Intra-annual dynamics of non-structural carbohydrates in the cambium of mature conifer trees reflects radial growth demands

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The presence of soluble carbohydrates in the cambial zone, either from sugars recently produced during photosynthesis or from starch remobilized from storage organs, is necessary for radial tree growth. However, considerable uncertainties on carbohydrate dynamics and the consequences on tree productivity exist. This study aims to better understand the variation in different carbon pools at intra-annual resolution by quantifying how cambial zone sugar and starch concentrations fluctuate over the season and in relation to cambial phenology. A comparison between two physiologically different species growing at the same site, i.e., the evergreen Picea abies Karst. and the deciduous Larix decidua Mill., and between L. decidua from two contrasting elevations, is presented to identify mechanisms of growth limitation. Results indicate that the annual cycle of sugar concentration within the cambial zone is coupled to the process of wood formation. The highest sugar concentration is observed when the number of cells in secondary wall formation and lignification stages is at a maximum, subsequent to most radial growth. Starch disappears in winter, while other freeze-resistant non-structural carbohydrates (NSCs) increase. Slight differences in NSC concentration between species are consistent with the differing climate sensitivity of the evergreen and deciduous species investigated. The general absence of differences between elevations suggests that the cambial activity of trees growing at the treeline was not limited by the availability of carbohydrates at the cambial zone but instead by environmental controls on the growing season duration.

Keywords: cambium, intra-annual analysis, Larix decidua, non-structural carbohydrates (NSC), Picea abies, phenology, tree-ring.

Introduction

Tree growth and survival depend not only on their capacity to produce and use carbohydrates, but also on efficient carbohydrate storage and remobilization. Carbohydrates are supplied by fixing atmospheric carbon (C) during photosynthesis and are vital in almost all plant physiological processes, including the maintenance of existing tissue, the formation and enlargement of organs and all associated metabolic processes. Investigating ecosystem productivity under changing climatic conditions requires understanding how various physiological and environmental factors constrain plant growth. In this regard, studies of non-structural carbohydrates (NSCs; defined here as soluble sugars + starch) have widely been used to assess the source–sink balance of trees (Fischer and Holl 1992, Barbaroux and Bréda 2002, Damesin and Lelarge 2003, Oberhuber et al. 2011, Woodruff and Meinzer 2011, Gruber et al. 2012, Michelot et al. 2012). Stored NSCs can be viewed as reservoirs that are refilled when the C demand for growth and maintenance is low and called upon during periods of high C requirements. Under this functional interpretation of NSCs,
accumulation of large C pools is inconsistent with the hypothesis of C supply limiting growth, and rather suggests ‘sink’ limitations (Körner 2003, Millard et al. 2007, Hoch and Körner 2012). Large pools of stored C have been observed in mature coniferous and deciduous trees with, for example, C reserves in deciduous trees sufficient to replace the entire canopy several times (Hoch et al. 2003). Trees growing under conditions of high environmental stress such as toward their cold thermal limits of growth (Hoch and Körner 2003, 2012, Hoch et al. 2003, Fajardo et al. 2013), following severe water stress (Breda et al. 2006, Gruber et al. 2012) or defoliation (Hoch 2005, Palacio et al. 2008) also showed high levels of stored NSCs associated with, or despite, reduced growth.

However, in the context of on-going climate change and concurrent increases in tree mortality, the role of C allocation has been revisited with several studies, concluding that significant amounts of mobile carbohydrates in various tree tissues may not be simply interpreted as a sink limitation (McDowell and Sevanto 2010, Millard and Grelet 2010, Sala et al. 2010, Ryan 2011, Wiley and Helliker 2012). Data obtained by interrupting the supply of new photosynthates via phloem girdling (Bhupinderpal et al. 2003), or following girdling and defoliation (Hoch 2005), have revealed significant NSC pools that remain unused by trees. This may reflect a condition intermediate to the ‘source’ and ‘sink’ limitations. Namely, trees appear physiologically capable of incorporating stored photosynthates into permanent tissues, yet do not do so. It is still unclear to what extent these data (i) indicate the inability of trees to supply the growing tissues with carbohydrates, (ii) reflect a prioritization of resources as a safety margin in the face of environmental stochasticity or (iii) suggest some other underlying mechanism. Regardless of the cause, active C storage competing with growth has been evidenced in different species (Chapin et al. 1990, Silpi et al. 2007, Genet et al. 2010). Consequently, if a significant fraction of the C pool is actively stored (thus competing with growth) or sequestered (i.e., unavailable for any further physiological processes), then the observed overabundance of C in trees is not a useful indicator for a sink limitation (Millard and Grelet 2010). Therefore, under a long-term perspective, competing requirements for NSCs (e.g., respiration, defense and export, maintenance of hydraulic integrity) might cause a source limitation (Epron et al. 2012, Sala et al. 2012, Wiley and Helliker 2012, Hartmann et al. 2013).

