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## Research paper

# Ecophysiological variation in two provenances of *Pinus flexilis* seedlings across an elevation gradient from forest to alpine

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Climate change is predicted to cause upward shifts in forest tree distributions, which will require seedling recruitment beyond current forest boundaries. However, predicting the likelihood of successful plant establishment beyond current species' ranges under changing climate is complicated by the interaction of genetic and environmental controls on seedling establishment. To determine how genetics and climate may interact to affect seedling establishment, we transplanted recently germinated seedlings from high- and low-elevation provenances (HI and LO, respectively) of *Pinus flexilis* in common gardens arrayed along an elevation and canopy gradient from subalpine forest into the alpine zone and examined differences in physiology and morphology between provenances and among sites. Plant dry mass, projected leaf area and shoot:root ratios were 12–40% greater in LO compared with HI seedlings at each elevation. There were no significant changes in these variables among sites except for decreased dry mass of LO seedlings in the alpine site. Photosynthesis, carbon balance (photosynthesis/respiration) and conductance increased  $>2\times$  with elevation for both provenances, and were 35–77% greater in LO seedlings compared with HI seedlings. There were no differences in dark-adapted chlorophyll fluorescence ( $F_v/F_m$ ) among sites or between provenances. Our results suggest that for *P. flexilis* seedlings, provenances selected for above-ground growth may outperform those selected for stress resistance in the absence of harsh climatic conditions, even well above the species' range limits in the alpine zone. This indicates that forest genetics may be important to understanding and managing species' range adjustments due to climate change.

**Keywords:** carbon balance, limber pine, Niwot Ridge, treeline, water relations.

## Introduction

Changes in high-elevation forest range limits, such as expansion of forest into subalpine and alpine meadows, require new tree establishment into these areas (review by Smith et al. 2009). In the alpine-treeline ecotone, survival of newly germinated seedlings appears to be a significant demographic bottleneck for tree establishment, and high mortality of conifer seedlings in these vegetation boundaries has been linked to physiological dysfunction, rather than predation or disease (Germino and Smith 1999, Maher and Germino 2006). Thus, understanding how ecophysiological factors limit tree seedling establishment and survival is critical for predicting changes in forest distribution.

Several lines of evidence suggest that young seedlings encounter problems maintaining a positive carbon balance when exposed to environmental stressors (Kohyama 1983, Canham et al. 1999, Bansal and Germino 2008, Kitajima and Myers 2008). Plant carbon balance is the net balance of carbon gain (gross photosynthesis), processing (respiration) and storage (mobile non-structural carbohydrate (NSC) pool; dry mass production). Factors limiting carbon acquisition have been implicated as the main limitation to seedling growth at high elevation (Smith et al. 2003). For example, in a high-elevation forest in Wyoming, Cui and Smith (1991) reported greater mortality in first-year seedlings of *Abies lasiocarpa* (Hooker) Nuttall compared with older seedlings, which was

linked with decreased photosynthesis and water use efficiency (photosynthesis/transpiration) in the younger seedlings. Similarly, reduced carbon gain in young seedlings of *A. lasiocarpa* and *Picea engelmannii* Parry ex. Engelm in open canopy vs. forest understory microsites was associated with low night-time temperatures and high sunlight exposure during the day (Johnson et al. 2004), factors that can cause photoinhibition for tree seedlings at alpine treeline (Germino and Smith 1999, 2001).

Seedlings often express adaptive traits that favor positive net carbon balance in order to tolerate stressful environmental conditions where seedlings germinate, and also to maximize carbon gain in desirable growing sites. For pioneer or shade-intolerant species that successfully establish into unforested areas (such as into the alpine zone), seedlings often exhibit allocation patterns and plant architecture that enhance carbon gain. This includes greater specific leaf area (SLA, ratio of leaf area to leaf mass), leaf area ratio (ratio of leaf area to whole-plant dry mass), leaf area:sapwood area and nitrogen concentrations in leaves of seedlings compared with older life stages (Reich et al. 1998, Thomas and Winner 2002, Sala 2006, Greenwood et al. 2008, Bansal and Germino 2010). For example, a meta-analysis by Thomas and Winner (2002) showed that SLA (inverse of leaf mass per unit area, which these authors used), which confers greater carbon gain on a leaf mass basis in seedlings, was consistently greater in seedlings compared with adult trees.

Observations of ecophysiological variation in tree populations along elevation gradients can provide insight into tree and forest responses to potential changes in climate. However, complex interactions between genotypic constraints and the environment limit the use of natural variation to predict plant responses to climate changes. For example, characteristics that affect plant carbon balance such as stomatal density, conductance, photosynthesis and respiration have been shown to be quite plastic in the field, and can increase, decrease or remain the same across elevations, depending on the species (Cordell et al. 1998, Hovenden and Brodrigg 2000, Hultine and Marshall 2000, Schoettle and Rochelle 2000, Premoli and Brewer 2007). On the other hand, several common garden studies have demonstrated that seedlings from provenances of tree species adapted to colder environments have greater photosynthetic capacity and leaf N, and an increased root:shoot ratio compared with those from warmer provenances when grown at the same elevation and under identical conditions (Reich et al. 1996, Cordell et al. 1998, Oleksyn et al. 1998, Premoli et al. 2007). The overall plant response may underscore adaptive strategies that balance selection for mean growth rate vs. tolerance of harsh environmental conditions (e.g., Grime 1974, Bazzaz 1979, Green 2005).

We designed a study to compare provenance-related (potentially genetic) and environmental constraints to seedling carbon

gain, morphology and survival within and beyond current ranges of subalpine forest. We measured morphological traits and ecophysiological performance in two provenances of first-year seedlings of limber pine (*Pinus flexilis* James) transplanted into common gardens arrayed along an elevation gradient ranging from subalpine forest into the alpine zone. The gradient included synchronous changes in elevation and canopy, because we were interested in the combined impacts of these factors as they jointly influence seedling success. Limber pine occupies a wide altitudinal range in the Rocky Mountains (1600–3300 m, stretching from lower subalpine forest to alpine treeline), and relies on birds for seed dispersal, suggesting little genetic variation among populations (Schoettle and Rochelle 2000). Previous studies on adult limber pine have shown that this species has a wide range of physiological tolerance across sites and elevations (Mooney et al. 1964, Lepper 1980, Letts et al. 2009), but has little variation in plant growth and needle characteristics (Schoettle and Rochelle 2000). Thus, we predicted low variation in seedling morphology and needle characteristics along our elevation gradient or between provenances. We predicted differences in physiology with elevation, and we hypothesized that these differences would vary according to local adaptation. Specifically, we expected greater carbon balance, dry mass gain and survival in seedlings from low-elevation compared with high-elevation seed sources at lower-elevation sites, and relatively greater carbon balance, dry mass and survival in high-elevation seedlings at higher-elevation sites.

