

# Carbon balance of conifer seedlings at timberline: relative changes in uptake, storage, and utilization

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**Abstract** Low rates of growth for conifers at high elevations may relate to problems in acquiring or utilizing carbon. A traditional hypothesis for growth limits of trees at alpine treeline is that low photosynthesis ( $A$ ) leads to insufficient supply of carbon for growth. Alternatively, the sink-limitation hypothesis questions the importance of low  $A$ , and suggests that trees at treeline have abundant carbon for growth as a result of greater decreases in carbon use [respiration ( $R$ ) and growth] than  $A$  at higher elevations. Concentrations of carbon intermediates (e.g., nonstructural carbohydrates,  $NSC$ ) have been used to support the sink-limitation hypothesis, with the assumption that  $NSC$  reflects the ratio of carbon source to sinks. Our objective was to determine elevation effects on carbon balance (whole-plant uptake, storage, and efflux of carbon) of tree seedlings during their critical establishment phase at timberline. Changes in  $A$  and  $R$  (of whole crowns),  $NSC$  (starches and simple sugars), and growth were measured in seedlings of a tree-line (*Abies lasiocarpa*) and nontreeline species (*Pseudotsuga menziesii*). Seedlings were outplanted at the lower (2,450 m) and upper (3,000 m) edges of the timberline zone in the Rocky Mountains, USA. At the upper compared with lower elevation, both species had 10–20% less root and

needle growth, 13–15% less  $A$ , 35–39% less  $R$ , and up to 50% greater  $NSC$ . *A. lasiocarpa* allocated more biomass to needles and had greater  $A$  but less  $NSC$  than *P. menziesii*. The greater effects of elevation on  $R$  compared with  $A$  led to a 1.3-fold increase in  $A:R$  at the upper elevation, and a corresponding increase in starch ( $r^2 = 0.42$ )—a pattern consistent with the predictions of the sink-limitation hypothesis. Nevertheless,  $A$  and dry mass gain were positively correlated ( $r^2 = 0.42$ ), indicating that variation in photosynthesis was related to growth of seedlings at timberline.

**Keywords** Photosynthesis · Respiration · Nonstructural carbohydrates · Growth · Timberline

## Introduction

A variety of photosynthetic adaptations are observed in plants inhabiting climates that are marginal for growth (Ackerly et al. 2000). Such adaptations indicate selection for carbon gain under abiotic stress, such as in desert and alpine environments (Ehleringer and Monson 1993; Smith et al. 2004). Nonetheless, direct evidence that inadequate carbon supply could hinder growth of establishing plants at the edge of their climate range is scarce, detracting from our broader understanding of the ecological importance of photosynthesis ( $A$ ). Specifically, few studies have explicitly compared photosynthetic carbon gain to growth (e.g., Poorter and Remkes 1990; Reich et al. 1998), especially for wildland plants in situ, and at the whole-plant level.

The ecotone at alpine treeline, which separates forest and alpine communities, is a distinctly climate-driven vegetation boundary. A traditional hypothesis for treeline formation is that net photosynthesis ( $A_{\text{net}}$ ) decreases with elevation, leading to a decrease in  $A:R$  below unity for trees in the alpine

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zone, precluding tree growth and establishment ('carbon-balance hypothesis'; Stevens and Fox 1991). Although several studies conclude that assimilation of carbon is reduced near treeline, the evidence for reduced  $A_{\text{net}}$  at treeline is equivocal. For example, the negative response of  $A_{\text{net}}$  to cool temperatures and high light at alpine treeline is well established (low temperature photoinhibition, LTP; Ball et al. 1991; Germino and Smith 1999). Yet the capacity for photosynthesis of trees (Piper et al. 2006) and alpine herbs (Körner and Diemer 1987) were similar at higher compared to lower elevations. Elevation trends in  $A_{\text{net}}$  can also be affected by carbon loss through respiration, which can be 20–70% of the carbon gained through photosynthesis (Tjoelker et al. 1999), and may be more sensitive to temperature than photosynthesis (Berry and Björkman 1980; Atkin et al. 2005).

Increased nonstructural carbohydrate concentrations (*NSC*) in trees near alpine treeline have been used as an indicator of reduced carbon use relative to carbon assimilation, which is an alternative explanation for limitations to tree growth at high elevations (e.g., 'sink-limitation hypothesis'; Körner 1998, 2003; Hoch et al. 2002; Hoch and Körner 2003, 2005; Piper et al. 2006; Shi et al. 2006, 2008). Whether *NSC* levels are passively controlled through simple source-to-sink flow, or are actively regulated for coping with high elevation climate (Ögren et al. 1997; reviewed by Janusz et al. 2001; Tolsma et al. 2007), is another consideration in interpreting *NSC*. Patterns of expression of genes that directly affect or respond to *NSC* indicate active regulation of *NSC* pools (reviews: Koch 1996; Rolland et al. 2002; Smith and Stitt 2007).

Tests of growth limitations in conifers at high elevations have been conducted on adult trees (Li et al. 2002; Hättenschwiler et al. 2002; Handa et al. 2005, 2006), although few studies have examined seedlings at and above their natural range. Under these conditions, rates of seedling establishment and growth are severely impaired and contribute to the scarcity of trees above treeline (Cuevas 2000; Germino et al. 2002; Smith et al. 2003; Maher et al. 2005; Maher and Germino 2006). Spatial patterns of establishment for tree seedlings (i.e., selection for microsites with overhead canopy) have matched photosynthetic sensitivities of seedlings to bright sunlight and low temperatures (LTP) across a suite of treelines in the Rocky Mountains, USA, and elsewhere (Ball et al. 1991; Germino and Smith 1999; Maher and Germino 2006; Bader et al. 2007). Newly germinated seedlings need to increase their carbon and dry mass many-fold to successfully establish and progress to maturity, and so could differ from adult trees in their sensitivity to carbon or growth limitations. In addition to their ecological relevance to treelines, tree seedlings are suitable for assessments of carbon balance because their small size allows whole-plant assessments, and they can be readily planted beyond their normal distribution to allow assessment of physiological tolerances.

