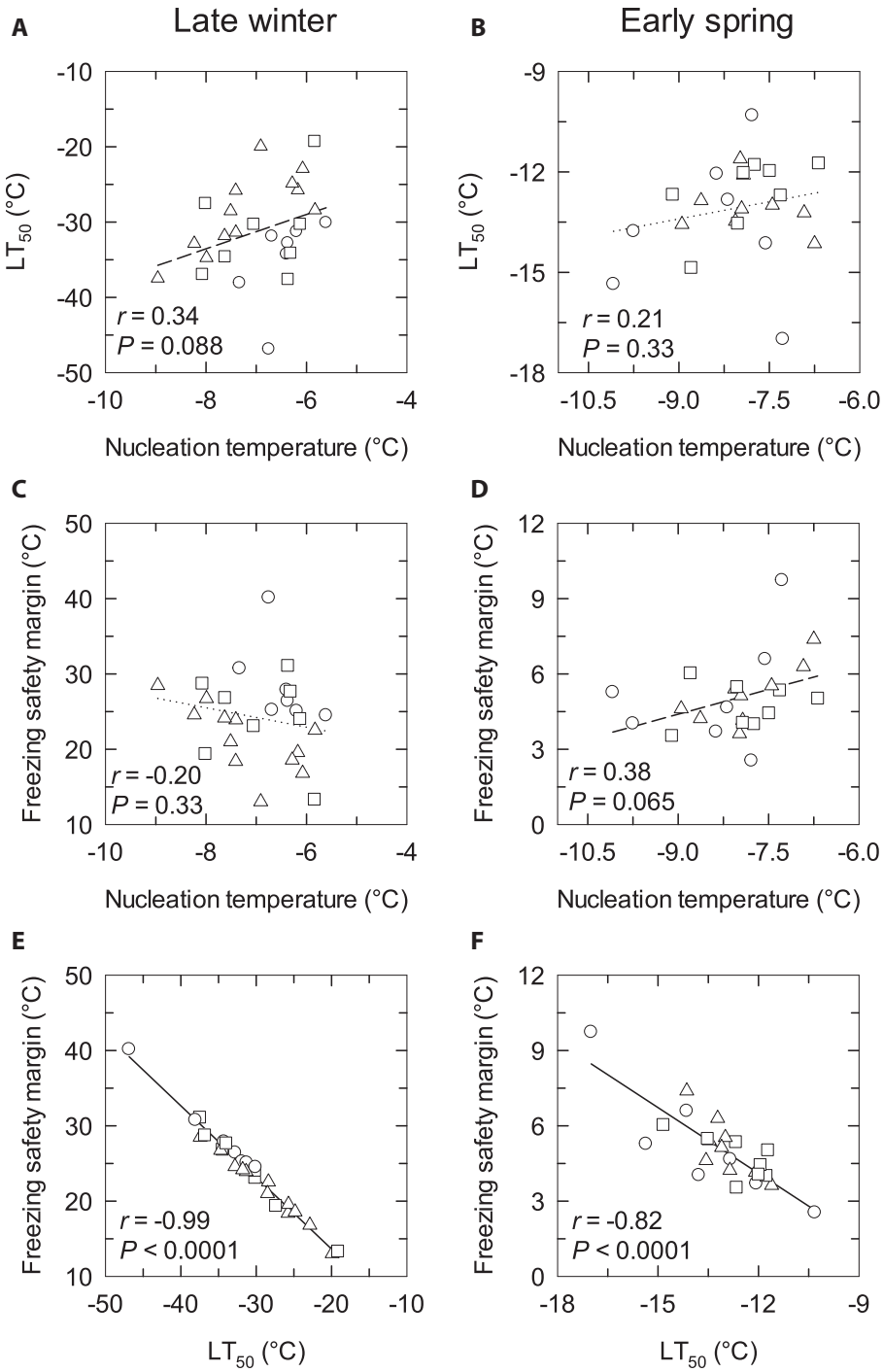

FIGURE 6. Correlations between LT_{50}, NT, and freezing safety margin (NT − LT_{50}) for individual plants (n = 28) at the Orchard common garden. Triangles = basin big sagebrush (subspecies *tridentata*); circles = mountain big sagebrush (subspecies *vaseyana*); squares = Wyoming big sagebrush (subspecies *wyomingensis*). Correlations shown by solid (significant, $P < 0.05$), dashed (marginally significant, $0.05 < P < 0.1$), or dotted (not significant, $P > 0.1$) lines.