Despite abundant literature on NSCs, the processes and pathways related to NSC allocation and storage within trees remain poorly understood (Epron et al. 2012, Sala et al. 2012, Wiley and Helliker 2012). Progress is hampered by the scarcity of field data necessary for model testing, with studies in natural mature forests particularly needed (Barbaroux and Bréda 2002, Hoch et al. 2003, Gough et al. 2009, Richardson et al. 2013). Non-structural carbohydrate concentrations have been measured in diverse tree organs, including stems, branches, foliage and roots (Fischer and Holl 1992, Uggl et al. 2001, Barbaroux and Bréda 2002, Damesin and Lefarge 2003, Oberhuber et al. 2011, Woodruff and Meinzer 2011, Gruber et al. 2012, Michelot et al. 2012). In these studies, a general conclusion was that high variation in the intra-annual NSC content was observed nearer to sites of active growth (e.g., apical and root meristems), while a low variation was recorded within the storage tissues (e.g., sapwood, coarse roots and ray parenchyma). Investigations at the primary sink location would be expected to yield better insights into the seasonal carbohydrate supply and demand in trees.

Owing to technical challenges associated with the sampling and isolation of NSCs in the cambial zone, very few studies have reported on C stock measurements where secondary growth occurs (Sundberg et al. 1993, Uggl et al. 2001, Deslauriers et al. 2009, Giovannelli et al. 2011). The cambial zone in tree stems is composed of a thin layer of meristematic cells and only recently has a procedure been developed to solve the challenge of separating this tissue for NSC extraction (Giovannelli et al. 2011).

Here, we use this new procedure to contribute to the debate on C dynamics in trees by supplying a detailed description of the intra-annual C fluctuation (soluble NSCs and starch content) in the cambial zone of mature conifer trees. We aim at elucidating the mechanisms controlling growth and at better understanding the effective sink strength of the cambium and its variability over time. Therefore, we perform measurements in a deciduous and an evergreen conifer species at specific phenophases of wood formation, as well as at two sites at contrasting elevations. In addition, we quantify the individual sugars, such as glucose, fructose, sucrose, raffinose, pinitol and starch, to better assign a functional meaning to the seasonal variations in NSC concentrations. This design allows us to address specific questions related to growth limitation and C demand, such as: (i) Is secondary growth limited by the availability of C or constrained by environmental conditions acting upon the sink function? (ii) How does C supply in the cambium respond to the C demand for stem growth and concurrent foliage production? (iii) Is there any species- and/or site-specific strategy in the production, storage and supply of NSCs/individual sugars in terms of C allocation to growth, management of reserves and protection against environmental stress?

Materials and methods

Study sites and field activities

Our study was performed in the Lötschental (46°23′40″N, 7°45′35″E), a southwest–northeast-oriented inner-alpine valley in the central Swiss Alps. The bottom of the valley is surrounded by steep forested slopes primarily composed of mixed, evergreen Norway spruce (Picea abies Karst.) and deciduous European larch (Larix decidua Mill.). The climate of
the region is cool and relatively dry, with a mean annual temperature of 6 °C, ranging from −3 °C (January) to 15 °C (July), and a mean annual precipitation >800 mm (data from MeteoSwiss for the period 1987–2006).

Field activities were conducted in 2010 at two sites, ~1 km apart from each other, with contrasting elevations. The high elevation site is located at the upper tree line at ~2200 m above sea level (a.s.l.), on a south-facing slope and consists solely of larch (this site is abbreviated S22L to reflect the aspect (South), elevation (2200 a.s.l.) and species (Larch)). The low elevation site is near the valley bottom close to the north-facing slope at 1300 m a.s.l. and is a mixture of larch and spruce (similarly abbreviated N13L and N13S). The mean temperature difference between sites, as monitored from April to October 2010, was 3.5 °C (average maximum and minimum temperature difference of 6 and 2.5 °C, respectively). Hydrological conditions are generally dryer at the low elevation site due to a combination of less precipitation and higher evaporative demand. The soils of both sites are ~60 cm deep podzolic cambisols characterized by significant coarse stone contents and low clay amounts. Field activities involved (i) collecting stem samples to follow NSC dynamics in cambial tissue and (ii) weekly monitoring of foliar and wood formation to document the progress of growth at the time of cambial sampling.

