

Responses of sapwood ray parenchyma and non-structural carbohydrates of *Pinus sylvestris* to drought and long-term irrigation

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Summary

1. Non-structural carbohydrates (NSC) play a crucial role in tree resistance and resilience to drought. Stem sapwood parenchyma is among the largest storage tissue for NSC in mature trees. However, there is a limited mechanistic understanding of how NSC reserves, stem parenchyma abundance and growth rates are interrelated, and how they respond to changing water availability.

2. We quantified NSC, ray parenchyma abundance and ring width along four successive 5-year radial sapwood segments of the stem of 40 mature *Pinus sylvestris* trees from a 10-year irrigation experiment conducted at a xeric site in Switzerland.

3. Percentage of ray volume (PERPAR) varied from 3.75 to 8.94% among trees, but showed low intra-individual variability. PERPAR responded positively to irrigation with a lag of several years, but was unrelated to %NSC. %NSC was lower in wider rings. However, wider rings still contained a larger NSC pool that was positively related to next year's ring growth.

4. Our results suggest that stem ray parenchyma does not limit NSC storage capacity, but responds to long-term environmental drivers with years of delay. The observed carbon allocation patterns indicate a prioritization of storage over growth independent of growth conditions, likely as a mechanism to ensure long-term survival. Furthermore, NSC pool size proved to be a determinant for the inter-annual autocorrelation in tree-ring growth. Our study highlights the importance of long-term multi-parameter studies to better understand tree responses to environmental variability at different time-scales.

Key-words: carbon allocation, drought stress, growth vs. storage, ring-width autocorrelation, structure–function relationships, tree-ring anatomy, xylem anatomy, xylem rays

Introduction

Ongoing global warming is enhancing stress across most of Earth's terrestrial biomes (IPCC 2013). Increases in the length, frequency, intensity and spatial extent of drought episodes are expected to reduce forest productivity and increase drought-induced tree mortality rates (Breda *et al.* 2006; Allen *et al.* 2010; Carnicer *et al.* 2011). Long-term

impacts include alterations in the global carbon cycling (Reichstein *et al.* 2013) and shifts in biomes and global biodiversity (Gonzalez *et al.* 2010; Mantyka-Pringle *et al.* 2015), with severe economic consequences (Hanewinkel *et al.* 2013). Understanding tree responses to drought is thus a very active field of plant science research (Lloret *et al.* 2013).

Two key processes play a major role in determining the processes of drought-induced stress and mortality: embolism-induced hydraulic failure and carbon starvation

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(McDowell *et al.* 2008). Yet, the current understanding of non-structural carbohydrate (NSC) mobilization during drought (Sala, Woodruff & Meinzer 2012), the recognized role of NSC in removing emboli from xylem conduits (Salleo *et al.* 2009; Johnson *et al.* 2012) and the assumed negative effect of water deficit on phloem transport (Hölttä, Mencuccini & Nikinmaa 2009; Sevanto 2014) points to the tight link between these two processes and their coupling with tree water and carbon balances (Hartmann 2015). Carbon starvation is postulated to occur during prolonged drought when long-term reduction of assimilation, typically in association with factors such as crown defoliation and mistletoe infestation, leading to NSC reserve depletion (Galiano, Martínez-Vilalta & Lloret 2011; Sala, Woodruff & Meinzer 2012; Adams *et al.* 2013; Jacquet *et al.* 2014; Yan *et al.* 2016). However, there are counterexamples showing no reduction or even increases in stored NSCs during drought (Sala & Hoch 2009; Muller *et al.* 2011; Anderegg & Anderegg 2013). Such discrepancies reveal the large variability of possible plant responses and the still modest understanding of the roles of NSCs for coping with water deficit (McDowell *et al.* 2011; Sala, Woodruff & Meinzer 2012; Mencuccini 2014). Furthermore, the allocation of carbon to NSC reserves competes with structural growth (Muller *et al.* 2011), posing interesting questions about carbon allocation priority during periods of limited carbon uptake (Sala, Woodruff & Meinzer 2012; Hartmann & Trumbore 2016). For example, the availability of older NSC pools to support future growth would alleviate direct allocation trade-offs between NSC storage and structural carbon (Richardson *et al.* 2015).

NSC concentrations are generally higher in actively growing tissues such as leaves, fine roots and the phloem (Hoch, Richter & Körner 2003; Pruyn, Gartner & Harmon 2005; Gruber, Pirkebner & Oberhuber 2013). Yet, the stem sapwood – owing to the large biomass – is the most important NSC storage organ for mature temperate trees, where it accounts for 25–41% of whole-tree NSC reserves (Jacquet *et al.* 2014). NSC concentrations are not evenly distributed in the sapwood, but typically show a decrease from the outer to the inner sapwood (Saranpää & Höll 1989; Hoch, Richter & Körner 2003; but see Würth *et al.* 2005 for other patterns). In stem sapwood, NSCs are exclusively stored in parenchyma cells, which in conifers are almost entirely confined to the rays (Plavcová & Jansen 2015), a specialized storage and transport tissue which radially integrates the sapwood with the cambium and phloem (Spicer 2014; Pfautsch, Hölttä & Mencuccini 2015). The activity of rays decreases from the outer to the inner sapwood and eventually completely stops in the heartwood as parenchyma cells die (Gartner, Baker & Spicer 2000). Rays are also important for providing carbohydrates and water to refill embolized conduits (Salleo *et al.* 2009; Brodersen & McElrone 2013; Spicer 2014), the formation of heartwood (Bamber 1976), the defence against pathogens (Hudgins *et al.* 2006), wounding responses (Arbellay, Stoffel & Bollschweiler 2010;

Arbellay, Fonti & Stoffel 2012) and contributing to the mechanical strength of the wood (Burgert & Eckstein 2001; Fonti & Frey 2002).