at Orchard in late winter had F_v/F_m values near 0.8 (Fig. 2A). This result indicates that they were not in the state of deep winter dormancy that is typical of many evergreen plants such as temperate and boreal conifers. This winter dormancy state is characterized by very low F_v/F_m resulting from a lack of photosynthetic capacity caused by low levels of the D1 and oxygen-evolving complex (OEC) proteins and high levels of xanthophylls in the photoprotective de-epoxidized state of their cycle (Zarter et al., 2006). Indeed, big sagebrush is known to photosynthesize during periods of mild winter temperatures (Gilmanov et al., 2004; M. J. Germino, unpublished gas-exchange measurements at the Orchard garden). However, the slow deep reductions in F_v/F_m to moderately low values (Fig. 4; Appendix S2A) without leaf blackening we observed in late winter with experimental freezing to temperatures much colder than field temperatures (less than −50°C) suggest that big sagebrush may be capable of reaching a state of deep winter dormancy via photoprotective downregulation. In contrast, the greater reductions of F_v/F_m (effectively complete loss; Fig. 4) and leaf blackening we observed at considerably higher freezing temperatures in new leaves in spring may indicate that new foliage lacks this capability. In other words, we may have observed a controlled and orderly shutting down of the photosynthetic apparatus (i.e., deformation or disassembly of D1 and OEC proteins; see Demmig-Adams and Adams, 2006) upon cooling to very low temperatures (less than −35°C) in winter when plants were acclimated to freezing. In contrast, cell damage and death were clearly evident upon cooling of actively growing new tissue in spring to (higher) low temperatures (less than −15°C).

Because we measured new spring leaves, our results do not distinguish between the possible roles of ontogeny and seasonal deacclimation/growth initiation in reduced spring freezing resistance. However, we also collected a small amount of the previous year's overwintering foliage in spring, concurrent with our collection of new foliage. The older, overwintering foliage lost cold hardiness from winter to spring but had greater cold hardiness than the new spring foliage (data not shown), suggesting that both seasonal deacclimation and ontogeny play a role in reduced spring freezing resistance after growth initiation. However, Loik et al. (2004) showed that overwintering foliage of experimentally warmed sagebrush that had new spring growth had greater freezing resistance than control plants that were not yet growing. Their results suggest that overwintering foliage need not lose freezing resistance upon initiation of new spring growth.

It is perhaps not surprising that winter and spring freezing resistance (LT_{50}) were uncorrelated with each other, given the large differences we observed in their physiology. However, it is very interesting that both were correlated in different ways with the climate of origin of the populations, indicating differences in natural

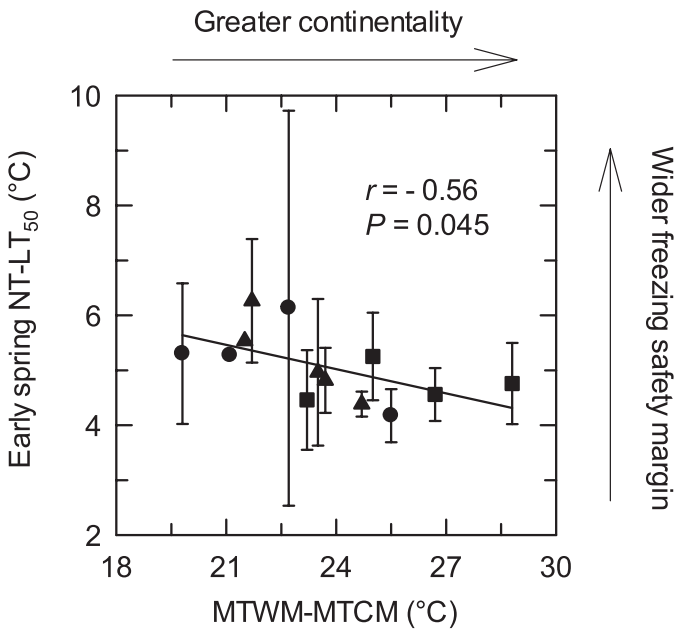

FIGURE 7. Correlation between freezing safety margin in early spring of each population (±SE, measured at the Orchard common garden) and continentality (MTCM-MTWM) of population origin. Triangles = basin big sagebrush (subspecies *tridentata*); circles = mountain big sagebrush (subspecies *vaseyana*); squares = Wyoming big sagebrush (subspecies *wyomingensis*). The climate variable with the best fit (highest |*r*|, lowest Akaike information criterion) is plotted here. All climate correlations for this freezing response are listed in Appendix S4. MTCM: mean temperature of the coldest month. MTWM: mean temperature of the warmest month).