Our goal was to determine how *A*, *R*, and *NSC* in conifer seedlings would respond to elevation within the timberline zone, and how those changes would relate to seedling growth. We studied seedlings of two conifer species (*Abies lasiocarpa* and *Pseudotsuga menziesii*) at the warm and cool edges of the timberline zone (2,450 and 3,000 m, respectively). Both species naturally occur at 2,450 m, but 3,000 m is near the elevational limit for *A. lasiocarpa* and is 300 m above the normal upper distribution of *P. menziesii*. We predicted that *A*, *R*, and growth would decline at the upper elevation, and that the negative effects of elevation would be greater for *P. menziesii*, the nontreeline species.

## Materials and methods

We conducted diurnal measurements of gas exchange on whole-crowns of seedlings, and then harvested the seedlings for *NSC* and growth analyses. Carbon balance and relationships among components of carbon balance were then compared between elevations and species. We experimentally held canopy and soil conditions similar between sites to emphasize microclimate variation that is most directly a function of elevation (e.g., temperature and atmospheric pressure).

### Study site and species

The study area was located on the west slope of Fred's Mountain in the Teton Range in the Rocky Mountains, USA (43°47.26'N, 110°57.52'W). Our study spanned an elevation gradient from the warm to cool edges of the timberline zone (2,450 and 3,000 m, respectively). Our lower site was in a meadow just above continuous and closed-canopy forest. Above this elevation, tree canopies became progressively sparser and primarily occurred in islands of upright trees to about 3,000 m, where our upper site was located. Mean annual snowfall at 2,750 m is about 1,300 cm, resulting in a snow depth of 400 cm by spring. The 3,000 m site typically has about 3–4 months without permanent snow cover, compared with 6–7 snow-free months at the 2,450 m site. Both *P. menziesii* and *A. lasiocarpa* are co-dominants of the forests in the Rocky Mountains. *A. lasiocarpa* occurs in the Tetons at elevations from the valley floor (1,800 m) up to alpine treeline (3,100 m), whereas *P. menziesii* typically does not occur above 2,700 m (300 m below our upper site).

### Seed source, growth medium, and propagation

Seeds of *A. lasiocarpa* were collected at approximately 3,000 m from San Isabel National Forest, about 3° latitude south in the Rocky Mountains of Colorado, USA (Dean

Swift Seed, Jaroso, CO, USA). Seeds of *P. menziesii* were obtained from U.S. Forest Service collections made at 2,125 and 2,425 m in the same genetic, geographic, and elevation seed zone as the study area. Seed sources of both species were from approximately 500 m below treeline elevation at their respective origins. Seed masses were similar between the two species (70–80 seeds/g).

In 2005, we conducted a pilot study with only needle tissues of *P. menziesii*. In 2006, we conducted a more sampling-intensive field season with whole-seedlings of *P. menziesii* and *A. lasiocarpa*, and with different potting conditions. For simplicity, only the 2006 data are treated in the text and statistics. The 2005 data are presented in the graphs to illustrate reproducibility of seasonal and elevational trends in gas exchange and NSCs of *P. menziesii*.

Seeds were germinated in plastic “conetainers” (Ray Leach Cells™, 3.8 cm diameter, 21 cm depth; Eugene, OR, USA). Soils were a commercial conifer potting mix (Promix, Wilbur Ellis, San Francisco, CA, USA) that were mycorrhizae-free, sterilized, and had peat moss, bark, dolomite limestone, and other small quantities of organic and mineral conditioners. Sterile soil was chosen because newly germinating seedlings of a variety of conifer species, including *A. lasiocarpa*, rarely become infected with mycorrhizae or other root symbionts during their first few months of growth (Hasselquist et al. 2005; Germino et al. 2006).

Seeds were germinated in greenhouse conditions at the University of Idaho Forest Research Nursery, Moscow, ID, USA (46°43.57'N, 116°59.01'W), at 800 m elevation. We transported the newly emerging seedlings to the study site within 1 week of germination for immediate outplanting. The date of outplanting occurred 2 weeks after snowmelt at 3,000 m (fourth week of June 2006). The seedlings were outplanted at ground level and remained rooted in the containers to facilitate recovery of entire root systems. Seedlings were watered to promote initial establishment following outplanting. Volumetric water content of soils was measured with handheld sensors in seedling root zones and in surrounding natural soils to allow supplemental watering adjustments to match the seasonal pattern of soil drying (12-cm probe, frequency-domain reflectometer; Hydrosense, Logan, UT, USA).

#### Plot layout, microclimate, and sampling

We established 2 sites, located at 2,450 m and 3,000 m elevation. Each site had 10 plots with 4 seedlings of each species, making a total of 80 seedlings per species. Plots were 1 × 2 m, and positioned at random distances along five 30-m transects (two plots/transect) within a 0.5-ha area having relatively flat topography and representative herb cover and soils. Plots were not chosen if they happened to occur in

microsites with excessively high (>80%) or low (<20%) canopy openness, based on where natural seedling germination would be expected (Gray and Spies 1996; Maher and Germino 2006). The extent of canopy openness for each plot, defined as the percent of the upper hemisphere comprised of open sky, was quantified using hemispherical photography and Gap Light Analyzer software (Canham 1988; Maher et al. 2005). Mean (±1 SE) canopy openness among all plots was not significantly different between the 2,450 and 3,000 m elevation sites (64.5 ± 6.1 and 61.7 ± 10.9% openness, respectively).

We placed three HOBO temperature data loggers (Model Pro 8; Onset, Bourne, MA, USA) at each site. The thermistor probes were positioned at 1 m above ground level in radiation shields, and the consoles with internal sensors were buried at 5 cm below the soil surface. Instantaneous temperature readings were recorded every 30 min. These data were used to calculate daily mean, maximum, and minimum temperatures of air and soil. Growing degree-days (°C) were determined from the average of maximum and minimum daily soil temperatures, minus 5°C.