Despite the crucial role that rays play in tree functioning, our knowledge about the factors that influence ray abundance is scarce. Based on the importance of rays for NSC storage, it could be hypothesized that variations in ray volume are closely linked to the changes in NSC storage requirements (Myer 1922; von Arx, Archer & Hughes 2012), which could be referred to as ‘storage space constraint’ hypothesis. If evidence supporting this hypothesis were found, i.e. a close relationship between percentage of NSC and the relative abundance of ray volume in the wood, time series of ray volume derived from quantitative wood anatomical analysis of tree-rings would allow the reconstruction of past NSC pools. This hypothesis is further supported by several studies suggesting that higher ray abundance could be connected to greater tree vigour and/or higher growth rate (see von Arx *et al.* 2015 for a review). Furthermore, ray abundance has been shown to depend on environmental influences including climate variability (Olano *et al.* 2013), site productivity (Fonti *et al.* 2015), flooding (Arbellay, Fonti & Stoffel 2012), fire injury (Arbellay *et al.* 2014) and water availability (von Arx, Archer & Hughes 2012; Esteban *et al.* 2012).

In this study, we investigated the impact of a decade-long irrigation on the abundance of ray parenchyma and the NSC reserves in mature *Pinus sylvestris* L. (Scots pine) trees growing at a xeric site in Switzerland. *Pinus sylvestris* closes stomata at relatively high xylem water potential (‘isohydric’; Irvine *et al.* 1998; Zweifel, Steppe & Sterck 2007) and therefore is a good candidate to address questions of carbon mobilization and depletion in response to drought (see Saranpää & Höll 1989; Galiano, Martínez-Vilalta & Lloret 2011; Zweifel *et al.* 2012; Poyatos *et al.* 2013). Our investigation was performed within the framework provided by the Pfynwald long-term irrigation experiment, where previous studies have reported increases in tree-ring width (Eilmann *et al.* 2011), shoot and needle length (Dobbertin *et al.* 2010), crown density (Eilmann, Dobbertin & Rigling 2013) and fine root growth and turnover rates (Brunner *et al.* 2009; Herzog *et al.* 2014). We measured NSC reserves and rays in 5-year blocks of tree-rings in the stem sapwood of 40 mature trees. Our aim was to quantify the relationship among ray abundance, NSC storage and growth, and to better understand how these relationships are affected by changes in water regime. In detail, we asked: (i) How do NSC levels and percentage of ray volume (PERPAR) respond to alleviation from long-term drought stress? (ii) How are NSC and PERPAR distributed along the stem sapwood? (iii) Are NSC levels related to PERPAR, i.e. is NSC storage constrained by PERPAR (‘storage space constraint’ hypothesis)? (iv) Does NSC availability affect secondary growth rates and does this relationship differ between irrigated and control trees?

Materials and methods

EXPERIMENTAL SETUP AND SITE CONDITIONS

The study was performed at the Pfywald long-term irrigation experiment, located in the driest part of the Swiss Rhone valley (46°18'N, 7°36'E, 615 m a.s.l.). The site is part of a xeric Scots pine (*P. sylvestris*) forest with some interspersed pubescent oak (*Quercus pubescens* Willd.) trees in the understorey. Stand density is 730 stems per hectare with a basal area of 27.3 m² ha⁻¹ and a homogeneous tree top height of about 11 m. The climate is continental with a mean annual temperature of 10.1 °C and mean annual precipitation of 603 mm (MeteoSwiss, Zürich, Switzerland; climate data for 1981–2010 from Sion, at 20-km distance from site). The soil is a skeletal pararendzina, with low water holding capacity. The experimental site consists of eight 25 × 40 m rectangular plots arranged side by side and separated by a 5-m buffer strip. Since 2003, four of the plots have been irrigated with sprinklers at night during the growing season (April to October). This irrigation corresponds to a supplementary rainfall of about 700 mm per year. The beginning of the irrigation treatment coincided with the extreme heat wave in summer 2003 (Schaer *et al.* 2004). Climate during the irrigated growing seasons (2003–2012) was on average warmer (+0.80 °C) and drier (–51 mm) compared to the pre-treatment period (1993–2002, Sion, MeteoSwiss). For each tree, the level of mistletoe infestation on a four-step scale and the percentage of crown defoliation (Dobbertin, Hug & Mizoue 2004) have been annually monitored.