**NSC sampling and biochemical analysis**

NSC sampling was performed on five different dates on 45 mature trees in total, with 15 trees per species and site (S22L, N13L and N13S). Sampling dates were selected to target five relevant phases of annual ring formation, i.e., (i) when cambial division/and earlywood cell enlargement are highly active, (ii) when earlywood cells are in both phases, enlargement and wall thickening, (iii) after cellular division has stopped but enlargement and wall thickening phases continue, (iv) when only latewood cells are conducting secondary wall thickening, and (v) during dormancy of cambium (Table 1). The sampling dates were estimated based on data from 2007 to 2009 (Moser et al. 2010; G. King et al. in preparation).

Two samples per tree and sampling date were taken at ~50 cm height using 37 mm diameter metal punchers. The samples, comprising phloem, cambium and xylem, were kept on ice during fieldwork, stored at −22 °C once in the laboratory and freeze-dried. Subsequently, the samples were prepared for biochemical analysis according to the protocol described in Giovannelli et al. (2011). Accordingly, samples were split along the tangential plane in the cambial zone and then the differentiating phloem and cambial cells from the phloem side sample were gently scraped with a razor blade to obtain a powder from the cambial tissue. Owing to the low amount of cambium powder per sample, blocks of five trees per site and species were pooled to obtain enough material for sugar extraction. After pooling and homogenizing, an equal amount of cambium powder per tree, a 40 mg subsample, was used for sugar extraction. Non-structural carbohydrates were extracted from the cambial powder using chemical procedures described in Giovannelli et al. (2011). The sugar content was determined by high-performance liquid chromatography analysis equipped with a SHODEX SUGAR Series SC 1011 8 × 300 mm column (Showa Denko, Germany) preceded by a pre-column Guard Pak Insert Sugar Pak II (Waters). The mobile phase was water, Milli Q grade, at 0.5 ml min−1. Soluble carbohydrate identification was verified using carbohydrate standards (Sigma-Aldrich, St. Louis, MO, USA) quantified by means of an internal standard. Concentrations of glucose, fructose, sucrose, pinitol and raffinose were thus obtained. The remaining pellet after soluble NSC extraction was used for starch quantification. The starch content was measured after an extraction procedure: the residual pellet was suspended in 1.5 ml of acetate buffer (pH 5), heated at 100 °C for 1 h in a sand bath and then cooled at room temperature. After incubation at 55 °C for 16 h with 150 µl amylglucosidase from Aspergillus niger (Fluka), samples were diluted with distilled water to 5 ml and three 0.25-ml aliquots of each sample were assayed colorimetrically using glucose oxidase (Sigma-Aldrich Saint Louis, Missouri, USA).

Seasonal changes in soluble NSCs and starch content were compared between elevation and species using repeated-measures analysis of variance (ANOVA) (Gumpertz and Brownie 1993, von Ende 1993). For within-subject analysis, a Huynh–Feldt corrected probability was used to overcome the sphericity assumption in the case of univariate repeated-measures analysis (von Ende 1993). Differences were considered significant at P < 0.05. When significant effects were found, mean comparisons by sampling date using ANOVA were performed to identify when the differences occurred. All ANOVA analyses were performed using the JMP® 8.0 software (SAS Institute, Inc.).

### Table 1. Days of the year (DOY) and corresponding dates of NSC sampling for each related phenological stage and site.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Period</th>
<th>N13, DOY (date)</th>
<th>S22, DOY (date)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highly active cambial division/EW cell enlargement</td>
<td>1</td>
<td>151 (31 May 2010)</td>
<td>174 (23 June 2010)</td>
</tr>
<tr>
<td>EW cell enlargement and wall thickening</td>
<td>2</td>
<td>186 (5 July 2010)</td>
<td>196 (15 July 2010)</td>
</tr>
<tr>
<td>LW cell wall thickening</td>
<td>4</td>
<td>258 (15 Sept 2010)</td>
<td>258 (15 Sept 2010)</td>
</tr>
<tr>
<td>Dormancy</td>
<td>5</td>
<td>68 (9 March 2011)</td>
<td>39 (8 Feb 2011)</td>
</tr>
</tbody>
</table>

EW, earlywood; LW, latewood.
Monitoring of foliar and wood formation

Foliar and cambial phenologies were monitored on a weekly basis over the entire growing season 2010. For this purpose, and to prevent any potential influence of the sugar sampling on growth, 12 additional trees were selected: four spruce and larch trees at N13 and four larch trees at S22. The date of budburst for each tree was defined when 50% of its buds were broken (Brügger and Vassella 2003). Exact dates were estimated by linear interpolation, with these values averaged per site and species for a site date.