## Materials and methods

Newly germinated seedlings of two provenances of limber pine were transplanted into common garden plots along an elevation gradient at Niwot Ridge, CO, USA in 2009. Survival, dry-mass gain, needle morphology, photosynthetic gas exchange, water relations, needle chlorophyll fluorescence ( $F_v/F_m$ ) and carbohydrate concentrations were compared between provenances and elevations during the first summer of growth. Differences in survival were compared with carbon relations, microclimate and plant water status along the gradient.

### Site description, plant materials and microclimate

Three sites were established in 2009 along an elevation gradient at the Niwot Ridge Long-Term Ecological Research Station, Nederland, CO, USA (40°3'N, 105°36'W). The sites were located in lower subalpine forest (LSA, 3080 m) at the upper subalpine-treeline ecotone (USA, 3425 m), and in the alpine tundra (ALP, 3545 m). The LSA site was an understory site, characterized by mature subalpine forest, containing a mixture of lodgepole pine (*Pinus contorta* Douglas), Engelmann spruce (*P. engelmannii* Parry ex. Engelm), subalpine fir (*A. lasiocarpa*

(Hooker) Nuttall) and limber pine with canopy gaps up to ~15 m in diameter and little to no herb cover. The USA site was in an open area having no overhead tree canopy but was surrounded by flagged krummholz mats and low tree islands. The ALP site was ~400 m upslope of timberline (contiguous forest). These sites span both an elevation and a canopy-closure gradient that is representative of current and future limber pine recruitment environments at Niwot Ridge.

At each site, 20 2 m × 2 m plots were arranged over ~0.5 ha area, and each plot was subdivided into four 1 m × 1 m quadrants (two quadrants for limber pine and two quadrants for Engelmann spruce (not evaluated in this study)). Into two of the quadrants, recently germinated (~2 weeks old) limber pine seedlings were transplanted within 2 weeks of snowmelt in 2009 ( $N = 48$  seedlings/plot). Seedlings were from local low-elevation ('LO', 3000–3200 m) and high-elevation ('HI', 3270–3345 m) seed sources ( $n = 24$  seedlings/plot for each provenance), located within a few hundred meters to 1 km of our study plots.

Seed handling followed the protocol for limber pine in Krugman and Jenkinson (1974). Briefly, cones were harvested in September and October 2008, dried in well-ventilated areas at <28 °C, and then stored at –20 °C. Following stratification at 4 °C, seeds were sown into 2.5 × 12 cm Ray Leach 'Cone-tainer' cells lined with coffee filter paper and filled with a mixture of washed sand and autoclaved potting soil designed to mimic the texture and organic matter content of native soil at each of the three sites. Seeds were sown in three waves in anticipation of different snowmelt dates at each site. Prior to transplanting, seedlings were placed in sheltered areas to acclimatize them to outdoor temperature and radiation regimes. Hardware cloth enclosures were positioned over plots to prevent seedling predation. Each seedling received 20 ml of water directly following transplantation, and then twice a week for the following 2 weeks.

Most microclimate data were obtained courtesy of the Niwot Ridge AmeriFlux and Niwot Ridge Long-Term Ecological Research (Saddle station) climate stations located near the LSA and ALP study sites, respectively (<500 m away, and approximately the same elevation). Data for air measurements at 2 m height above ground and soil measurements 0–10 cm below ground were used from these climate stations. Photosynthetically active radiation (PAR, 400–700 nm) was measured at each site using a LI-190 sensor mounted at 2 m, and connected to a datalogger (model CR1000, Campbell Scientific Inc., Logan, UT, USA). Data were recorded every 60 s and averaged over 15 min periods. Photosynthetically active radiation sensors were not fully operational in summer 2009, so summer 2010 PAR data were used to characterize site sunlight regimes. Snow presence/absence surveys were conducted visually at the sites every day from mid-May until early July (end of meltout for all sites).

### Seedling survival, dry mass and morphology

Seedling presence/absence surveys began when seedlings were transplanted and continued approximately weekly until the end of the growing season. To quantify dry mass and morphology, a subset of the transplants ( $N = 51$  total;  $n = 5$ –12 per provenance per site) were excavated after measuring gas exchange using a small spade, taking care to harvest the entire seedling and its root system. Roots and shoots were then placed in plastic bags, kept on ice in a cooler, and transported to a lab where they were microwaved for ~30 s to stop metabolic activity. Projected leaf area, needle dry mass of whole crowns and root dry mass were determined on all harvested transplants. The projected leaf area for each seedling was determined by removing all needles of the whole crown and laying them flat, digitally photographing the needles and then quantifying the area using image processing software (Image J, Scion Co., Fredrick, MD, USA). Following area measurements, shoots (needles plus stems) and roots were dried for 48 h at 70 °C and then weighed for dry mass to  $\pm 0.1$  mg. Specific leaf area was calculated as projected leaf area divided by leaf mass. Leaf widths were small (<0.1 mm), and there was no variation in cross-sectional geometry of needles.