Sampling events started 10 days after outplanting, and were repeated about every third week until winter snowpack began accumulating, for a total of 4 sampling events. All sampling events occurred on relatively clear-sky conditions. During each sampling event, we randomly selected 6 of the 10 plots at each elevation for seedling measurements, and then 1 seedling per species per plot was randomly selected for a diurnal course of gas exchange measurements. After gas exchange measurements, we harvested whole seedlings for NSC and growth analyses.

#### Response variables

##### *Photosynthesis, respiration, and growth*

We measured gas exchange of entire crowns of seedlings in natural sunlight, and at ambient levels of CO<sub>2</sub>, relative humidity, and temperature using a portable photosynthesis instrument equipped with a conifer chamber and CO<sub>2</sub> controller (LI-6400; Li-Cor Biosciences, Lincoln, NE, USA). Following measurements of instantaneous net photosynthesis ( $A_{\text{net}}$ ,  $\mu\text{mol CO}_2 \text{ s}^{-1}$ ) in sunlight, the chamber was shaded until gas exchange rates stabilized (usually 2–4 min) and dark respiration ( $R$ ) was measured (photosynthetically active radiation = 0). Gas exchange of each individual was measured 4–6 different times over each diurnal sampling event, with at least one of the measurements of  $R$  at night. Three to five days were required per sampling event to attain sufficient replication for each seedling. Daily-integrations of CO<sub>2</sub> uptake and efflux ( $A_{\text{wp}}$  and  $R_{\text{crown}}$ , respectively,  $\mu\text{mol CO}_2 \text{ day}^{-1}$ ) for individual seedlings were determined from the means of consecutive readings of

instantaneous CO<sub>2</sub> exchange rates, multiplied by the time between the readings, and then summed over all readings throughout the day. Total moles of net CO<sub>2</sub> uptake between sampling events (i.e., net carbon gain per individual per sampling interval) were determined from the means of consecutive  $A_{wp}$  ( $\mu\text{mol CO}_2 \text{ day}^{-1}$ ), multiplied by the number of days between the consecutive sampling events ( $\mu\text{mol CO}_2 \text{ individual}^{-1} \text{ sampling interval}^{-1}$ ). Gas exchange per individual was assumed to be minimal ( $A_{wp} = 0$ ) immediately upon germination, due to the small size of the plants. With this assumption plus measurements on four subsequent dates, a total of four intervals were generated. A total of 508 gas exchange measurements were conducted on 90 seedlings throughout the field season. Survivorship could not be assessed because most of the outplanted seedlings were destructively sampled.

Gross photosynthesis ( $A$ ) was calculated as the sum of net CO<sub>2</sub> uptake and efflux ( $A_{\text{net}} + R$  in the dark).  $R$  under sunlit conditions may be less than in darkness (reviewed by Krömer 1995), and consequently our calculation of gross  $A$  may be overestimated. We assumed that differences in  $R$  in the light compared to dark were similar among our populations (which had similar light conditions), and emphasized relative changes in gross  $A$  and  $R$  among populations.

Following the diurnal measurements of gas exchange, we extracted whole seedlings with entire-root systems and chilled them on ice, and then microwaved them for 30 s at 650 W within 2 h of collection to stop carbohydrate enzymatic reactions (Hoch et al. 2002). Needle and non-needle (stem and root) tissues were separately laid flat and photographed, with a reference object for scale (model Coolpix 990; Nikon, Memphis, TN, USA), and projected needle area was calculated with image-processing software (Image J; National Institute of Mental Health, Bethesda, MD, USA). Gas exchange was calculated on a whole-plant (individual<sup>-1</sup>), projected needle area (m<sup>-2</sup>), or needle mass basis (mg<sup>-1</sup>); and on an instantaneous (s<sup>-1</sup>), daily (day<sup>-1</sup>), or integrated between sampling events (sampling interval<sup>-1</sup>) basis.

Following area determinations after each sampling event, tissues were dried for 48 h at 75°C. Needle and non-needle tissues were then weighed for plant dry mass to  $\pm 0.1$  mg. Growth was determined as the increase in mass between sampling events. Relative growth rate (RGR, mg g<sup>-1</sup> day<sup>-1</sup>) was calculated as:  $\text{RGR} = (\ln W_{n+1} - \ln W_n) / (T_{n+1} - T_n)$ , where  $W$  was dry mass of whole plants,  $T$  was the day number, and  $n$  and  $n + 1$  were consecutive sampling events (Zhang et al. 1996). Preliminary measurements of dry mass at the time of germination were used to calculate incremental mass gain and RGR between the time of germination and the first sampling event. Incremental mass gain and RGR could not be calculated for either species between the third and fourth sampling events at 2,450 m due to cotyledon loss.

### Nonstructural carbohydrate analysis

$NSC$  is defined as starch plus simple sugars (glucose, fructose, and sucrose); our method followed Hoch et al. (2002). Root and stem  $NSC$  concentrations (both simple sugars and starch) were within  $0.25 \pm 0.05$  % (dry mass) of each other in seedlings of *P. menziesii* grown indoors, and therefore these tissues were combined as non-needle tissue. We ground the dried needle and non-needle tissues to a fine powder and then weighed to  $\pm 0.1$  mg. Dried powder was boiled in water to extract simple sugars into aqueous solution and then enzymatically treated with invertase (to convert sucrose to glucose), phosphoisomerase (to convert fructose to glucose), and hexokinase (to convert glucose to 6-Phosphogluconate; Sigma Diagnostics, St. Louis, MO, USA). Oxidation of the simple sugars to 6-Phosphogluconate resulted in an equimolar reduction of NAD to NADH, which increased the absorbance of the solution at 340 nm (Synergy Microplate Reader; Biotek Instruments, Winooski, VT, USA) and was directly proportional to simple sugar concentrations. The original solution was then additionally treated with a high activity fungal alpha-amylase (“Clarase G-Plus” from the fungus *Aspergillus oryzae*; Genecore International, Rochester, NY, USA) to metabolize starch to glucose. The solution was re-analyzed for total  $NSC$  (starch and simple sugars) with the procedure described previously. Starch was calculated as total  $NSC$  minus simple sugars. Starch, sucrose, fructose, and glucose standards were used on every 96-well microplate analysis.