SAMPLING AND DATA COLLECTION

Twenty mature pine trees per treatment (40 trees total) of similar age and size were randomly selected while controlling for equal numbers per plot and similar average degree of mistletoe infestation (Table 1). Thus, anomalously vital or suffering trees based on crown defoliation data were not considered to avoid results that might only apply to extreme situations. Tissue for NSC measurement was sampled from all trees by taking four 5-mm stem cores at breast height from the north, east, south and west side of each

Table 1. Characterization of the sampled *Pinus sylvestris* trees from the control and irrigated plots at the Pfywald experimental site (means ± SE; bold face, significant differences between treatments based on *t*-test, *P* ≤ 0.05)

Treatment	Control	Irrigation
Number of trees	20	20
Tree age (years)	83.3 ± 7.0	95.2 ± 3.5
Stem diameter (DBH) 2003 (cm)	19.3 ± 1.3	22.0 ± 1.1
Crown defoliation 2003–2012 (%)	28.8 ± 1.6	28.9 ± 2.6
Mistletoe infestation level 2003–2012 (levels: 0–3)	1.48 ± 0.21	1.71 ± 0.21
Number of sapwood rings	51.8 ± 3.7	57.2 ± 2.5
Sapwood area (cm ²)	187.9 ± 22.7	258.4 ± 27.3
Mean tree-ring width 1993–2002 (mm)	1.08 ± 0.12	0.78 ± 0.12
Mean tree-ring width 2003–2012 (mm)	0.71 ± 0.09	1.22 ± 0.13
NSC concentration in all sapwood (%)	2.83 ± 0.07	2.87 ± 0.05
NSC pool size in all sapwood	5.30 ± 0.66	7.42 ± 0.82
% Ray surface 1993–2002	6.01 ± 0.25	5.99 ± 0.25
% Ray surface 2003–2012	5.79 ± 0.27	5.92 ± 0.24

tree on 5 March 2013, i.e. before the onset of bud break to avoid influences by differences in phenological stage (Palacio *et al.* 2008). In any case, sampling date is not assumed to influence the outcome of the study as NSC pools in the stem sapwood of Scots pine has been shown to be comparably stable throughout the year (Fischer & Höll 1992; Hoch, Richter & Körner 2003; Oberhuber *et al.* 2011). Cores were bagged and put on dry ice in a cooler immediately after sampling. To eliminate enzymatic activity, after returning to the laboratory, cores were microwaved at 600 W for >90 s (Popp *et al.* 1996) and subsequently air-dried in an oven at 65 °C for 3 days. In May 2013, one 10-mm increment core was extracted adjacent to the NSC samples to quantify ring width and ray abundance. After the 2013 growing season (December), one additional micro-core was collected per tree to quantify ring width of 2013. A single core for growth analysis was assumed sufficiently representative due to the concentric ring growth in this flat stand. Ring widths were measured using a LinTab measuring system (Rinntech, Heidelberg, Germany). The exact calendar years for all individual rings were assigned via tree-ring crossdating and subsequently verified using COFECHA (Grissino-Mayer 2001).

RAY QUANTIFICATION

Rays were quantified in high-resolution images of anatomical samples from all 40 cores. Therefore, cross-sections of <15-µm thickness perpendicular to the axially oriented tracheids were cut from the 10-mm cores with a WSL Lab-microtome (Gärtner, Lucchinetti & Schweingruber 2015), placed on a slide, and stained with Alcian blue (1% solution in acetic acid) and safranin (1% solution in ethanol). This staining procedure results in blue unlignified (parenchyma) and red lignified (tracheid) cells. Afterwards, the cross-sections were dehydrated using a series of ethanol solutions of increasing concentrations, washed with xylol, and then permanently preserved by embedding them into Eukitt glue (Kindler GmbH, Freiburg, Germany; Gärtner & Schweingruber 2013). Overlapping images covering the entire samples were captured with a Nikon D90 digital camera mounted on a Nikon Eclipse 50i optical microscope with 100× magnification and merged to a single image using PTGUI v8.3.10 Pro (New House Internet Services B.V., Rotterdam, the Netherlands; von Arx *et al.* 2016). All rays of the outermost 20 years (comprising 10 years before and 10 after the onset of irrigation) were then quantified in the merged images as described previously (von Arx *et al.* 2015). In short, rays and the annual ring borders were manually outlined using a tailored clone of ROXAS v1.6 (von Arx & Dietz 2005; von Arx & Carrer 2014), a specialized image analysis tool based on Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA). The final sample width ranged from 7 to 9 mm, resulting in mean PERPAR values being within ±5–6% (rel. 95% confidence interval) of the true values (von Arx *et al.* 2015). The automatic output included the percentage of ray surface (PERPAR) for each year, which is a good estimate of the percentage of ray volume (von Arx *et al.* 2015).

NSC MEASUREMENTS

Increment cores designated for NSC measurements were cut in 5-year segments starting from the bark for the outer 20 years (resulting in two pre- and two post-treatment segments), and a fifth segment including all remaining sapwood rings (between 7 and 57 rings). Five-year segments were chosen to provide sufficient wood material for reliable NSC measurements, because of the generally small growth rates. The width of the sapwood segments from outer to inner sapwood were (mean ± SE) 3.6 ± 0.4, 3.4 ± 0.5, 5.1 ± 0.6, 5.7 ± 0.6 and 28.7 ± 2.8 mm in control trees, and 6.1 ± 0.6, 6.1 ± 0.8, 3.3 ± 0.5, 4.5 ± 0.7 and 31.3 ± 2.6 mm in irrigated trees. Corresponding segments from different cores of the

same tree were then pooled and milled. Extraction of NSC followed the anthrone technique (Olano, Menges & Martinez 2006). This technique provides an estimate of NSC content per wood dry weight (%NSC), distinguishing between the contribution of soluble mono- and oligosaccharides and non-soluble carbohydrates (starch). % NSC was calculated for each tree and radial segment by adding soluble and insoluble carbohydrate contribution. A proxy for total NSC content or pool size (TNC) per sapwood segment was calculated by multiplying the basal area of each 1-cm wide sapwood segment by %NSC of the corresponding sapwood segment. TNC measurement units are thus $\text{g g}^{-1} \text{cm}^2$, which could be converted into g by multiplying by wood density and considering segment height. We use it here without units for the sake of simplicity.