### Gas exchange, water relations, chlorophyll fluorescence and tissue carbohydrates

Instantaneous gas exchange (photosynthesis, transpiration, stomatal conductance of H<sub>2</sub>O) was measured using a portable photosynthesis system (model LI-6400, Li-Cor Biosciences, Lincoln, NE, USA) equipped with a CO<sub>2</sub> controller and a red–blue LED chamber (model LI-6400-02b). Gas exchange variables were measured on entire seedlings and are reported on a silhouette leaf area basis according to Smith et al. (1991). Silhouette leaf area was determined by digitally photographing the seedling and objects of known size (for calibration) from the angle of the LI-6400 light source, and then quantified using image processing software (Image J). Gas exchange was measured between 1000 and 1600 h local time in 3 days in late June and 4 days in late August. In June, it was not possible to take measurements at the USA site, because it was still under snow and no seedlings had been transplanted. The total number of gas exchange measurements during both campaigns was  $N = 122$  (LSA),  $N = 58$  (USA, August only) and  $N = 34$  (ALP).

During all gas exchange measurements, CO<sub>2</sub> gas concentration was set to 385 ppm, and relative humidity and temperature were matched to ambient conditions ( $22.2 \pm 5.0\%$  and  $22.7 \pm 4.0$  °C, respectively; mean  $\pm$  SE). Chamber temperature and humidity during gas exchange measurements were similar between provenances (see Table S1 available as Supplementary Data at *Tree Physiology* Online). Light-saturated photosynthesis ( $A_{\text{net}}$ ) was measured with the chamber light source set at an intensity of 1200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Dark respiration ( $R_{\text{d}}$ ) was then

measured by darkening the chamber (light intensity of  $0 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and waiting until gas exchange fluxes stabilized (total coefficient of variation of  $\text{CO}_2$  and  $\text{H}_2\text{O}$  fluxes in time <5%).  $R_d$  under sunlit conditions may be less than in darkness (Krömer 1995), and consequently our calculated  $A_{\text{net}}:R_d$  may be overestimated. However, we assumed that differences in  $R_d$  in the light compared with dark were similar among provenances at each site, and emphasized relative changes in  $A_{\text{net}}$  and  $R_d$  among populations. All measurements are reported as plot-level means.

Pre-dawn (0400–0600 h) seedling water potential ( $\Psi$ ) was measured on the seedlings harvested for dry-mass measurements using a Scholander-type pressure bomb (model PMS-1000, PMS Instruments, Corvallis, OR, USA). Measurements were made in early July ( $N = 18$ ) and in early August ( $N = 35$ ). For both gas exchange and water potential measurements, measurements were made on roughly equal numbers of seedlings from both provenances.

Needle chlorophyll fluorescence was measured in the field using a portable chlorophyll-*a* fluorometer (model MINI-PAM, Heinz Walz GmbH, Effeltrich, Germany) on 35 seedlings (18 HI and 17 LO over all sites). Needles were dark adapted for 25 min using leaf clips provided with the fluorometer. The ratio of variable to maximum fluorescence ( $F_v/F_m$ ) was determined for the dark-adapted needles by measuring the fluorescence emission from photosystem II (PSII) under a weak measurement light ( $F_o$ ) and following a pulse of saturated light ( $F_m$ ). From this,  $F_v$  was determined as  $F_m - F_o$  (Maxwell and Johnson 2000).

Non-structural carbohydrates are defined as starch plus soluble sugars (glucose, fructose and sucrose); our protocol followed Bansal and Germino (2009). Following measurements of dry mass, whole plants (root + shoot) were ground to a fine powder and weighed to  $\pm 0.1$  mg. To measure soluble sugars, 2 ml of water was added to each sample, which was heated to  $100^\circ\text{C}$  with steam for 30 min to extract soluble sugars into aqueous solution, and then a 200  $\mu\text{l}$  aliquot of each sample was enzymically treated with invertase, phosphoisomerase and glucose hexokinase to convert sucrose, fructose and glucose to 6-phosphogluconate (Sigma Diagnostics, St Louis, MO, USA). Oxidation of the soluble sugars to 6-phosphogluconate resulted in an equimolar reduction of NAD to NADH, increasing absorbance of the solution at 340 nm, which was measured using a spectrophotometer (Synergy Microplate Reader, Biotek Instruments, Winooski, VT, USA), and was directly proportional to soluble sugar concentrations. To obtain total NSC (starch plus soluble sugars), the original sample (powder plus water) was also treated with a high activity fungal  $\alpha$ -amylase from *Aspergillus oryzae* (Clarase G-Plus, Genecore International, Rochester, NY, USA) to metabolize starch to glucose and re-analyzed for total NSC with the procedure described previously. Starch was calculated as total NSC minus soluble sugars. All NSC data were normalized for dry mass of needle tissue.

Due to the small size of the seedlings ( $\sim 35$ – $63$  mg), it was only possible to determine NSC concentrations for whole plants. Sampling time-of-day does not significantly affect NSC concentrations (Bansal and Germino 2009).

### Statistical analysis

The experimental design was a split-plot. Initially, there were equal numbers of transplants from high- and low-elevation seed sources planted in each plot. However, seedling survival was unevenly distributed among plots, with many plots having no surviving seedlings of one provenance or another. The resulting layout could thus not be analyzed as a split-plot, and so was analyzed as a two-factor analysis of variance, with site and provenance as the main factors and plot as the main unit of replication (JMP v8.0.1, SAS Institute, Cary, NC, USA). There were no significant differences in physiological or morphological measurements between the sampling dates ( $P > 0.13$  and  $P > 0.69$ , respectively, for all comparisons), and plots were not re-measured (except for three instances). Therefore, the data from the 2 months were pooled for analysis. Before analysis, all data sets were analyzed for statistical assumptions of normality and homoscedasticity. Leaf area data were log transformed before analysis. Effects were considered significant at the  $P = 0.05$  level. The Tukey–Kramer multiple-comparisons method was used for post hoc evaluation of differences between individual means. Site-level differences in survival were analyzed using a  $\chi^2$  test.

## Results

### Microclimate

Snowpack persistence and ablation rate varied by site, with the understory subalpine forest (LSA) site melting out earliest and the alpine-treeline ecotone site (USA) melting out latest (Table 1). Incident solar radiation above canopies was similar at the weather stations near LSA and ALP. Mean summer (June–September) air temperature at the Saddle station (ALP site) was  $1.3^\circ\text{C}$  cooler than at the Ameriflux station (LSA site). Mean summer soil temperature at ALP was  $0.5^\circ\text{C}$  cooler than at the LSA site.