Cambial phenology was quantified weekly on the same trees. The forming annual ring was monitored for each tree by analyzing tracheid formation from microcores collected weekly between April and November after Moser et al. (2010). Microcores were collected from the stem at 1–2 m height using a Trephor (Rossi et al. 2006a), preferentially perpendicular to the slope to avoid reaction wood. Sampling was conducted along an oblique line and 3–5 cm apart to minimize wound reactions caused by earlier samplings (Forster et al. 2000). Microcores were placed for 24 h in diluted acetic acid and ethanol to preserve forming cells from degradation, and then stored in a 70% alcohol solution. Samples were prepared for cellular analysis by cutting 20- to 30-μm-thick transversal microsections using a sliding microtome. Microsections were stained with safranin and astrablue, and fixed to microscope slides with Canada balsam. Ring formation was analyzed at a magnification of ×400–600 and the number of tracheids in the different phases of cell development (i.e., enlargement, wall thickening and maturity) assessed by averaging the counting along three radial files of each microsection. Enlarging cells were characterized by thin primary cell walls with a radial diameter roughly two or more times larger than that of dividing cambial cells. Polarized light was used to discriminate between enlarging and wall-thickening tracheids. Mature cells were recognized by completely lignified secondary walls and empty cell bodies (Rossi et al. 2006b, 2007). Dates representing critical phenological stages of wood formation were calculated for each site and species based on the cell counts.

Results

Growth dynamics and timing of NSC sampling

At the bottom of the valley, larch budburst occurred on days of the year (DOY) 135 ± 3.5 (mean ± standard deviation) and xylem formation (defined in this work as the timing of the first observed enlarging xylem cells) on DOY 138 ± 3. Budburst and xylem formation in spruce occurred later, on DOYs 149 ± 3.5 and 143 ± 0, respectively. Budburst in larch occurred before or close to the first observations of xylem cells entering the enlargement phase. In contrast, spruce initiated growth of new xylem tracheids before the emergence of current-year needles. These observations indicate that spruce and larch adopt different sequential arrangements in the timing of foliar and xylem growth resumption. At S22L, in comparison with larch growing 900 m lower or at a 3.5 °C warmer site, budburst was delayed by ~2 weeks (DOY 149 ± 3.5) and xylem formation onset by ~3 weeks (DOY 159 ± 3.5) (Figure 1, Table 2). The timing of growth resumption significantly differed (Table 3) between species (~6 days; P = 0.01) and elevation (~22 days; P < 0.0001).

The onset of wall thickening, the formation of fully mature cells, as well as the maximum number of cells observed in the phase of enlargement and wall thickening occurred earlier at N13S than N13L (Figure 1). The first wall thickening and mature cells were both observed 8 days later for spruce. Despite their delay in the onset of wall thickening (9 days) and mature cells (12 days), S22L soon reached similar levels of cell production as found at N13L. The total number of xylem cells in the 2010 ring varied among larch (ranging from 10 to 30) and spruce (from 25 to 70), but was, in general, higher for spruce.

The differences between both species and elevation decreased towards the end of the growing season. Significant differences in the end of xylogenesis (DOY 308 on average)
between N13L and N13S disappeared, although larch at the treeline stopped xylogenesis a few days earlier. Thus, the shorter total duration of xylogenesis at S22L compared with N13L (18 days, \( P = 0.0004 \)) and for N13S compared with N13L (8 days, \( P = 0.02 \)) was mainly due to a difference in the onset of xylem differentiation (Table 3).

### Non-structural carbohydrate concentrations

Significant seasonal variations in NSCs in the cambial zone were observed (Figure 2). In general, the soluble fraction peaked between July and August, corresponding to the times of high rates of cell division and enlargement (Period 2) and when many cells were in the wall thickening phase (Period 3; Table 1). Total soluble NSC concentrations increased by >50% between the onset of the growing season and the period of maximum cell division. In September, during latewood cell wall thickening (Period 4), the soluble carbohydrate concentrations decreased, and increased again during the subsequent dormant season (Period 5). Larch and spruce displayed similar seasonal variations, with particularly high NSC concentrations during Periods 2 and 3, but the NSC concentrations in spruce peaked during the dormant season. S22L and N13L showed similar seasonal patterns.

The starch concentrations for all sites and species were high towards the end of the growing season (Periods 3 and 4) and decreased dramatically during dormancy. However, lower starch concentrations were measured at N13L during the early growing season (Period 1). On average, cambium sugars consist of around 40% glucose, 35% fructose, 10% starch, 10% pinitol, 5% sucrose and <1% raffinose (Figure 2). However, these proportions slightly vary in time, between species and elevations. Total NSC concentrations closely follow those of glucose and fructose together, showing similar patterns and accounting for up to nearly 80% of the growing season soluble NSCs (Figure 2) and leading to high hexose (glucose + fructose)-to-sucrose ratios (on average from 15 to 30 during the growing season). A fructose-to-glucose ratio of \( \sim 1 \) was also observed throughout the whole growing season.

Glucose, fructose and sucrose concentrations for spruce tend to be lower during the growing season and higher during the dormant season; however, fructose concentration was not found to be species dependent like the other sugars.