STATISTICAL ANALYSIS

We established a linear mixed-effect model to assess the irrigation effect on PERPAR in the outermost sapwood segment (2008–2012). We only evaluated this segment because we considered it unaffected by any transition effects immediately after irrigation started. The fixed term included treatment, %NSC, mean ring width of the outermost segment (as a proxy for tree growth rate), stem diameter (a surrogate for tree size), mistletoe infestation level, percentage of crown defoliation (both as indicators for tree health status), and the interactions of treatment with %NSC, mean ring width and stem diameter. Plot identity was considered as a random effect to account for pseudo-replication on plot level (Appendix S1).

The factors determining PERPAR variability at annual scale were tested with a linear mixed-effect model. The fixed term included treatment and period (before vs. after onset of treatment) and its interactions as well as annual tree-ring width (as a proxy for tree growth rate) and stem diameter in 2012 (as a surrogate for tree size). The considered random effect terms were plot and tree identity, the latter to account for multiple measurements per tree. The initial model also included a first-order autocorrelation structure to consider year-to-year dependence of PERPAR within individual trees (Appendix S1). Furthermore, as a measure for the inter-annual variability and thus sensitivity of PERPAR within individuals, we calculated the 'mean sensitivity', i.e. the mean relative change of PERPAR from year to year (Cook & Pederson 2011); and as a measure for the consistency of yearly variability in PERPAR among individuals, we calculated the mean Pearson's correlation between the individual series (r_{bt} ; Cook & Pederson 2011).

We performed a linear mixed-effect model to test the NSC 'storage space constraint' hypothesis and the potential drought-release response on the outermost sapwood segment (2008–2012). The rationale behind this approach was that NSC mobilization and depletion in the outer sapwood were expected to be larger than in the inner segments (Richardson *et al.* 2013). The fixed term included PERPAR, treatment, mean ring width of the outermost segment, stem diameter, mistletoe infestation level, percentage of crown defoliation, and the interactions of treatment with PERPAR, mean ring width and stem diameter. Plot identity was considered as a random effect (Appendix S1).

The radial distribution of %NSC (total, soluble, insoluble) at the sampling date was analysed with linear mixed-effect models. The fixed terms included segment position with five levels and treatment. The random terms included plot and tree identity. No autocorrelation structure was considered in this analysis because NSC is mobile (Appendix S1).

We followed the same two-step approach to select the best model for all linear mixed-effect models following Zuur *et al.* (2009). First, we compared different random terms to choose the random component that best fitted the data. The fixed part of the mixed-effect model was then selected by sequentially removing

non-significant terms using dredging methods. All possible reduced models fitted by maximum likelihood were then compared based on AICc to obtain the 'optimal' model. The optimal model was refitted by the restricted maximum likelihood method to obtain estimates and significances of effects. When several models showed $\Delta\text{AICc} \leq 2$, the simplest model was chosen for the sake of parsimony (see Appendix S1 for details on model selection). Homoscedasticity and normality of residuals were verified graphically.

Finally, we analysed how a high vs. low level of TNC stored in the outermost segment (2008–2012) affected tree-ring growth of the following year (2013). In this analysis, it was necessary to account for individual tree growth trends, and for the strong collinearity between TNC and mean tree-ring width ($r = 0.865$). Individual tree growth trends were removed by dividing the ring width of 2013 by the mean ring width of the outermost segment (2008–2012). To account for the collinearity between TNC and tree-ring width, we performed the classification into low and high TNC level separately for ring width classes of 0.25 mm on the basis of the respective median TNC value. The class size of 0.25 mm was chosen to balance a high spatial resolution with a robust classification of TNC level. We then compared the obtained relative growth rate between the two TNC levels using Student's *t*-test.

Statistical analyses were performed in R version 3.1.3 (R Development Core Team 2015) including the packages 'NLME' (Pinheiro *et al.* 2016) and 'MuMIn' (Barton 2016). Details about model selection are available in Appendix S1 in Supporting Information.

Results

Trees from both treatments did not differ in age, stem diameter and number of sapwood rings (Table 1). Tree-ring width was larger in the irrigated than the control trees in the irrigation period (2003–2012; *t*-test, $t = 3.21$, $P = 0.003$), while this relationship tended to be inversed for the pre-irrigation period (1993–2002, $t = 1.82$, $P = 0.077$). This change was related to a positive treatment response in irrigated trees ($t = 2.49$, $P = 0.017$), whereas growth in control trees decreased during the treatment period ($t = 2.55$, $P = 0.015$; Fig. 1a). Furthermore, irrigated trees contained larger TNC at the end of the treatment ($t = 2.11$, $P = 0.042$; Table 1).