### Survival, dry mass and morphology

At all sites throughout the summer, survival was at least 20% greater in LO compared with HI transplants ( $\chi^2$ ,  $P < 0.0001$  for all sites). Seasonal survival trends were similar in the LSA and ALP sites, declining to  $\sim 40\%$  (HI) and  $60\%$  (LO) at the end of the season at both sites (Figure 1). End of season survival was slightly lower at the alpine-treeline ecotone site (USA), with survival declining to  $\sim 24\%$  (HI) and  $41\%$  (LO) at the end of season.

There was no statistical difference in dry mass, leaf area or shoot:root among sites ( $P > 0.38$  for all variables; Table 2;

Table 1. Summary of microclimate data for summer 2009 (June 1–September 30) for LSA (3080 m), USA (3425 m) and ALP (3545 m) sites. Microclimate data except for snowmelt dates are from Ameriflux and LTER climate stations located near the LSA and ALP study sites, respectively (<500 m away, and approximately the same elevation). Snowmelt dates indicate the plot-level range in Julian days of snowmelt at study sites. Solar radiation ( $R_{\text{solar}}$ , 300–3000 nm), cumulative PAR (400–700 nm), air ( $T_{\text{air}}$ ), minimum air ( $T_{\text{min}}$ ), maximum air ( $T_{\text{max}}$ ) and soil ( $T_{\text{soil}}$ ) temperatures, and air VPD are summer daily means.

	Snowmelt dates	$R_{\text{solar}}$ ( $\text{W m}^{-2}$ )	PAR ( $\text{mol m}^{-2} \text{d}^{-1}$ )	$T_{\text{air}}$ ( $^{\circ}\text{C}$ )	$T_{\text{min}}$ ( $^{\circ}\text{C}$ )	$T_{\text{max}}$ ( $^{\circ}\text{C}$ )	$T_{\text{soil}}$ ( $^{\circ}\text{C}$ )	$T_{\text{night}} < 0$ ( $^{\circ}\text{C}$ ) <sup>1</sup>	VPD (kPa)
LSA	147–154	429 (5)	30 (1)	9.2 (0.1)	2.6 (0.4)	16.4 (0.8)	7.2 (0.1)	16	0.80 (0.01)
USA	170–180		52 (1)						
ALP	140–171	441 (8)	51 (1)	7.9 (0.3)	3.5 (0.3)	13.4 (0.4)	6.7 (0.2)	16	0.43 (0.01)

<sup>1</sup> $T_{\text{night}} < 0$   $^{\circ}\text{C}$  is the number of nights during the summer when air temperatures dropped below 0  $^{\circ}\text{C}$ . PAR data are from 2010, as sensors were not operational during summer 2009. Numbers in parentheses are SE.

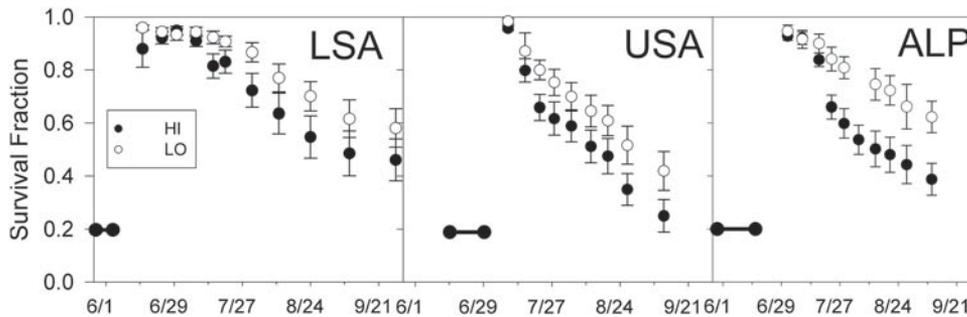


Figure 1. Fraction of transplanted seedlings present by survey date for *P. flexilis* from high-elevation (HI) and low-elevation (LO) seed sources ( $\chi^2$ ,  $P < 0.0001$  for all sites). Data are means ( $\pm$ SE) of plot data for LSA, USA and ALP sites ( $N = 14$ , 12 and 12 plots, respectively). The horizontal black bars indicate the dates of snowmelt across each site.

Table 2. Summary of ANOVA results for the effects of site (LSA, USA and ALP), provenance (LO and HI), and site  $\times$  provenance interactions for response variables dry mass, projected leaf area (PLA), SLA, shoot:root ratio (S:R), net photosynthesis ( $A_{\text{net}}$ ), dark respiration ( $R_{\text{d}}$ ), carbon flux balance ( $A_{\text{net}}:R_{\text{d}}$ ), total NSC, pre-dawn water potential ( $\Psi$ ), stomatal conductance ( $g$ ), internal to external  $\text{CO}_2$  concentration ( $C_i:C_a$ ) and water use efficiency (WUE).

Effects	Growth			PLA			SLA			S:R		
	$df$	$F$	$P$	$df$	$F$	$P$	$df$	$F$	$P$	$df$	$F$	$P$
Site	2.47	0.92	0.91	2.31	0.10	0.90	2.31	0.65	0.53	2.47	0.88	0.42
Provenance	1.48	13.63	<b>0.0006</b>	1.32	1.34	0.25	1.32	5.09	<b>0.03</b>	1.48	3.3	0.08
S $\times$ P	2.47	3.23	<b>0.05</b>	2.31	1.52	0.23	2.31	0.30	0.74	2.47	0.21	0.80
Effects	Carbon balance			$R_{\text{d}}$			$A_{\text{net}}:R_{\text{d}}$			NSC		
	$df$	$F$	$P$	$df$	$F$	$P$	$df$	$F$	$P$	$df$	$F$	$P$
Site	2.109	44.06	<b>0.0001</b>	2.109	19.26	<b>&lt;0.0001</b>	2.109	8.21	<b>0.0005</b>	2.51	3.92	<b>0.03</b>
Provenance	1.110	4.86	<b>0.03</b>	1.110	0.96	0.33	1.110	1.74	0.19	2.52	4.19	<b>0.03</b>
S $\times$ P	2.109	1.85	0.12	2.109	0.71	0.49	2.109	1.04	0.36	2.51	0.13	0.87
Effects	Water relations			$g$			$C_i:C_a$			WUE		
	$df$	$F$	$P$	$df$	$F$	$P$	$df$	$F$	$P$	$df$	$F$	$P$
Site	2.44	8.65	<b>&lt;0.0001</b>	2.109	59.65	<b>&lt;0.0001</b>	2.109	10.25	<b>&lt;0.0001</b>	2.109	31.46	<b>&lt;0.0001</b>
Provenance	1.45	3.82	<b>0.05</b>	1.110	3.63	0.06	1.110	0.15	0.70	1.110	0.67	0.41
S $\times$ P	2.44	1.16	0.32	2.109	2.72	0.07	2.109	0.27	0.72	2.109	0.29	0.76

Numbers in  $df$  columns indicate treatment and error degrees of freedom. Numbers in bold are significant at the  $P < 0.05$  level.