### Statistical analysis

We used a three-factor, mixed-model ANOVA with repeated measures (PROC MIXED, SAS version 9.1; SAS Institute, Cary, NC, USA) to determine species and elevation effects on response variables over the growing season. The between-subject fixed factors included tree species identity (*A. lasiocarpa* or *P. menziesii*) and elevation of site (2,450 or 3,000 m), and the within-subject fixed factor was date-of-measurement (four sampling events). Plots were a random factor in each test upon which repeated measures occurred throughout the season. The sampling of 6 of 10 available plots for physiological measurements on each sampling event resulted in missing values in the variance-covariance matrix. The restricted maximum likelihood estimation method of variance in PROC MIXED is less sensitive to unbalanced replication compared with traditional linear models (see example 3.4 in Littell et al. 1996). Response variables included daily-integrated photosynthesis ( $A$ ) or respiration ( $R$ ) (on a leaf area or mass basis, or over the whole crown), nonstructural carbohydrates ( $NSC$ ), and growth. Tests of between-subject effects from the repeated-measures analyses are equivalent to comparisons

**Fig. 1** Means ( $\pm 1$  SE) of physiological and morphological traits throughout the growing season for *Pseudotsuga menziesii* (left column, square and triangular symbols show 2006 and 2005, respectively) and *Abies lasiocarpa* seedlings (right column, round symbols) grown at 2,450 m (solid) and 3,000 m (open). Gas exchange variables (a–c) were calculated from the time-integrated summation of five instantaneous readings per each of six seedlings during each 24-h sampling event. Traits from top to bottom panels are: **a** gross carbon gain (net photosynthesis plus dark respiration) plus aboveground dark respiration of whole crowns (top and bottom, respectively), **b** gross carbon gain (net photosynthesis plus dark respiration) per unit needle mass, **c** aboveground dark respiration per unit needle mass, **d** projected needle area (both cotyledons and primary needles combined) for whole crowns, **e** needle:non-needle tissue ratios, and **f** needle starch concentrations (% dry mass). Errors are smaller than symbols where none appear

among treatments, integrated throughout the growing season. Root area and root mass were log transformed to meet the assumptions of normality and homoscedasticity. Effects were considered significant at  $\alpha = 0.05$ . The two *P. menziesii* seedlots did not differ in masses ( $F_{1,12} = 0.03$ ,  $P = 0.86$ ), *A* ( $F_{1,12} = 0.11$ ,  $P = 0.75$ ), or *NSC* ( $F_{1,12} = 1.33$ ,  $P = 0.27$ ) throughout the experiment, and consequently data were pooled for statistical analyses.

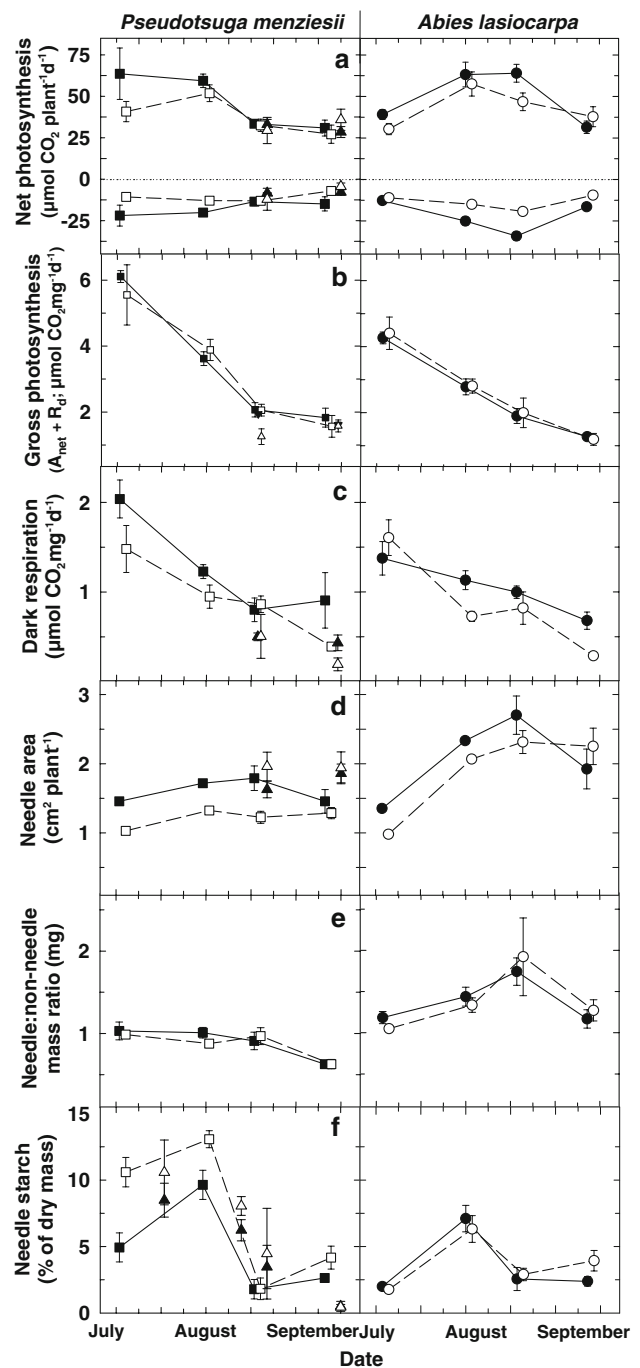
Relationships of gas exchange or *NSC* with growth were determined from least-squares regressions. To accomplish this,  $A_{net}$  (individual<sup>-1</sup>) was integrated or *NSCs* and *A:R* were averaged between consecutive sampling events (i.e. during a sampling interval) for each of the 4 populations (*P. menziesii* and *A. lasiocarpa* at 3,000 and 2,450 m), and the resulting values were compared with corresponding increases in mass or RGR during the same sampling intervals. Correlations of *A:R* to *NSC* were determined from least-squares regression with daily values for each variable, where each datum was a mean value of each of the 4 populations per sampling event.