DETERMINANTS OF VARIABILITY IN PERPAR ACROSS THE SAPWOOD

In absolute terms, ray abundance increased strongly with TNC in the outermost 5-year segment (2008–2012; Fig. 2a) due to the collinearity of both variables with ring width. However, the optimal linear model for the percentage of ray surface (PERPAR) in the outermost segment did not reveal any significant relationships with treatment, %NSC, ring width, stem diameter, mistletoe infestation level or percentage of crown defoliation (intercept \pm SE: 5.796 ± 0.196 , $t = 29.590$, $P < 0.001$; Figs 2b and 3a). Nevertheless, at an annual time-scale, PERPAR significantly increased in irrigated trees during the treatment period (2003–2012) relative to the control trees (Fig. 1b, Table 2). This treatment response occurred with a temporal lag of several years (Fig. 4b). Annual PERPAR was also strongly negatively

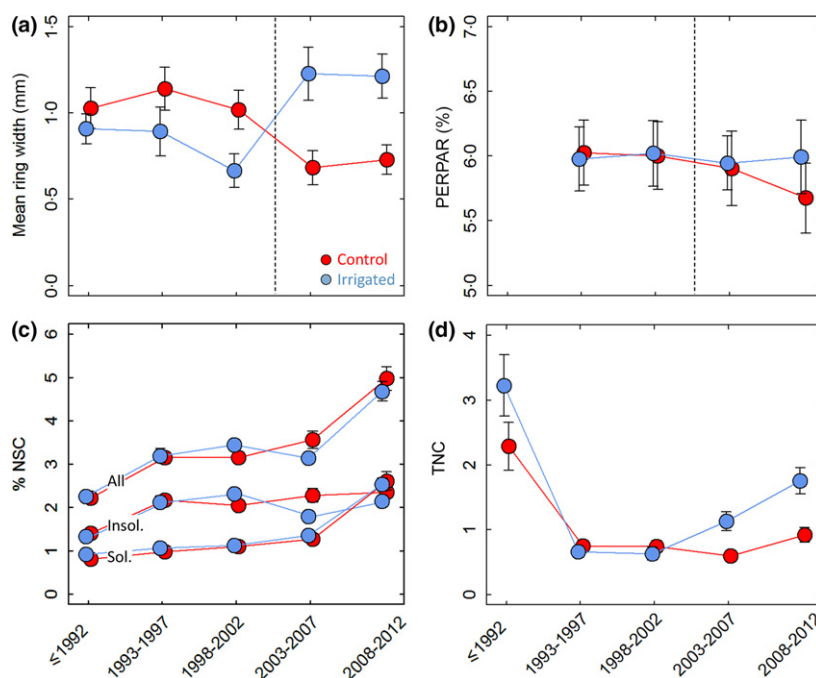


Fig. 1. Mean tree-ring width (a), percentage of ray surface (PERPAR) (b), non-structural carbohydrate (NSC) concentration (%NSC) (c), and NSC pool size (TNC) (d) in the stem sapwood of *Pinus sylvestris* growing at the Pfywald irrigation experiment (Salgesch, Switzerland) in radial 5-year segments from 1993 to 2012 and all remaining sapwood (≤ 1992 ; control: 31.8 ± 3.7 years, irrigated: 37.2 ± 2.5 years). The dashed vertical lines indicated the onset of the irrigation treatment (2003). Values are means of 20 individuals per treatment ± 1 SE. Check Table 3 for significance of segment differences.

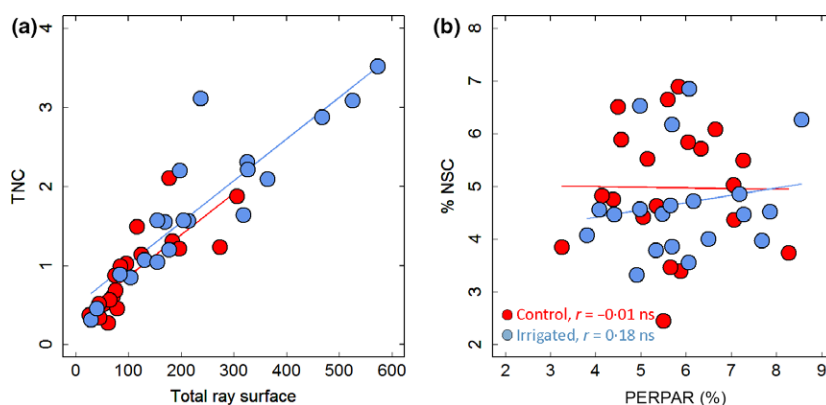


Fig. 2. Relationship between non-structural carbohydrate (NSC) levels and ray abundance in the outmost sapwood (2008–2012) of *Pinus sylvestris* growing at the Pfywald irrigation experiment (Salgesch, Switzerland). (a) NSC pool size (TNC) vs. total ray surface, (b) NSC concentration (%NSC) vs. percentage of ray surface (PERPAR). Each symbol represents data from one tree ($n = 20$ for each treatment). r , Pearson's correlation coefficient. No correlation coefficient reported for TNC vs. total ray surface because both are based on basal segment area.

related to ring width (Table 2). Interestingly, in control trees, this negative relationship between PERPAR and ring width was superimposed by a concomitant decrease in both variables during the treatment period, i.e. the short- (annual) and long-term (decadal) responses showed opposing patterns (Table 2, Fig. 4b). The variability in PERPAR from year to year was not significantly correlated between the control and irrigated trees (Pearson's $r = 0.37$, $P = 0.104$) when considering the entire study period (1993–2012), but it significantly co-varied during the pre-treatment period (1993–2002; Pearson's $r = 0.64$, $P = 0.045$; Fig. 4b) when all trees experienced the same conditions. Despite these significant relationships, it has to be noted that the average relative change of PERPAR from 1 year to the next was rather low with a mean sensitivity of only 0.11 in both treatments, and the consistency among individuals was also low as reflected by the mean correlation between annual time series (r_{bt}) of only 0.052 for control and 0.027 for irrigated trees.