Figure 2). Whole-plant dry mass of LO transplants was >20% greater than HI transplants at LSA and USA sites, but there was no difference in plant dry mass at ALP (provenance  $\times$  site  $P = 0.05$ ; Table 2; Figure 2a.). There was no difference in projected leaf area between provenances (Figure 2b). Compared with HI seedlings, LO seedlings had 12–42% greater shoot:root mass ratios (mostly due to increases in shoot dry mass relative to root dry mass) at all sites, and up to 37% lower SLA at the

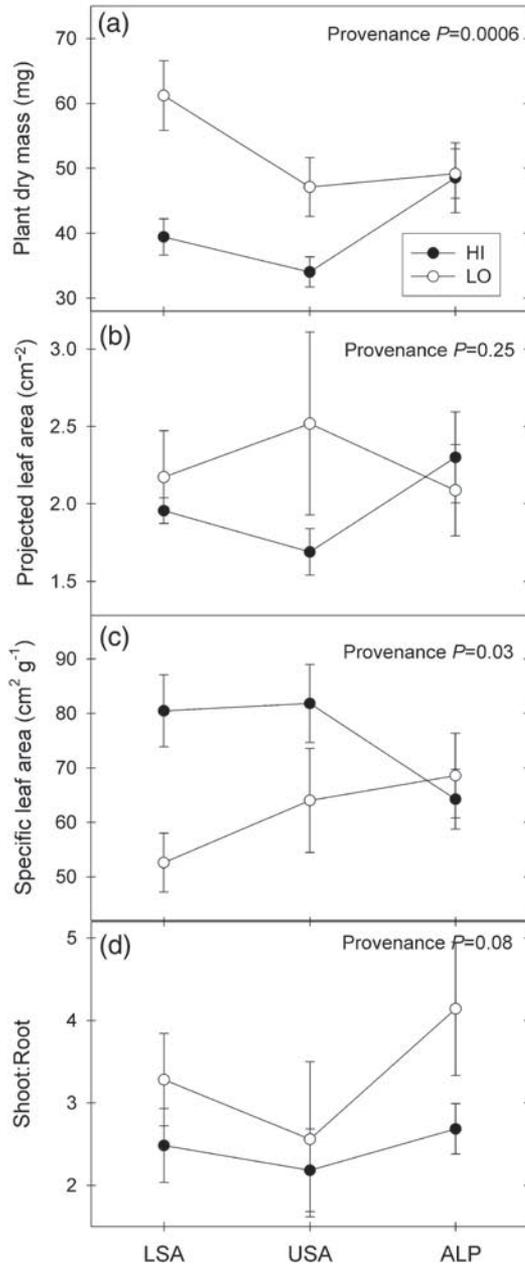


Figure 2. Comparison of (a) whole-plant dry mass, (b) projected leaf area, (c) specific leaf area and (d) shoot:root ratios of *P. flexilis* seedling transplants from HI and LO seed sources across the three study sites. There were no significant differences among sites ( $P > 0.38$  for all variables); between-provenance test  $P$  values are indicated on each panel. There was a site  $\times$  provenance interaction for dry mass ( $P = 0.05$ , panel a). Data are seasonal means  $\pm$  SE.

LSA and USA, but not ALP, sites ( $P = 0.03$ ) (Table 2, Figure 2c and d).

### Photosynthetic gas exchange, carbohydrates, water relations and chlorophyll fluorescence

Photosynthesis under saturating light ( $A_{\text{net}}$ ) increased more than threefold with elevation for both LO and HI seedlings (Table 2; Figure 3a), and was positively associated with air