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### Table 2. Onset and duration of the growing season, and budburst for larch growing at the valley bottom and at the treeline (1300 m a.s.l. and 2200 m a.s.l., respectively) and for spruce growing at the valley bottom.

<table>
<thead>
<tr>
<th>Elevation (m)</th>
<th>Species</th>
<th>Onset of xylem production (DOY)</th>
<th>End of xylem differentiation (DOY)</th>
<th>Duration of xylogenesis (days)</th>
<th>Budburst (DOY)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1300</td>
<td><em>Picea</em></td>
<td>143 (0.0)</td>
<td>305 (6.9)</td>
<td>162 (6.9)</td>
<td>149 (3.5)</td>
</tr>
<tr>
<td>1300</td>
<td><em>Larix</em></td>
<td>138 (3.0)</td>
<td>311 (0.0)</td>
<td>173 (3.0)</td>
<td>135 (3.5)</td>
</tr>
<tr>
<td>2200</td>
<td><em>Larix</em></td>
<td>159 (3.5)</td>
<td>302 (6.0)</td>
<td>143 (7.9)</td>
<td>149 (3.5)</td>
</tr>
</tbody>
</table>

Numbers in parenthesis are the standard deviation of the mean (\( n = 4 \)).

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### Table 3. ANOVA (F- and \( P \)-values) of the onset, end and duration of xylem differentiation, and budburst, for differences between species and elevation.

<table>
<thead>
<tr>
<th>Source</th>
<th>Onset of xylem differentiation</th>
<th>End of xylem differentiation</th>
<th>Duration of xylogenesis</th>
<th>Budburst</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( F )</td>
<td>( P )-value</td>
<td>( F )</td>
<td>( P )-value</td>
</tr>
<tr>
<td>Species</td>
<td>13.44</td>
<td>*</td>
<td>3.00</td>
<td>ns</td>
</tr>
<tr>
<td>Elevation</td>
<td>85.00</td>
<td>***</td>
<td>9.00</td>
<td>*</td>
</tr>
</tbody>
</table>

ns, not significant.  
* \( P < 0.05 \).  
** \( P < 0.001 \).  
*** \( P < 0.0001 \).
Our intra-annual NSC measurements sampled directly in the stem cambial zone of mature trees growing in the subalpine zone allow us to improve understanding of carbohydrate variation during a complete annual cycle. The dynamics of the different mobile carbohydrates observed at both elevations and for the two species reflects the changing requirements for storage, mobilization and use of C resources needed to sustain growth as well as protecting vital tissue from harsh (e.g., winter freezing and summer drought) environmental conditions.

**The annual cycle**

In temperate regions with a distinct seasonal cycle and a dormant period for vegetation in winter, the onset of wood formation is usually temperature driven (Moser et al. 2010). The photoperiod may also provide secondary control for the growth onset, at least for the primary meristem (Chuine et al. 2010, Körner and Basler 2010). Wood formation is terminated in late fall when the chain of maturation processes is completed (Rossi et al. 2012). These notions of the annual cycle are broadly reflected in the cellular developmental stages (Figure 1) and concentrations of the different types of NSCs (Figure 3) for both larch and spruce.

The general paralleling of the NSC compounds in the cambium and the annual dynamics of wood formation have also been observed in the other few studies with comparable approaches, i.e., for poplars (Populus × canadensis Moench ‘I-214’ and Populus deltoides Marsh. ‘Divina’) (Deslauriers et al. 2009, Giovannelli et al. 2011), Scots pines (Pinus sylvestris L.) (Sundberg et al. 1993, Uggla et al. 2001) and eucalyptus (Eucalyptus regnans F. Muell.) (Stewart et al. 1973). Collectively, these studies suggest that the dynamics of NSC concentrations in the cambium of different species, habitats and angiosperm versus gymnosperm lineages follow a similar seasonal pattern.

During the growing season, NSCs sustain the metabolic processes involved in the formation of new cells within the cambium. We observed very high amounts of soluble sugars (primarily glucose and fructose, collectively named hexose) in the cambial zone of both larch and spruce. While lower at the beginning and the end of the growing season, soluble sugar concentration increased rapidly and peaked when the resource demand was highest, i.e., when a greater number of cells were in the enlargement and cell wall thickening phases. Such high concentrations of hexose are unusually high compared with what is normally observed in other tissues such as stem wood (Damesin and Lelarge 2003, Gruber et al. 2011, Streit et al. 2013). In our study of larch and spruce, we found similar concentrations of fructose and glucose within the cambial zones. Glucose-to-fructose ratios, approximately equal to unity, were similarly reported for Scots pine by Uggla et al. (2001), who also observed strongly decreasing sucrose concentration gradients (yet, strongly increasing glucose and fructose levels) from functional phloem to developing xylem. Relative to sucrose, high levels of hexose within the cambial zone are consistent with the high metabolic activity of the dividing and rapidly growing cells.