The small high-frequency variability within individuals (mean sensitivity) contrasted with the large differences among individuals: PERPAR averaged over the 1993–2012 rings varied from 3.75 to 8.94%, with a mean \pm SD of $5.93 \pm 1.07\%$.

DETERMINANTS OF VARIABILITY IN NSC ACROSS THE SAPWOOD

The optimal linear model for %NSC in the outermost five tree-rings only included a negative effect of tree-ring width (estimate \pm SE: -0.876 ± 0.302 , $t = -2.904$, $P = 0.006$), but no significant influence by treatment, DBH, mistletoe infestation and crown transparency. The slope of this relationship thus translates into a decline in %NSC of nearly 0.9% for each millimetre of tree-ring width (Fig. 3b). In contrast, TNC significantly increased with tree-ring width (Fig. 3c), which is clearly due to the dependency of TNC from basal segment area.

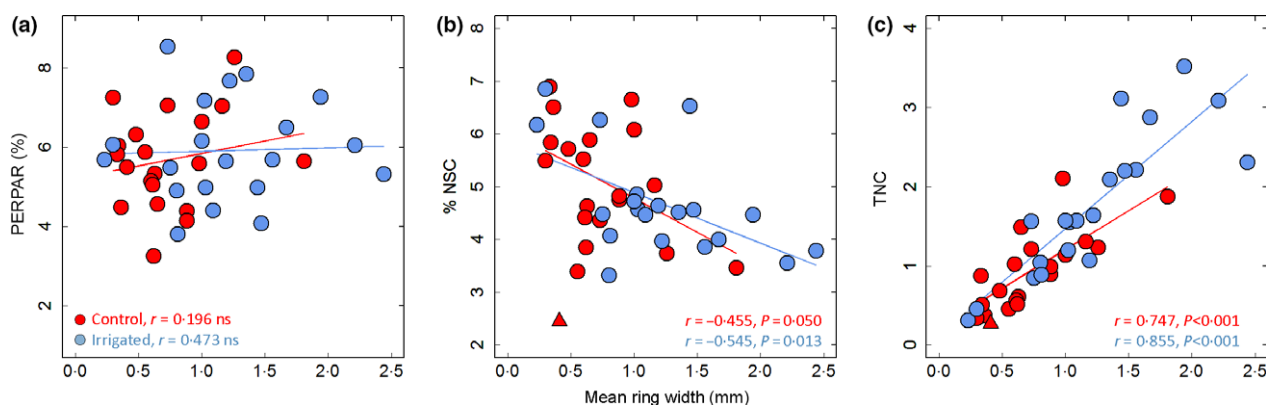


Fig. 3. Relationships between percentage of ray surface (PERPAR; a) NSC concentration (%NSC; b) and NSC pool size (TNC; c) with mean tree-ring width in the outmost sapwood (2008–2012) of *Pinus sylvestris* growing at the Pfynwald irrigation experiment (Salgesch, Switzerland). Each symbol represents data from one tree ($n = 20$ for each treatment). The triangle symbol in (b, c) indicates an outlier tree that was excluded from analyses because it showed abnormally lower %NSC in the outer compared to the middle segments. r , Pearson's correlation coefficient.

Table 2. Results of the linear mixed-effect model to explain the annual percentage of ray surface (PERPAR) in the period 1993–2012 for *Pinus sylvestris* trees growing at the Pfynwald experimental site

Fixed effects	Estimates	SE	P	Random	SD	Phi
Intercept	0.811	0.020	<0.001	Tree ID	0.083	0.471
Treatment (irrigation)	−0.012	0.027	0.653			
Period (2003–2012)	−0.033	0.008	<0.001			
Ring width	−0.041	0.006	<0.001			
Treat. \times Period	0.046	0.012	<0.001			

PERPAR log-transformed to meet normality criteria.

SE, standard error of fixed effect estimates; SD, standard deviation of random factor; Phi, autocorrelation coefficient.

The optimal linear mixed-effect model revealed a decrease in %NSC from the outmost (2008–2012) to the innermost (<1993) sapwood segment independent from treatment (Fig. 1c). Higher %NSC levels in the outer sapwood segment were mainly related to a drop in the soluble NSC fraction, whereas the low values in the innermost sapwood reflected a drop in insoluble NSC (Fig. 1c, Table 3).

In absolute terms, the innermost sapwood segment had the largest contribution (43%) to TNC in both control (mean \pm SE: $42.4 \pm 6.8\%$) and irrigated trees ($43.5 \pm 6.3\%$) (Fig. 1d). This is primarily a consequence of the greater number of tree-rings and the associated large basal segment area, despite the lower %NSC. The contribution to TNC by the four outer sapwood segments was rather homogeneous in the control trees (average in three middle segments: $13.3 \pm 1.0\%$, outmost segment: $17.7 \pm 2.2\%$), but was relatively larger in the outer sapwood in irrigated trees (average in three middle segments: $10.9 \pm 0.9\%$, outmost segment: $23.7 \pm 2.7\%$), mainly because of the increase in ring width (Fig. 1a).

INFLUENCE OF NSC POOL SIZE ON GROWTH

TNC in the annual rings of 2008–2012 (sampled prior to the growing season in early March 2013) was strongly related to tree-ring width in the next growing season

(sampled in December 2013; Fig. 5a). When relative changes in tree-ring growth vs. TNC were considered, tree-rings formed in 2013 were significantly wider when TNC pool size in the previous five annual rings of 2008–2012 was larger ($t = 2.608$, $P = 0.014$; Fig. 5b).