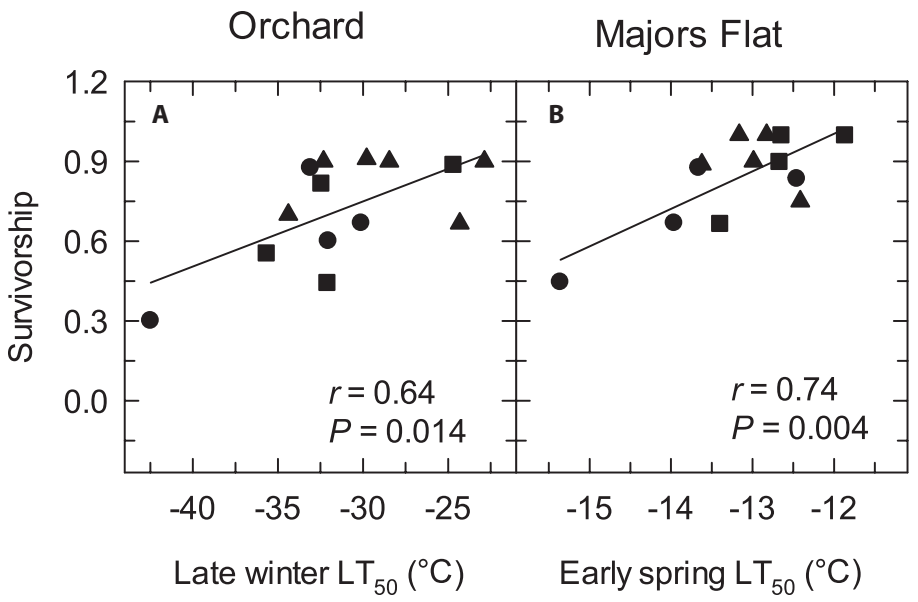

FIGURE 8. Relationship of population survival to the most explanatory freezing-response variable (measured at Orchard) for each garden. Triangles = basin big sagebrush (subspecies *tridentata*); circles = mountain big sagebrush (subspecies *vaseyana*); squares = Wyoming big sagebrush (subspecies *wyomingensis*).

selection on freezing resistance in different seasons (Fig. 5A, B). Greater winter freezing resistance (lower LT_{50}) appears to have been selected in populations from places with later onset of spring warmth (D100), greater depth and duration of low temperatures (DD0), cooler summers, and higher growing season precipitation (Fig. 5A; Appendix S4). In such places, populations are perhaps more likely to encounter temperatures low enough to require photosynthetic dormancy and less likely to have the opportunity or the need to photosynthesize with mild mid-winter temperatures. In contrast, greater spring freezing resistance (lower LT_{50}) appears to have been selected in populations from places with cooler summers that are less continental (Fig. 5B; Appendix S4). Freezing temperatures are extremely rare in summer, even in the coolest parts of the range of big sagebrush. Thus, we do not interpret this pattern as selection for maintenance of freezing resistance where summer frosts are likely, but instead a need to quickly lose freezing resistance in order to be more productive in spring in places where summers are hotter. This seasonal variation in selection on freezing resistance is a novel discovery that has major implications for the understanding of freezing responses. In support of the concept, spring and fall cold hardiness in Douglas fir (*Pseudotsuga menzisii*) have been shown to be uncorrelated and controlled by different genes (O'Neill, 1999; Anekonda et al., 2000; Jermstad et al., 2001).

Carbon gain when moisture is available may be critical to survival

Greater cold hardiness has often been linked to slower growth among populations within a species (e.g., Loehle et al., 1998; Darychuk et al., 2012; Prada et al., 2016), but the connection between cold hardiness and survival is poorly characterized, particularly in warm environments. We observed greater mortality in populations with greater winter freezing resistance in a warm garden and greater spring freezing resistance in a colder garden. These patterns in mortality suggest that in both cases excess freezing resistance carried a survival cost that we propose may be associated with lower carbon gain during periods of moisture availability. Carbon gained during cooler wetter periods may assist plants in avoiding carbon limitation during drought periods, which could reduce the ability of plants to recover from stresses such as aphid feeding on phloem, defoliation due to agora moths, and browsing by wild ungulates, all of which are common on sagebrush (McDowell et al., 2010; Poyatos, 2013). The vital importance of high productivity during periods of early spring moisture availability is particularly likely for species such as big sagebrush that have been observed to operate closer to the isohydric end of the stomatal control spectrum (Dobrowolski et al., 1990; Naithani et al., 2012; but see Kolb and Sperry, 1999b). Although big sagebrush is a desert species, its highest rates of growth and carbon gain occur during periods of abundant water availability (DePuit and Caldwell, 1973; Miller and Shultz, 1987; Ryel et al., 2010; Germino and Reinhardt,

2014), which exposes those processes to the freezing nights that co-occur in spring. A survival cost to freezing resistance related to carbon gain may help explain why Wyoming big sagebrush in two 27-yr-old common gardens showed greater mortality in populations that originated in places with lower minimum winter temperatures than the gardens (Germino et al., 2019).