In addition to serving as the building blocks for growth itself, sugars play an important role as signaling molecules and/or as global regulators of gene expression (Koch 2004, Eveland and Jackson 2012). Glucose and fructose in spruce and larch probably originated from the cleavage of sucrose as suggested by

# Table 4. ANOVA (F- and P-values) for testing the differences in the carbohydrate compounds.

<table>
<thead>
<tr>
<th>Carbohydrates</th>
<th>Source</th>
<th>F</th>
<th>P-value</th>
<th>Source</th>
<th>F</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>S</td>
<td>5.85</td>
<td>ns</td>
<td>E</td>
<td>9.27</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>29.03</td>
<td>***</td>
<td>P</td>
<td>17.33</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>S*P</td>
<td>7.64</td>
<td>**</td>
<td>E*P</td>
<td>2.81</td>
<td>ns</td>
</tr>
<tr>
<td>Fructose</td>
<td>S</td>
<td>0.24</td>
<td>ns</td>
<td>E</td>
<td>0.04</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>4.25</td>
<td>*</td>
<td>P</td>
<td>3.57</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>S*P</td>
<td>2.57</td>
<td>ns</td>
<td>E*P</td>
<td>3.56</td>
<td>ns</td>
</tr>
<tr>
<td>Sucrose</td>
<td>S</td>
<td>2.76</td>
<td>ns</td>
<td>E</td>
<td>0.28</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>60.53</td>
<td>***</td>
<td>P</td>
<td>11.38</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>S*P</td>
<td>14.71</td>
<td>**</td>
<td>E*P</td>
<td>1.38</td>
<td>ns</td>
</tr>
<tr>
<td>Raffinose</td>
<td>S</td>
<td>100.76</td>
<td>**</td>
<td>E</td>
<td>0.24</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>412.71</td>
<td>***</td>
<td>P</td>
<td>59.73</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>S*P</td>
<td>100.76</td>
<td>**</td>
<td>E*P</td>
<td>0.24</td>
<td>ns</td>
</tr>
<tr>
<td>Pinitol</td>
<td>S</td>
<td>40.82</td>
<td>*</td>
<td>E</td>
<td>7.48</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>8.37</td>
<td>**</td>
<td>P</td>
<td>1.78</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>S*P</td>
<td>2.64</td>
<td>ns</td>
<td>E</td>
<td>2.9</td>
<td>ns</td>
</tr>
<tr>
<td>NSC total</td>
<td>S</td>
<td>4.39</td>
<td>ns</td>
<td>E</td>
<td>0.53</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>15.40</td>
<td>***</td>
<td>P</td>
<td>9.74</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>S*P</td>
<td>3.47</td>
<td>*</td>
<td>E*P</td>
<td>2.49</td>
<td>ns</td>
</tr>
<tr>
<td>Starch</td>
<td>S</td>
<td>2.11</td>
<td>ns</td>
<td>E</td>
<td>6.4</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>20.37</td>
<td>***</td>
<td>P</td>
<td>15.22</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>S*P</td>
<td>5.41</td>
<td>**</td>
<td>E*P</td>
<td>0.77</td>
<td>ns</td>
</tr>
</tbody>
</table>

Tests include differences between species (S), period (P) and their interaction (S*P); and for elevation (E), period (P) and their interaction (E * P). P-values for repeated-measures ANOVA are presented with Huynh–Feldt corrected probabilities. 

ns, not significant. 

*P < 0.05. 

**P < 0.001. 

***P < 0.0001.
high levels of sucrose-cleaving enzymes such as acid invertase (AI) and, to a lesser extent, sucrose synthase (Susy), in the cambial zone of Scots pine (Uggla et al. 2001). The relative ratios of hexose-to-sucrose concentrations are maintained by these various enzymes, which collectively coordinate and fine-tune growth during key phases of development (Koch 2004, Eveland and Jackson 2012). Hexose is regarded to have a greater signaling potential in promoting growth and cell proliferation, whereas sucrose is typically associated with differentiation and maturation (Koch 2004, Eveland and Jackson 2012). While we did not measure enzyme concentrations, we found that particularly the hexose-to-sucrose ratio in the cambium zone of spruce and larch generally decreased during the growing season, as the cambial phenology shifted from rapidly dividing cells peaking around DOY 161 (10 June 2010) to the wall-thickening phases peaking ~2 months later (Figure 1).