Discussion

RAY ABUNDANCE RESPONDS WITH DELAY TO MOISTER CONDITIONS, BUT IS UNRELATED TO %NSC

The lack of a pronounced treatment effect on the mean percentage of ray surface (PERPAR) contrasts with the strong response of other morphological and anatomical tree traits such as tree-ring growth (Fig. 4a), shoot and needle length (Dobbertin *et al.* 2010; Feichtinger *et al.* 2015), needle density (Feichtinger *et al.* 2015), fine root growth (Brunner *et al.* 2009; Herzog *et al.* 2014), resin duct production (Rigling *et al.* 2003), cell lumen diameter and cell wall thickness (Eilmann *et al.* 2009) to irrigation in different experimental sites in the study region. In contrast, PERPAR seemed to scale mostly allometrically with other tissues to maintain a certain level of integration within the xylem (Fonti *et al.* 2015). Yet, in the control trees, PERPAR declined in the last years of the experiment, simultaneously with a decrease in tree-ring width,

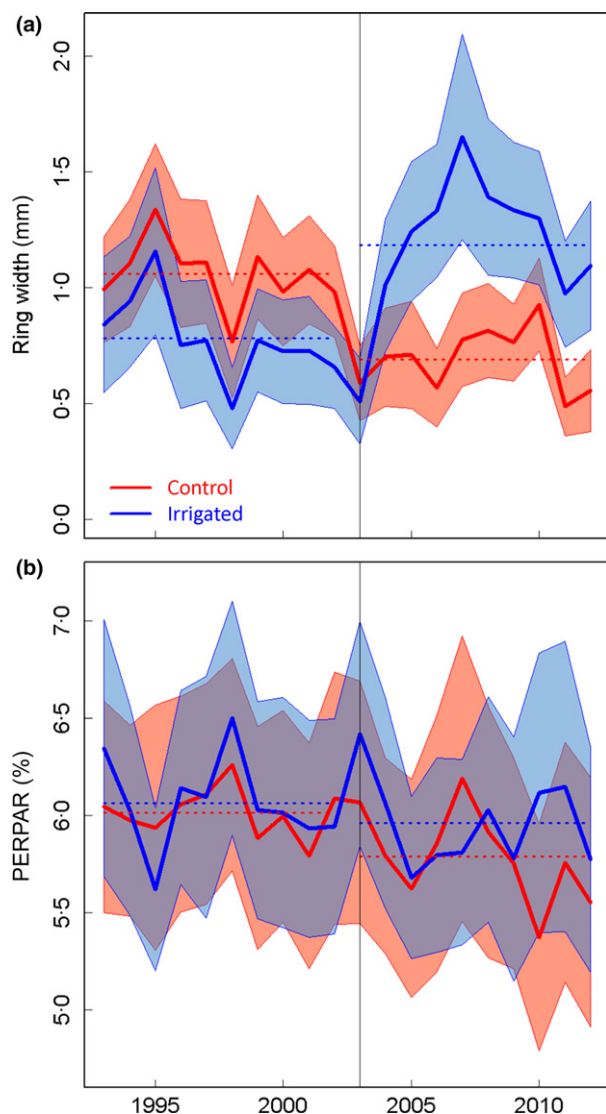


Fig. 4. Chronologies of ring width (a) and percentage of ray parenchyma (PERPAR; b) $\pm 95\%$ confidence intervals for the 10 years before (1993–2002) and the 10 years after (2003–2012) the onset of irrigation for *Pinus sylvestris* growing at the Pfynwald irrigation experiment (Salgesch, Switzerland). Means are based on 20 individuals for each treatment. Note the truncated y-scale in (b). Vertical lines separate the pre- from the post-irrigation period. Horizontal lines give mean values for each treatment and period. Ring width showed a significant decrease (control) and increase (irrigated) from the pre- to the post-treatment period (*t*-test, $P \leq 0.001$), whereas PERPAR decreased significantly only in the control trees (*t*-test, $P = 0.020$).

while it remained unchanged, and thus relatively increased, in the irrigated trees (Table 2, Figs 1b and 4b). This lagged response of PERPAR in the control trees to naturally warmer and dryer conditions possibly initiated by the extreme year of 2003 could indicate that the mean level of PERPAR might adjust to changes in general growth conditions only at the mid-term, possibly along with changes in tree vitality (Gartner, Baker & Spicer 2000). Furthermore, and in contrast to the multi-year time-scale, PERPAR and tree-ring width were negatively related on an annual time-

scale (Table 2). This may indicate different determinants depending on the time-scale, i.e. contrasting short-term responses to weather conditions driving PERPAR and ring width, and similar long-term responses related to conditions improving tree vigour and thus both PERPAR and ring width (see also Fonti *et al.* 2009).

Our results do not support the hypothesis that the need for NSC storage space determines the relative amount of ray parenchyma in the stem ('storage space constraint' hypothesis). This outcome was unexpected considering (i) the importance of ray parenchyma for NSC storage, (ii) the fact that species with higher %NSC in their stem sapwood such as the angiosperms (Hoch, Richter & Körner 2003) also show a greater PERPAR (Spicer 2014), and (iii) the observation from other studies that more vital trees usually have a greater PERPAR (Gartner, Baker & Spicer 2000) and, likely, also a greater NSC pool (Carbone *et al.* 2013). In contrast, our findings suggest for Scots pine that NSC storage space in the stem is usually not limited, and that PERPAR in the stem might indicate, at best, the maximum potential NSC storage capacity (von Arx, Archer & Hughes 2012; Plavcová & Jansen 2015).