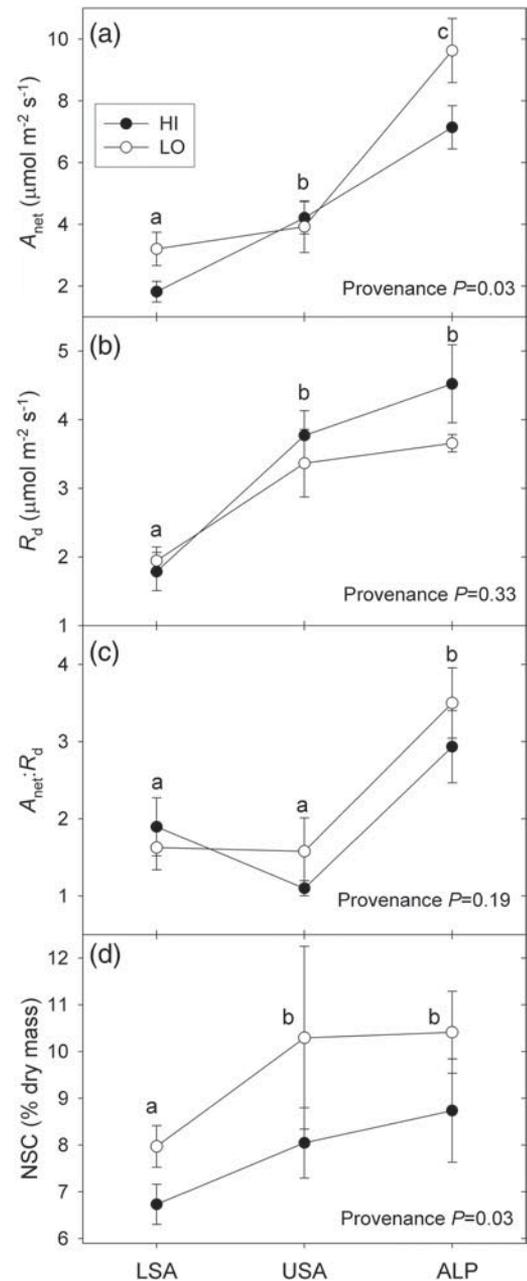


Figure 3. Comparison of (a) net photosynthesis ( $A_{\text{net}}$ ), (b) dark respiration ( $R_d$ ), (c) carbon flux balance ( $A_{\text{net}}:R_d$ ) and (d) NSC in *P. flexilis* seedling transplants from HI and LO seed sources across the three study sites. Different letters show significant differences ( $P < 0.05$ ) between sites. Between-provenance test  $P$  values are noted on each panel. Data are seasonal means  $\pm$  SE.

humidity (greater  $A_{\text{net}}$  with decreasing leaf-to-air vapor pressure difference; see Figure S1 available as Supplementary Data at *Tree Physiology* Online).  $A_{\text{net}}$  in LO transplants was greater than in HI transplants by 77% at the LSA site and by 35% at the ALP site. Dark respiration ( $R_d$ ) doubled along the elevation gradient, with no difference between HI and LO transplants (Table 2; Figure 3b).  $A_{\text{net}}:R_d$  at the LSA and USA sites was similar, but increased by ~75% at the ALP site (Table 2; Figure 3c).

Total NSC of whole plants increased linearly with elevation for both LO and HI transplants (Figure 3d), and were >25% greater in LO compared with HI seedlings (Table 2).  $A_{\text{net}}:R_d$  did not explain variation in whole-plant dry mass for either provenance ( $P = 0.43$ ,  $r^2 = 0.02$ ; Figure 4a), but increases in dry mass were associated with increases in both  $A_{\text{net}}$  ( $P = 0.004$ ,  $r^2 = 0.12$ ; Figure 4b) and NSC ( $P = 0.003$ ,  $r^2 = 0.20$ ; Figure 4c).

Stomatal conductance ( $g$ ) increased fourfold with increasing elevation for both provenances, but was significantly greater in LO transplants at LSA and ALP (Table 2; Figure 5a). This trend was accompanied by increases in mesophyll  $\text{CO}_2$  concentrations with elevation (Figure 5b). August pre-dawn water potentials ( $\Psi$ ) were similar at LSA and ALP (between  $-0.4$  and  $-0.8 \pm 0.1$  MPa), but were significantly higher at the USA site (about  $-0.3 \pm 0.1$  MPa; Table 1; Figure 5c). Mean  $\Psi$  across all sites was more negative in HI than in LO transplants, but only by a small margin ( $-0.6 \pm 0.1$  compared with  $-0.5 \pm 0.1$  MPa, respectively; Table 2). Water use efficiency (net photosynthesis/transpiration) was not different between provenances, but was significantly different among sites, with greatest values at the ALP site compared with the lower-elevation sites (Table 2; Figure 5d).

Maximal efficiency of PSII ( $F_v/F_m$ ) was similar ( $\sim 0.79$ ) in both provenances ( $F_{1,34} = 1.30$ ,  $P = 0.26$ ), but was significantly different among sites ( $F_{1,34} = 5.12$ ,  $P = 0.01$ ). Low-elevation seedlings had significantly lower  $F_v/F_m$  ( $\sim 0.72$ ) at the USA site (site  $\times$  provenance interaction,  $P = 0.03$ ; Figure 6).

## Discussion

### Effect of elevation and canopy on survival, dry mass and plant morphology

We observed no significant differences in survivorship, seasonal dry-mass gain or needle morphology among sites, even

though they spanned a gradient of >500 m from subalpine forest understory to treeline ecotone to alpine meadow (Figures 1 and 2). The constancy of seedling dry mass and needle morphology across the gradient in our study may have been due to the similarity in air and soil temperatures across sites during the period of our study. Relatively small differences in air and soil temperatures between the highest and lowest elevations were observed during our study. Seasonal mean, maximum and air temperatures differed by  $<1.3$  °C between our highest and lowest sites (Table 1). These differences in temperatures were much less than predicted by previously determined lapse rates for Niwot Ridge ( $\sim 0.6$  °C/100 m during the growing season, translating to an expected differential of  $\sim 2.8$  °C between ALP and LSA sites; Greenland 1989, Pepin and Losleben 2002). Thus, there may not have been enough of a difference in thermal forcing to cause significant changes in morphology in our study. This is supported by the lack of variation of  $F_v/F_m$  along the gradient, which is often low in the alpine due to low-temperature photoinhibitory stress (e.g., Ball et al. 1991, Germino and Smith 1999, Hughes et al. 2009).

The lack of within-provenance morphological variation with altitude we report here may also be related to low phenotypic plasticity in *P. flexilis*. For example, Schoettle and Rochelle (2000) reported very little morphological variation in adult limber pine stands across 12 sites that spanned 1630–3330 m in elevation and  $>10$  °C in mean air temperature. These findings suggest that limber pine may differ from other species of pine in which substantial morphological variation does occur with changes in elevation (e.g., Poulos and Berlyn 2007, Gebauer et al. 2010). Widely distributed, bird-dispersed tree species such as limber pine may rely on broad physiological tolerances rather than phenotypic plasticity in morphological traits for success in the wide range of environments in which they become established (Mooney et al. 1964, Lepper 1980, Letts et al. 2009). Another consideration is that first-year conifer seedlings are mostly cotyledonous, and have little primary needle tissue. Cotyledon needle structure is generally considered to be constrained, with low variation within species ('canalized'), but little research has directly addressed this issue (see, for example, Conner and Agrawal 2005, Tucić et al. 2005, Breznenová et al. 2010).