**Results**

Date-of-measurement, as evaluated by the within-subject factor effects, explained most variation in nearly all response variables (Fig. 1; all *F*-values  $\geq 6.24$  and *P*-values  $\leq 0.002$ ). However, the effects of elevation and species identity on seedling physiology changed throughout the season, leading to date-by-elevation and date-by-species interactions.

**Microclimate variation**

Daily averages of mean, maximum, and minimum air temperatures were cooler at 3,000 m compared with 2,450 m (2.4, 2.3, and 3°C cooler at the upper site, respectively; see Electronic supplementary material S1 for actual means).



Daily maximum soil temperatures were 2.6°C warmer at 3,000 m (S1). No temperatures  $< 0^\circ\text{C}$  were recorded at the lower elevation during the months in which seedlings were measured, and only one frost (31 August 2006) was recorded at the upper elevation. Total growing degree-days were similar between the lower and upper elevations, both during the study period (550 and 517 degree days, respectively) and when calculated across all snow-free days at each respective elevation (666 and 601 degree days, respectively). This occurred despite 6 extra snow-free weeks in

spring and 3 extra weeks in autumn at the 2,450 m site compared with the 3,000 m site (i.e., only 10% more growing degrees despite more than 40% more snow-free days). Also, during the extra snow-free periods at the lower elevation, soil and air temperatures regularly dropped below freezing (minimum temperatures down to  $-4^{\circ}\text{C}$  recorded after our final data collection).

#### Photosynthesis and respiration

Seedlings of *A. lasiocarpa* and *P. menziesii* had 13–15% less photosynthesis at the whole-plant level ( $A_{\text{wp}}$ ) at the upper compared with lower elevation (Fig. 1; both species combined,  $A_{\text{wp}}$ ,  $F_{1,17} = 4.71$ ,  $P = 0.044$ ). Leaf-level  $A$  (i.e. photosynthesis per unit needle mass or area) did not differ between elevations for either species ( $A$  per unit mass, Fig. 1; summary data of  $A$  per unit area, S2), and therefore less  $A_{\text{wp}}$  was attributable to less needle production (mass and area) at the upper elevation (Fig. 1; both species combined: needle mass,  $F_{1,17} = 8.12$ ,  $P = 0.011$ ; needle area,  $F_{1,17} = 19.22$ ,  $P = 0.0004$ ). Seedlings of *P. menziesii* had relatively greater leaf-level  $A$  compared with *A. lasiocarpa* (Fig. 1;  $A$  per unit mass,  $F_{1,15} = 18.80$ ,  $P = 0.0006$ ).

Seedlings of both *A. lasiocarpa* and *P. menziesii* had 35–39% less respiratory carbon loss at the whole-crown level ( $R_{\text{crown}}$ ) at the upper compared with lower elevation (Fig. 1; both species combined,  $R_{\text{crown}}$ ,  $F_{1,17} = 25.08$ ,  $P = 0.0001$ ).  $R_{\text{crown}}$  decreased with elevation more than  $A_{\text{wp}}$  because, unlike leaf-level  $A$ , leaf-level  $R$  was about 20% less at the upper elevation in both species ( $R$  per unit mass Fig. 1;  $R$  per unit area S2;  $R$  per unit mass,  $F_{1,17} = 16.86$ ,  $P = 0.0007$ ). Leaf-level  $R$  was similar between species, but *A. lasiocarpa* seedlings had 22% more  $R_{\text{crown}}$  compared with *P. menziesii* seedlings (Fig. 1;  $F_{1,15} = 9.60$ ,  $P = 0.0073$ ) because of relatively greater needle area (Fig. 1;  $F_{1,15} = 21.49$ ,  $P = 0.0003$ ). Based on the difference between  $A_{\text{wp}}$  and  $R_{\text{crown}}$  plus carbon mass gain, an approximate, and plausible, 20% of gross carbon gain could have effluxed through the roots (S3 for calculations).

#### Nonstructural carbohydrates

Nonstructural carbohydrates (NSC) ranged from 2 to 12% dry mass, and at times were up to 50% greater at 3,000 m compared with 2,450 m for both species (S2; whole-plant NSC,  $F_{1,17} = 4.84$ ,  $P = 0.042$ ). NSC variation among the elevations and species was primarily attributable to differences in starch and not simple sugars; although simple sugars increased throughout the season in both needles and roots to become 60–70% of whole-plant NSC by the end of the growing season (S2). Starch was greater in both needles and roots at 3,000 m compared with 2,450 m (needles,

Fig. 1; roots, S2; both species combined: needles,  $F_{1,17} = 9.15$ ,  $P = 0.008$ ; roots,  $F_{1,17} = 14.03$ ,  $P = 0.0016$ ). NSC, and specifically starch, was greater in *P. menziesii* than in *A. lasiocarpa* (S2; whole-plant NSC,  $F_{1,15} = 26.73$ ,  $P = 0.0001$ ; whole-plant starch,  $F_{1,15} = 28.62$ ,  $P < 0.0001$ ), especially during the early season (time by species interaction,  $F_{3,12} = 16.35$ ,  $P = 0.0002$ ).