The freezing safety margin concept

The need for sagebrush to be productive during brief periods of spring moisture availability may also explain why we observed narrower freezing safety margins (FSMs) in new spring growth in populations originating in more continental locations (Fig. 7). The FSM can be considered a relative measure of where a population or species "falls" on a continuum from freezing avoidance to freezing tolerance. Individual plant variation in FSMs in late winter was driven entirely by LT_{50} values (Fig. 6C, E); small NT differences among individuals were inconsequential relative to the very large LT_{50} differences among individuals. In contrast, narrower FSMs for new growth in spring were caused by both a higher LT_{50} and lower NT (Fig. 6D, F). The greater importance of LT_{50} than NT could indicate that in late winter when plants are not growing, nucleation temperature is of little consequence to a plant's overall freezing resistance; instead, the ability to tolerate extracellular ice is critical. Differences in FSM among individuals or populations are not especially meaningful during winter when all plants show a high degree of freezing tolerance. LT_{50} values in both seasons were always lower temperatures than NTs and thus reflected physiological tolerance of extracellular ice (Fig. 6A, B). Nevertheless, the importance of both NT and LT_{50} in determining the spring FSM and the clear evidence for selection on the level of the FSM for new growth in early spring suggests that there are important genetic differences in physiological response to the degree of freezing during active spring growth. A potential physiological mechanism underlying the spring FSM variation (aside from the mechanisms that dictate LT_{50} and NT) could include enhancing freezing survival with more time for rapid physiological adjustments that bolster "tolerance" once ice has formed in leaves of plants having greater FSM. Variation in FSM could also result from physiological differences among populations in their responses to desiccation, which can result in lower NTs (Rada et al., 2001; Sierra-Almeida et al., 2009). Whatever the underlying mechanism, plants with smaller FSMs rely more on avoidance than tolerance of extracellular ice formation. Smaller FSMs confer a growth advantage by liberating the plant from the greater costs-to-growth of improving freezing tolerance, but smaller FSMs also confer a greater risk of freezing damage or death at temperatures closer to the NT.

Implications for big sagebrush response to climate shifts

Big sagebrush cover and productivity have historically decreased at the warm end and increased at the cold end of its range in response to warming trends (Kleinhesselink and Adler, 2018), which is consistent with our finding that cold-adaptation confers a fitness cost under warmer conditions. Our finding is also consistent with species-distribution model and ecohydrological model predictions that big sagebrush will contract at the warm end and expand at the cold end of its range with climate warming (Schlaepfer et al., 2012; Still and Richardson, 2015). Our results furthermore suggest a potential mechanism for these observations. Specifically, excess freezing resistance may prevent optimum carbon gain during critical periods of moisture availability, leading to lower productivity and eventual death. Moreover, throughout its range, big sagebrush could be negatively impacted by a mismatch between levels of freezing resistance and warming climate over time. Plasticity in freezing resistance and the ability of populations to move or exchange genes quickly enough to keep pace with warming rates are unmeasured factors that would strongly affect whether a warming-induced mismatch between freezing resistance and minimum temperatures results in big sagebrush mortality. Data of this nature form the basis for recommendations of assisted migration of populations or species (e.g., Gray et al., 2011; Buma and Wessman, 2013; Richardson and Chaney, 2018). Notably, these findings may help explain the tendency for low establishment success for the many big sagebrush restoration trials in which seed was moved from cooler to warmer environments (Germino, 2014).

Our measurements were taken after only 5 years of growth, and additional mortality of maladapted populations of big sagebrush in our gardens is likely according to the findings and guidelines of Germino et al. (2019). However, measuring threshold freezing responses in addition to survival allowed us to capture potential influences of climate extremes that might take decades to shape survival patterns. Additionally, Germino et al. (2019) found that the minimum temperature of populations' origins similarly explained their differences in survival in both short- and long-term observations. Still, our results may be more relevant to the establishment phase, an important demographic bottleneck for big sagebrush populations (James et al., 2011). The establishment phase is of particular importance when considered in the context of restoration after megafires, which affect more than half of big sagebrush's range (Miller et al., 2011).

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AUTHOR CONTRIBUTIONS

M.J.G. and B.E.L. conceived of the research questions and experimental design, using the common gardens established by B.A.R. B.A.R. and M.J.G. measured survivorship, and B.E.L. measured the physiological responses and performed statistical analyses. B.E.L. and M.J.G. wrote the manuscript, and B.A.R. provided comments and editorial advice.

DATA ACCESSIBILITY

Data from this study are available through Figshare at https://figshare.com/articles/Sagebrush_freezing_responses/8059814.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. *Artemesia tridentata* populations: lat./long., elevation, and climate of population origin.

APPENDIX S2. Time courses of dark-adapted F_v/F_m.

APPENDIX S3. Climate variables and their definitions.

APPENDIX S4. Correlations between Orchard garden freezing responses and climate of population origin.

APPENDIX S5. Correlations between Orchard garden freezing responses and survival in all three gardens.

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