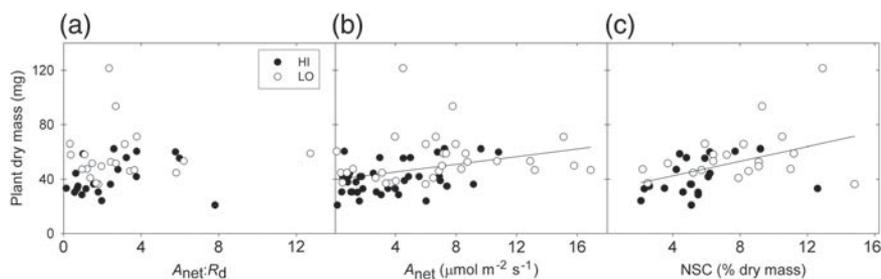


Figure 4. Comparison of plant dry mass with (a)  $A_{\text{net}}:R_d$ , (b)  $A_{\text{net}}$  and (c) NSC in seedlings of *P. flexilis* from high-elevation (HI) and low-elevation (LO) seed sources. Regression lines are linear (panel a:  $r^2 = 0.02$ ,  $P = 0.43$ ; panel b:  $r^2 = 0.12$ ,  $P = 0.004$ ; panel c:  $r^2 = 0.13$ ,  $P = 0.003$ ).

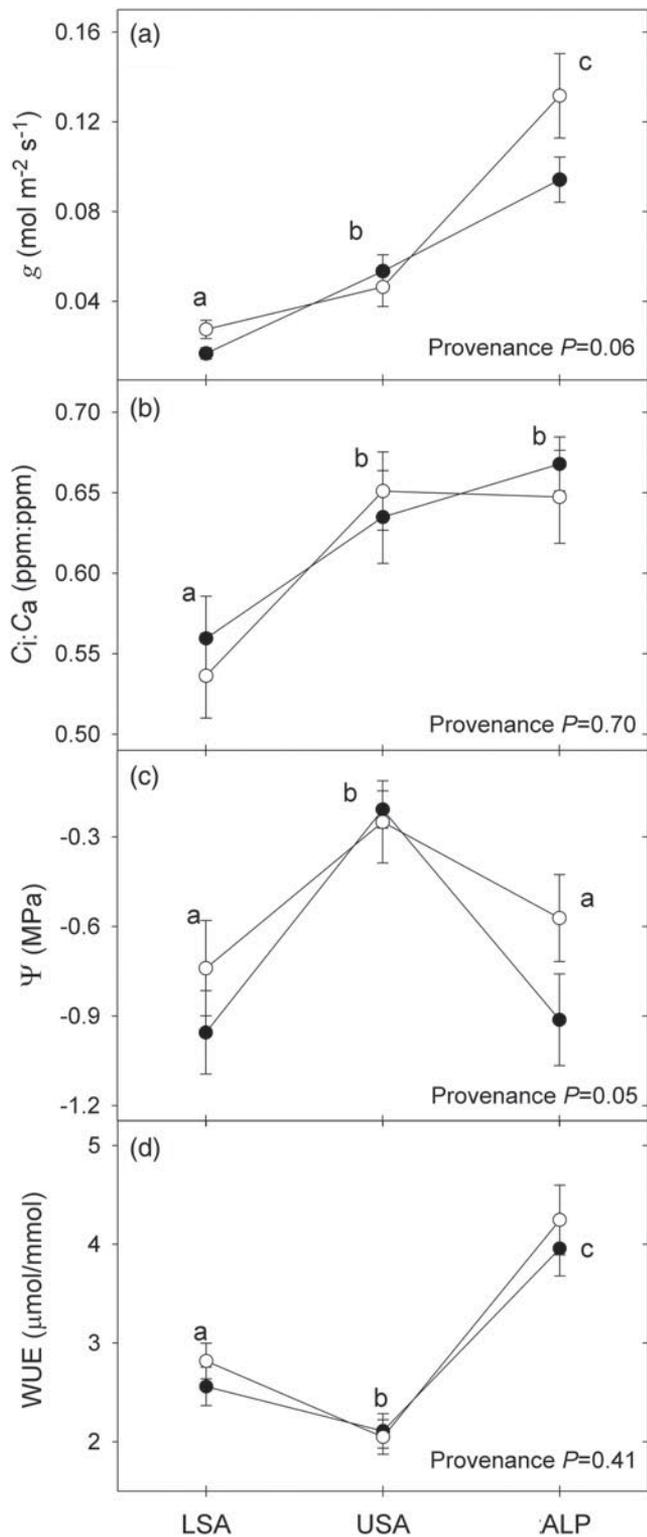


Figure 5. Comparison of (a) stomatal conductance ( $g$ ), (b) ratio of internal to ambient external  $\text{CO}_2$  concentration ( $C_i:C_a$ ), (c) water potential ( $\Psi$ ) and (d) water use efficiency (WUE, photosynthesis/transpiration) in *P. flexilis* seedling transplants from high-elevation (HI) and low-elevation (LO) seed sources. Different letters show significant differences ( $P < 0.05$ ) between sites. No significant differences between provenances across all sites existed for these variables. Between-provenance test  $P$  values are noted on each panel. Data are seasonal means  $\pm$  SE.

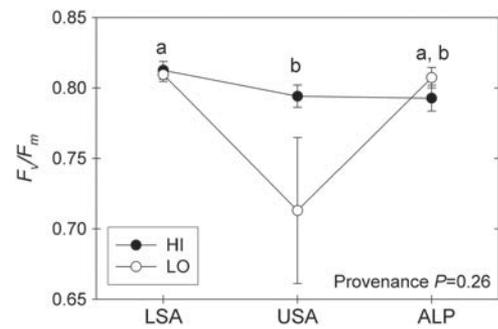


Figure 6. Pre-dawn maximal photochemical efficiency of PSII ( $F_v/F_m$ ) in *P. flexilis* seedling transplants from high-elevation (HI) and low-elevation (LO) seed sources. There was a significant site  $\times$  provenance interaction ( $P = 0.01$ ). Different letters show significant differences ( $P < 0.05$ ) between sites. Data are seasonal means  $\pm$  SE.