Starch concentration commonly shows a considerable seasonal variation in the stems and branches of temperate zone trees. Lower reserves at budburst, a late summer maximum as growth slows down and starch hydrolysis to sugar in autumn when days are short and nights are cold are all patterns previously reported (Kozlowski 1992, Gruber et al. 2011). Decreasing levels of starch during the summer in tree stem, branches and cambial zone have also been observed (Sundberg et al. 1993, Hoch et al. 2003, Deslauriers et al. 2009). In comparison with reports from poplar (Deslauriers et al. 2009) or Scots pine (Sundberg et al. 1993) cambial zones, we found that starch levels in the cambial zone of spruce and larch at all the three sites remained relatively constant during the growing season (Figure 3). Similarly, Geisler-Lee et al. (2006) found little expression of genes related to starch metabolism in comparison with carbohydrate-related enzymes (e.g., Susy and cellulose synthase) in the cambium of poplar during xylogenesis.

Large amounts of starch are consumed during cambial reactivation, with reserves replenished only sometime after the onset of xylem differentiation (Begum et al. 2013). The concentrations we observed tended to be lower than those reported for Scots pine (Sundberg et al. 1993) and higher than those reported for poplar (Deslauriers et al. 2009). Our first sampling campaign (DOY 151, N13; DOY 171, S22) was possibly too late to catch the minimum starch level as cambium reactivation, xylem differentiation and budburst had already occurred. During reactivation of the cambium, starch is used as the main source of energy, but later on, the continuation of cambial activity seems to require a continuous supply of sucrose (Oribe et al. 2003) for cell wall biosynthesis. The low variation in starch during the growing season suggests a constant supply of fresh assimilates to the cambium of larch and spruce at our sites.

For all of the tree groups investigated in our study, we found starch breakdown during the cold season, and synthesis from
soluble sugars in late winter-springtime (Figure 3). Our findings support previous observations of α-amylase activation and starch synthase genes in dormant poplar tissues during cold periods supporting starch breakdown for cryoprotection purposes (Geisler-Lee et al. 2006). Similarly, resynthesis of starch in late winter (Kozlowski 1992) was documented in needles (Hansen and Beck 1994, Hoch et al. 2003, Bansal and Germino 2009, Chen et al. 2012) and in the trunk (Fischer and Holl 1992, Hansen and Beck 1994, Hoch et al. 2003, Michelot et al. 2012) of various tree species. Our results demonstrate that these starch dynamics also apply to the cambial region.

Raffinose and pinitol are both compatible solutes (i.e., osmotically active compounds) that help cells survive osmotic stress (Bachmann et al. 1994, Bohnert and Shen 1998). While increased concentrations of raffinose and pinitol can decrease the osmotic potential of cells to maintain the water balance, their main function might be to stabilize proteins, protein complexes or membranes by scavenging radical oxygen species (ROS) that build up during environmental stress (e.g., cold, drought, high salinity) (Orthen et al. 1994, Bohnert and Shen 1998). Levels of raffinose increased during winter (Figure 3), thereby presumably protecting cell membranes from damage during frost-induced dehydration by detoxifying ROS that accumulates at low temperatures (Nishizawa et al. 2008). Pinitol was present year-round in both species; however, concentrations peaked in the cambial zone of spruce when growth processes were most active. Streit et al. (2013) observed higher pinitol concentrations in the branch bark and branch wood of larch growing at another tree line location in Switzerland in comparison with larch from lowland (500 m a.s.l.) and interpreted this difference in terms of long-term adaptation to high levels of ROS in response to low temperature. Similar pinitol concentrations to Streit et al. (2013) in larch growing both at the bottom of the valley (1300 m a.s.l.) and the tree line (2200 m a.s.l.) suggest that long-term adaptation responses to high levels of ROS already occur at 1300 m a.s.l., and that pinitol concentrations may not increase linearly with elevation. The conclusions for greater environmental stress towards the upper elevations and the bottom of the valley are similarly supported by analyses of the climatic sensitivity of radial growth variations along ~900-m elevational transect (King et al. 2013b).

Species and climatic controls on NSCs

The general trends in the dynamics of NSC concentrations in this study were similar between the species and elevations. Differences relate primarily to the absolute concentrations: the protective sugars raffinose (during dormancy) and pinitol (during the warmest period corresponding to July/August), as well as glucose, fructose and sucrose (during dormancy) differed in spruce and larch. The species-specific concentrations are potentially related to the differing climatic sensitivities of larch and spruce, also observed in an Europe-wide multi-species tree-ring network (Babst et al. 2013), to cold (in winter) and drought (in summer). Himesley et al. (1992) observed links between the raffinose content in the foliage of different conifers and their level of cold hardness. A similar relationship was also found for the presence of other NSCs (glucose, fructose, sucrose and raffinose) in the shoots of shrubs and in tree stems (Morin et al. 2007, Lee et al. 2012). Trees with thinner bark like spruce are more sensitive to frost damage in the cambium zone (Gurkaya and Shiyatov 2006) and might thus need a different strategy for additional protection. Higher levels of soluble NSCs in the cambial zone of spruce, in comparison with larch, in a period where the risk of freezing injuries is high would meet this requirement. The lower level of raffinose in particular, and total soluble NSCs in general, we observed in larch might also reflect a higher degree of spring de-hardening compared with spruce in early March (sampling period 5). This hypothesis is further supported by the earlier bud break and onset of xylem differentiation of larch.