#### Growth

Mean ( $\pm 1$  SE) dry mass of seedlings of both species was  $17 \pm 3\%$  less at 3,000 compared with 2,450 m, over the first 3 sampling events and prior to partial senescence of cotyledons in September (S2;  $F_{1,17} = 8.75$ ,  $P = 0.009$ ). Also during the first 3 sampling events, mean ( $\pm 1$  SE) mass of needles was  $18 \pm 3\%$  less, and needle area was  $23 \pm 3\%$  less at 3,000 compared with 2,450 m, in both species (needle area, Fig. 1; needle mass, S2). For both species, root mass and area were  $9 \pm 4\%$  and  $15 \pm 5\%$  less at the upper elevation, respectively (S2; both species combined: root mass,  $F_{1,17} = 36.81$ ,  $P < 0.0001$ ; root area,  $F_{1,17} = 7.41$ ,  $P = 0.015$ ). Despite greater decreases in needles compared with roots at the upper elevation, the ratio of needle to non-needle tissue within each species was not significantly different between elevations (Fig. 1).

Relative growth rates (RGRs) generally declined through the growing season and were correlated to incremental mass gain of whole-plant (calculated from data in S2;  $F_{1,15} = 13.85$ ,  $r^2 = 0.46$ ,  $P = 0.0022$ ). RGRs were approximately 42% less at the upper elevation during the first sampling interval for both species, but there were no overall species or elevation differences when integrated over the growth season ( $P = 0.99$ ).

Whole-plant mass decreased between the third and fourth sampling events, likely from loss of cotyledons, and was most apparent at 2,450 m in both species (Fig. 1 and S2; S. Bansal, personal observation). If the fourth sampling event was included in the analysis, then whole-plant and needle mass did not differ between elevations for either species.

Mean ( $\pm 1$  SE) seedling dry mass was  $14 \pm 8\%$  greater on average in *A. lasiocarpa* than in *P. menziesii* during the first 3 sampling events (S2;  $F_{1,15} = 15.58$ ,  $P = 0.0013$ ), primarily because of a greater and longer period of needle mass production (Fig. 1;  $F_{3,14} = 4.41$ ,  $P = 0.022$ ). The ratio of needle to non-needle tissue was significantly greater in *A. lasiocarpa* compared with *P. menziesii* (Fig. 1; by mass,  $F_{1,15} = 21.49$ ,  $P = 0.0003$ ). Specific leaf area (needle area divided by needle mass, SLA) decreased similarly for both species at both elevations throughout the growing season, and did not differ among populations (S2).

## Relationships among photosynthesis, respiration, nonstructural carbohydrates, and growth

Greater decreases in  $R$  compared to  $A$  with elevation led to a 20–30% greater mean  $A:R$  at the upper elevation in both species (calculated from data in S2;  $F_{1,17} = 13.32$ ,  $P = 0.002$ ). Specifically, mean ( $\pm 1$  SE)  $A:R$  integrated over the growing season at 3,000 and 2,450 m was  $3.8 \pm 0.3$  and  $2.9 \pm 0.3$ , respectively, for both species combined. Despite large seasonal changes in both  $A$  and  $R$ ,  $A:R$  remained relatively constant over the course of the study period, for both species within each elevation. The ratio of incremental dry mass gain to  $R_{\text{crown}}$  ( $Gr:R$ ) was 1.5 times greater at the upper compared with lower elevation throughout the growing season in both species ( $4.1 \pm 0.7$  and  $2.6 \pm 0.5$  mg dry mass gain per mg  $\text{CO}_2$  efflux per sampling interval at 3,000 and 2,450 m, respectively;  $F_{1,13} = 7.28$ ,  $P = 0.0194$ ). The ratio of incremental dry mass gain to whole-plant carbon gain ( $Gr:A$ ) did not significantly differ between elevations for either species ( $1.4 \pm 0.17$  and  $1.1 \pm 0.15$  mg dry mass gain per mg  $\text{CO}_2$  uptake per sampling interval at 3,000 and 2,450 m, respectively;  $F_{1,13} = 2.72$ ,  $P = 0.13$ ).

Incremental mass gain of whole-plants (e.g., growth) and net  $A_{\text{wp}}$  (individual $^{-1}$ ) were positively correlated over both species and elevations combined (Fig. 2;  $F_{1,13} = 8.72$ ,  $r^2 = 0.42$ ,  $P = 0.0121$ ). Day-to-day variation in climate between sampling events (e.g., the few days with cloud cover and precipitation) could have increased variation in our estimate of integrated  $A_{\text{wp}}$ , thereby reducing the  $r^2$  of our correlation. Incremental carbon gain was greatest during the second interval for both species at both elevations due to relatively high leaf-level  $A$  and total leaf area (Fig. 1). Carbon gain decreased following the second interval for all four populations (despite seedlings increasing in

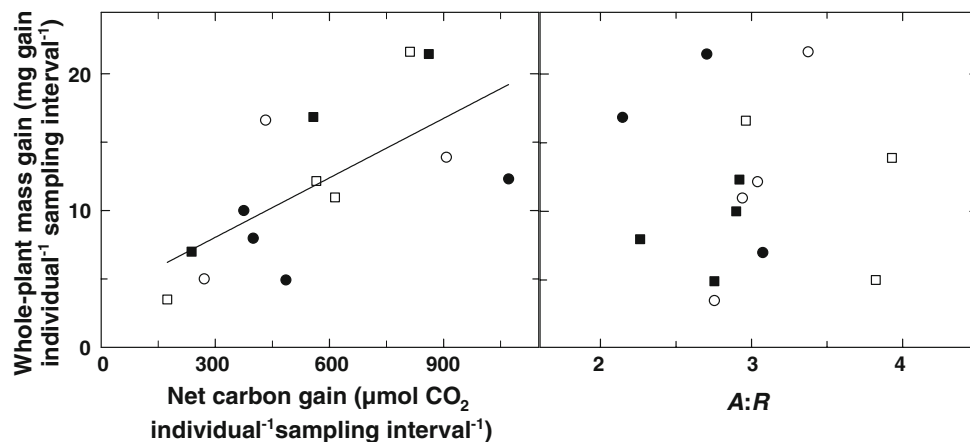
size) due to a relatively large decline in leaf-level  $A$  in combination with a relatively small increase in needle area. Consequently, larger plants did not always have greater carbon gain, and leaf-level  $A$  alone did not explain a significant amount of variation for either incremental mass gain or RGR. Growth was not related to  $A:R$  (Fig. 2; for both species combined:  $F_{1,13} = 0.07$ ,  $r^2 = 0.00001$ ,  $P = 0.99$ ).