### Effect of elevation and canopy on physiology

Photosynthetic carbon relations ( $A_{\text{net}}$ ,  $R_d$ ,  $C_i:C_a$ ) and conductance ( $g$ ) increased with elevation (Figure 3), and increases were associated with decreases in vapor pressure deficit (see Figure S1 available as Supplementary Data at *Tree Physiology* Online), as has been shown for adult limber pine (Fischer et al. 2002, Letts et al. 2009). Additionally, limber pine is considered shade intolerant (Rebertus et al. 1991), and greater sunlight at the upper sites compared with the understory LSA site may also have allowed for greater photosynthetic capacity and carbon balance in seedlings at the USA and ALP sites (Figure 3a and c). Photosynthesis in the alpine zone was similar to that reported for first-year seedlings of whitebark pine (*Pinus albicaulis*, a closely related species) growing at the alpine treeline in Wyoming (Maher et al. 2005).

Greater  $A_{\text{net}}$  and  $A_{\text{net}}:R_d$  at the ALP compared with other sites may also have been due to enhanced water relations. Mean midday VPD at the ALP site was about half that at the LSA site (Table 1), though there did not appear to be any difference in soil moisture between these two sites as indicated by plant water potentials (Figure 5c). This may have contributed to less stomatal regulation of photosynthesis at the upper sites compared with the lower LSA site, as suggested by greater  $g$  and  $C_i:C_a$  values (Figure 5a and b).  $A_{\text{net}}:R_d$  and water use efficiency (WUE) were greater at the ALP site compared with both LSA and USA sites, even though both ALP and USA were open canopy sites (Figures 3c and 5d). Lower  $A_{\text{net}}:R_d$  and WUE at USA compared with ALP may be related to the later snowmelt date at USA, which may have suppressed photosynthesis through midsummer due to cool soil temperatures (Day et al. 1989). Also, the late transplant date of seedlings at USA may have exposed them to warm, dry midsummer (July) atmospheric conditions that were conducive to transplant shock, compared with earlier transplantings at LSA and ALP during cooler, cloudier May and June. This could have hindered physiological performance at USA compared with the ALP site in August despite

similar canopy cover and higher water potentials also resulting from later snowmelt (Figure 5c).

### Carbon relations and growth limitations in young conifer seedlings

Growth limitation in conifer tree species at high elevations has been ascribed to imbalance of carbon gain, processing and growth (Stevens and Fox 1991, Körner 1998, Smith et al. 2003). We found that factors limiting carbon acquisition (i.e.,  $A_{\text{net}}$ ), and not processing (i.e.,  $A_{\text{net}}:R_{\text{d}}$ ,  $R_{\text{d}}$ ), best matched growth patterns. Seedling dry-mass gain was positively associated with  $A_{\text{net}}$  and tissue NSC, and not  $A_{\text{net}}:R_{\text{d}}$  or  $R_{\text{d}}$  (Figure 4; data not shown for  $R_{\text{d}}$ ). This has been reported by other researchers investigating conifer seedlings near the alpine treeline (e.g., Cui and Smith 1991, Germino and Smith 1999, 2001, Johnson et al. 2004), but is contrary to some findings for adult trees near the treeline in which carbon processing is considered to be more limiting than carbon acquisition (e.g., Hoch and Körner 2003, Körner and Paulsen 2004, Shi et al. 2006). Under scenarios that limit carbon use, though, low-temperature constraints to carbon use are postulated to result in greater NSC, and ultimately negative relationships between growth and NSC—patterns that were clearly the opposite of our findings. While we did observe increases in NSC with elevation, this was likely due to greater  $A_{\text{net}}$  with elevation (Figure 3), and not to decreased carbon processing resulting from increasing environmental stress (see physiology subsection above). Greater NSC consequently resulted in greater plant dry-mass gain, which has been reported previously in conifer tree species in temperate studies (Oleksyn et al. 1998, 2000). Our results also highlight that leaf-level, and not just mass-based  $A_{\text{net}}$  may be related to plant growth for small seedlings where total leaf area is small (see also Bansal et al. 2010).

### Provenance-level variation and implications for changes in limber pine range limits

It has been suggested that morphological and physiological variation in limber pine is more affected by environmental factors, as opposed to genetic factors, as might be expected in a bird-dispersed tree species (Rehfeldt 1983, Rebertus et al. 1991). Populations of adult limber pine trees display little variation in morphology (Schoettle and Rochelle 2000), and instead seem to rely on physiological variation in order to persist in a broad range of environments (Mooney et al. 1964, Letts et al. 2009). However, we observed significant differences in both morphology and physiology between newly germinated seedlings of different provenance in our study, even though seed sources were located relatively close to each other (within ~200 m elevation). This may highlight ontogenetic differences in the degree of inter-provenance ecophysiological variation between juvenile seedling and adult life stages.

Low-elevation seedlings had greater carbon gain, produced more NSC, accumulated more dry mass and had greater

survival compared with HI seedlings across the entire elevation gradient, with few exceptions (Figures 1–3). The better physiological performance and abundance of LO seedlings can be attributed in part to the greater shoot:root mass ratio and leaf area of this provenance relative to HI seedlings (Figure 2b and c), which allowed for greater photosynthetic carbon gain and NSC production, and consequently greater dry mass. This could reflect an adaptive growth-oriented strategy of this provenance (see Grime 1974), which, in our study, outperformed a stress-oriented provenance (HI). On the other hand, maternal effects may also have influenced seedling physiology and survival. For example, although LO seeds were ~10% smaller than HI seeds (data not shown), they germinated more successfully and quickly in the greenhouse. This may have contributed to faster growth rates or greater growing-season length that resulted in the greater dry mass in LO compared with HI seedlings that we observed in this study.

The greater performance of LO compared with HI seedlings across all sites may have important implications for changes in limber pine range limits due to climate change. Reductions in harsher microclimatic conditions at the highest elevations (e.g., greater sunlight, cooler temperatures, greater frost frequency) due to increased greenhouse gases or cloud cover could allow for 'leapfrogging' of growth-adapted provenances beyond stress-adapted provenances to higher elevations. Bird dispersal of seed could facilitate this pattern. Greater advances of growth-adapted provenances into previously unoccupied areas such as subalpine meadows and the alpine tundra would enhance the predicted upslope migration of treeline due to climate change, either directly or through facilitation of shade-adapted species such as subalpine fir and Engelmann spruce.

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## Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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