During June and July, pinitol concentrations were found to be higher in spruce compared with larch (on average 1.5- to 2.5-fold) at the valley bottom, while glucose levels were lower. Glucose has been described as one of the precursors of pinitol synthesis (Obendorf et al. 2008) and, therefore, the lower levels of glucose relative to pinitol in spruce suggest that some glucose was directed towards pinitol synthesis. However, why the pinitol levels significantly increased in the cambial zone of spruce during the summer months, while remaining constant (and at similar levels) in larch at both the valley and tree line sites, is uncertain. These patterns might reflect a greater need for maintaining turgor potential (Aranjuelo et al. 2011) within the cambial zone of spruce to sustain cell enlargement, or may result from a higher sensitivity of spruce to environmental stress leading to enhanced generation of ROS (Orthen et al. 1994). A recent investigation showed that spruce growing at our study site exploit their internal stem water reserves more quickly during dry conditions compared with larch (King et al. 2013a).

While total NSC concentrations remained similar throughout the growing season in our study, higher concentrations of the soluble sugar fraction (except pinitol) during dormancy were observed in spruce compared with larch. While winter respiration of living tissue of the dormant deciduous trees depends exclusively on reserves (starch + soluble sugars), the higher levels in our evergreen species might reflect photosynthesis (Kozlowski 1992) and phloem transport (Blechschmidt-Schneider 1990) during mild winter days.

Despite a temperature difference of 3.5 °C and differences in the length of the growing season of 30 days between the treeline and valley bottom larch sites, we found similar seasonal variations in the NSC concentrations. This similarity probably has to do with our sampling keyed into expected cambial activity rather than only calendar dates. Notable differences between elevations occurred only for starch concentrations in
the early growing season (Period 1), although no statistically significant differences were found (Table 4). Starch concentrations were already near their maximum in early summer at the treeline, whereas at the valley bottom they only peaked at the beginning of July. Attributing these differences to particular mechanisms remains speculative. However, higher respiration losses due to warmer temperatures at the valley bottom in addition to active growth of competing sinks while needles are still not fully photosynthetically functional (Kozlowski 1992) would be consistent with our intra-seasonal NSC data.

Is secondary growth allocation C or sink limited?

Our data, although focused on one growing cycle and limited to only the cambial zone, contribute to a better understanding of the potential functions on NSC allocation and storage within trees. Notables for our investigation are the direct observations of NSC concentrations at the sites of secondary growth and the ability to link these changes with cambial zone phenology. The reduced growth (i.e., number of tracheids produced) observed for larch in comparison with spruce growing at the same site and for S2L compared with N13L cannot be explained by temporary limitation in NSC supply in the cambium. The high similarities in the NSC concentrations during the growing season of trees at different elevations, together with the delayed start of cambial activity and xylem cell production at the tree line, support the hypothesis of Körner (2003) that tree line trees are not C limited. Factors affecting carbohydrate conversion to new tissues, rather than carbohydrate availability, seem to control cambial activity (Kozlowski 1992, Sundberg et al. 1993). Similarly, Hoch et al. (2002) observed higher levels of mobile C pools in needles, branches, stems and roots of trees with increasing elevation, and concluded that sink activity of trees growing at high elevations was limited by low temperature rather than C availability. Similar conclusions were reached by Fajardo et al. (2013), who compared deciduous and evergreen treeline species across elevational gradients. Although no significant increase in NSC concentration in the cambium zone was observed with elevation in our study, we did observe a higher contribution of starch to the total NSC pool in larch at the tree line ($P = 0.03$, data not shown). Our data thus support growth limitation at the tree line in response to sink activity rather than the inability of trees to supply growing tissues with carbohydrates.

In conclusion, our first study of intra-seasonal NSC dynamics at the cambial level in subalpine forests indicates that carbohydrate fluctuations at the sink level are closely linked to cambial activity (C sink demand) and the metabolic needs associated with cell formation, rather than to species or climate (elevation). Variation in the concentration of NSC with more specific functions such as raffinose or pinitol seems to be, however, species dependent. Observations on larch growing across an elevational gradient of nearly 1000 m suggest that temperature limits the cambial activity of trees and not the availability of carbohydrates within the cambium.

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Conflict of interest

None declared.

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