Whole-plant starch was positively correlated with  $A:R$  (Fig. 3; both species combined,  $F_{1,15} = 8.06$ ,  $r^2 = 0.42$ ,  $P = 0.007$ ), but  $NSC$  or simple sugars were not related to  $A:R$ .  $NSC$ , starches, or simple sugars in whole-plant, needles, or roots were not significantly correlated to incremental mass gain or RGR, with the exception of a negative relationship between needle sugars and RGR ( $F_{1,13} = 7.05$ ,  $r^2 = 0.37$ , slope =  $-2.02$ ,  $P = 0.021$ ).

## Discussion

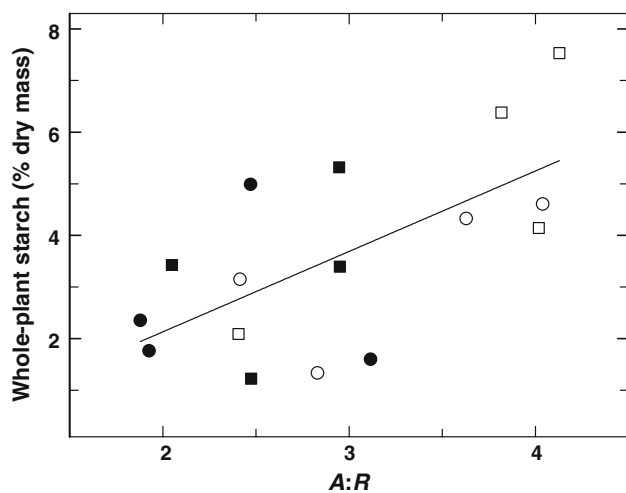
Studies of the carbon-balance and sink-limitation hypotheses for treeline formation have generally measured components of carbon balance at the tissue-level, and primarily in adults. Our study is unique because we assessed photosynthesis ( $A$ ), respiration ( $R$ ), nonstructural carbohydrates ( $NSC$ ), and growth at the whole-plant level, and during the seedling establishment phase.  $A$ ,  $R$ , and growth were all less at the upper elevation, but  $R$  decreased the most, in seedlings of both *A. lasiocarpa* and *P. menziesii*.  $NSCs$ , specifically starch concentrations, were greater at the upper elevation, and 40% of the variation in starch concentrations was explained by the ratio of  $A$  to  $R$ .

Based on the biogeographic range differences between species, we expected the treeline species (*A. lasiocarpa*) to be less responsive to the elevation gradient we evaluated at



**Fig. 2** Comparison of mean increases in whole-plant biomass between consecutive sampling events (mg dry mass gain individual $^{-1}$  sampling interval $^{-1}$ ) with net carbon gain ( $r^2 = 0.42$ ; left panel,  $\mu\text{mol CO}_2$  individual $^{-1}$  sampling interval $^{-1}$ ) or mean photosynthesis:respi-

ration ( $r^2 = 0.0001$ ;  $A:R$ , right panel) between corresponding sampling events for *Pseudotsuga menziesii* (squares symbols) and *Abies lasiocarpa* seedlings (round symbols) grown at 2,450 m (solid) and 3,000 m (open)



**Fig. 3** Comparison of mean photosynthesis:respiration ( $A:R$ ) with mean whole-plant starch concentrations (% dry mass) at each sampling event for *Pseudotsuga menziesii* (square symbols) and *Abies lasiocarpa* seedlings (round symbols) grown at 2,450 m (solid) and 3,000 m (open),  $r^2 = 0.42$

timberline compared to the nontreeline species (*P. menziesii*). Moreover, our upper site was above the natural range of *P. menziesii* in the study area, which could have further exacerbated the effects of elevation for it compared to *A. lasiocarpa*. However, both species experienced similar variation in  $A$ ,  $R$ , and growth between the upper and lower elevation. The notable difference between species was a greater change in  $NSC$  in seedlings of *P. menziesii* compared to *A. lasiocarpa*, which may indicate that  $NSC$  concentrations are relatively more sensitive to the effects of elevation compared to  $A$ ,  $R$ , and growth. A more substantial difference in carbon balance between species was apparent as greater allocation of biomass to needles in *A. lasiocarpa*, which contributed to greater carbon flux at the whole-crown level. Increased needle area favored acquisition of aboveground resources (e.g.,  $CO_2$ ) in seedlings of the tree-line compared with the nontreeline species.

#### Expected compared with observed variation in photosynthesis

Comparisons with several other studies of tree seedlings in the alpine-treeline ecotone indicate that the negative effects on carbon uptake for our seedlings growing at the upper “cool edge” of timberline reported here might be conservative. Less whole-plant  $A$  at upper elevation in our study was more attributable to less needle production than to lower leaf-level  $A$ , in contrast to previous studies with conifer seedlings (e.g., Johnson et al. 2004). Leaf-level  $A$  of conifer seedlings in other treelines (including *A. lasiocarpa*) was negatively affected by frost-sunlight interactions and low-temperature photoinhibition (LTP; Ball et al. 1991; Germino and Smith

1999), but we observed only one frost event. We also selected plots at each elevation to experimentally eliminate variation in overhead canopy, which entailed relatively more overhead cover for seedlings at upper timberline and possibly less LTP in the current compared with other studies (e.g.,  $F_v/F_m$  values  $> 0.60$  in our seedlings; S4).

#### Variation in nonstructural carbohydrates

Increases in  $NSC$  of trees at treeline have been interpreted as support for reduced carbon sink relative to source activity, and for the inference that carbon supplies were in excess of the growth needs of trees growing at high elevations (‘sink-limitation hypothesis’; Körner 1998; Hoch and Körner 2003; Handa et al. 2005; Piper et al. 2006). We also observed greater  $NSC$  concentrations when  $R$  decreased relatively more than  $A$  at the upper site; however, increased  $NSC$  did not appear to indicate excess carbon supply for growth in our seedlings. If elevated  $NSC$  concentrations resulted from excess carbon, then some indication of feedback inhibition of increased  $NSC$  on leaf-level  $A$  might be expected, such as a negative relationship between the two variables (Sharkey et al. 1986; Koch 1996; Cross et al. 2006; reviewed by Paul and Foyer 2001; Rolland et al. 2002; Smith and Stitt 2007). Also, if  $NSC$  were accumulating because of low rates of biosynthesis, then we would expect greater  $NSC$  during periods of low RGR. However,  $NSC$ ,  $A$ , and RGR all followed similar seasonal patterns (i.e., all decreased throughout the growing season), suggesting that  $NSCs$  were not feeding back on  $A$  nor were a passive response of slow growth. Nevertheless, our correlative data cannot be used to unequivocally dismiss the possibility of feedback between  $NSC$  and  $A$  (e.g., genetic, post-transcriptional, or post-translational links between carbon availability, storage, and growth; Paul and Foyer 2001; Rolland et al. 2002; Smith and Stitt 2007).

The seasonal variations in  $NSC$  among roots and shoots that could not be attributed to  $A:R$  (i.e., 60% of variation) may be related to functional strategies to cope with high-elevation conditions (Mooney and Billings 1960; reviewed by Janusz et al. 2001; Tolsma et al. 2007). Over the course of the study, needle starch concentrations dropped to  $< 2\%$  in all seedlings, indicating that the ‘excess’ starch was eventually metabolized and converted to simple sugars (as starch is not transported through the phloem) by the end of the season. In contrast to the shoots, storage of starch in root tissue dramatically increased at the end of the growing season, especially at the upper elevation. The metabolism, transport, and re-synthesis of starch indicate active regulation of the  $NSC$  pool (e.g., residual starch concentrations not explained by  $A:R$ ). Unlike starch concentrations, there was no correlation between simple sugar concentrations and  $A:R$ , and the increase in simple sugars near the end of



the growing season and prior to winter was likely related to cold-hardening (Ögren et al. 1997; Gilmour et al. 2000; Janusz et al. 2001).

The relationships among *NSC*, gas exchange, and growth that we observed in seedlings may not hold true for adult trees. Conifers typically have needles from previous growing seasons that can provide photosynthate to developing tissues (Hansen and Beck 1994; Li et al. 2002), a benefit that only germinating seedlings lack. High growth requirements for successful establishment combined with only a single cohort of needles may contribute to greater resource limitations of first-year seedlings compared to older trees. Consequently, the relationship between gas exchange and growth to *NSC* may be different for first-year seedlings compared to older age classes.

#### Evidence for treeline-formation hypotheses

The carbon-balance hypothesis for treeline formation states that net photosynthesis decreases with elevation, leading to a decrease in *A:R* below unity and insufficient carbon for growth (Stevens and Fox 1991). However, we hypothesized that if trees near alpine treeline were carbon limited, then we may expect compensatory physiological adjustments to improve carbon use efficiency, such as an increase in the ratios of carbon uptake and utilization (growth) per unit carbon loss (*A:R* and *Gr:R*, respectively), similar to an increase in water use efficiency in plants adapted to drought (Donovan and Ehleringer 1994). We did indeed observe greater *A:R* and *Gr:R* in our seedlings at the upper elevation; however, the increased carbon use efficiency was not enough to compensate for less needle area, and consequently net carbon gain and growth were still less at the upper elevation in our seedlings. Therefore, our seedlings may have been carbon limited (consistent with the carbon-balance hypothesis), but not from a decrease in *A:R*.

Although the effects of cold temperatures at treeline on root growth have been emphasized in the literature (Körner and Hoch 2006; Alvarez-Uria and Körner 2007 and citations within), the positive feedback between needle growth and carbon gain at the upper elevation appeared to be an important constraint to growth in our subject seedlings. Less needle growth at the upper site was apparent by the first sampling event (i.e., only 10 days after outplanting; Fig. 1) in both species. Following the first sampling event, the differences in needle mass and area between elevations remained relatively constant (except at the end of the growing season during cotyledon senescence; Fig. 1 and S2). Therefore, elevation effects on early development may have had a relatively large effect on final leaf area (Cookson et al. 2005).

The sink-limitation hypothesis states that carbon sinks decrease more than carbon sources in trees at high eleva-

tions (Körner 1998), but the hypothesis does not differentiate the roles of growth and *R* as carbon sinks. Our separation of carbon sinks into growth and *R* revealed *R* to be a more dynamic carbon sink than growth, and also a major driver of variations in net carbon gain among elevations and species. Variation in *R* compared with *A* or growth along elevation gradients could result from relatively greater temperature sensitivity of *R*, shifts in the expression of different *R* pathways (e.g., shifts in the cyanide-resistant pathway with temperature; Grabelnych et al. 2004; Cambell et al. 2007), or variable inhibition of *R* by irradiance (reviewed by Krömer 1995; Atkin et al. 2000; Zaragoza-Castells et al. 2007). Given the role of *R* in the variation of net carbon gain among elevations and species, future inquiry on *R* along elevation gradients appears necessary for understanding growth limitation in trees at high elevations.

The sink-limitation hypothesis has stimulated critical tests of assumptions on the ecological importance of photosynthesis—in this instance for trees near their high-elevation limit. The sink-limitation hypothesis proposes that trees at alpine treeline have surplus carbon for growth, and therefore suggests that the decreases in photosynthesis with elevation would have little effect on growth. The positive correlation between carbon gain and biomass gain that we observed shows photosynthesis to be important to growth, though the possibility of feedbacks between *NSC* and *A* hinder our ability to conclude that carbon source or sinks were most limiting growth. Moreover, sink limitation on needle growth would also lead to a decrease in carbon gain, as we observed in our study. These linkages confound attempts to separate the carbon-balance and sink-limitation hypotheses. Further consideration of hypotheses for treeline formation may thus be necessary.

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