More generally, we were not able to explain the considerable variability in mean PERPAR among individuals. However, considering it was not related to tree size, growth rates, crown condition, mistletoe infestation and %NSC, we speculate that genetic differences among individuals may be responsible for differences in PERPAR among individuals, as already observed in other anatomical traits (Fichot *et al.* 2009). The rather low year-to-year variability within individuals might reflect the fact that once initiated, rays grow and extend to keep the connection with the cambium and phloem (Fischer & Höll 1992; Spicer 2014). The only way to control PERPAR from one year to the next is therefore by changing ray height and width and/or the initiation rate of new rays (von Arx *et al.* 2015). However, the presence of a common inter-annual variability between irrigated and control trees before treatment, but not after the onset of irrigation, is in line with the high-frequency response of ray parenchyma to environmental factors found by Olano *et al.* (2013).

RADIAL NSC STORAGE PATTERNS IN SAPWOOD INDICATE A GRADIENT IN RAY ACTIVITY AND USE OF NSC

In line with previous studies in conifers and angiosperms (Saranpää & Höll 1989; Hoch, Richter & Körner 2003; Pruyn, Gartner & Harmon 2005), %NSC decreased from the outer to inner sapwood (Fig. 1a). This pattern may reflect different activities of the sapwood (Pruyn, Gartner & Harmon 2002) and therefore different requirements for NSC. In the outer sapwood, NSC requirements are highest for respiration and growth (Carbone *et al.* 2013), and probably also for other demands including maintaining hydraulic integrity (Sala, Woodruff & Meinzer 2012;

Table 3. Results of the linear mixed-effect models explaining NSC concentration (%NSC; overall, soluble, insoluble) in the sapwood of *Pinus sylvestris* trees growing at the Pfywald experimental site

Response	Fixed effects	Levels	Estimates	SE	P	Random	SD
%NSC	Intercept	–	0.347	0.013	<0.001	Tree ID	0.031
	Segment	Segment $\leq 1992^a$	0	0.013	<0.001		
		1993–1997 ^b	0.147	0.017			
		1998–2002 ^b	0.168	0.017			
		2003–2007 ^b	0.171	0.017			
		2008–2012 ^c	0.326	0.017			
%NSC soluble	Intercept	–	–0.071	0.018	<0.001	Tree ID	0.047
	Segment	Segment $\leq 1992^a$	0	0.018	<0.001		
		1993–1997 ^b	0.071	0.023			
		1998–2002 ^b	0.113	0.023			
		2003–2007 ^c	0.176	0.023			
		2008–2012 ^d	0.455	0.023			
%NSC insoluble	Intercept	–	0.130	0.016	<0.001	–	–
	Segment	Segment $\leq 1992^a$	0	0.016	<0.001		
		1993–1997 ^{bc}	0.189	0.023			
		1998–2002 ^{bc}	0.202	0.023			
		2003–2007 ^b	0.165	0.023			
		2008–2012 ^c	0.213	0.023			

All response variables log-transformed to meet normality criteria. Different superscript letters for segment estimates indicate significant differences at $P < 0.05$.

SE, standard error of fixed effect estimates; SD, standard deviation of random factor.

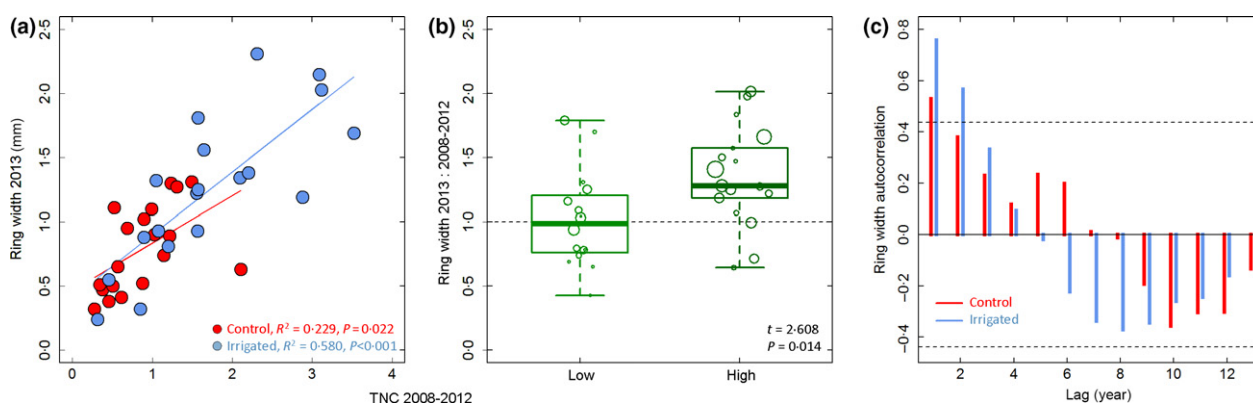


Fig. 5. Relationship of tree-ring width 2013 to total NSC content (TNC) as quantified in the previous year in the outermost sapwood rings (2008–2012). (a) Tree-ring width 2013 vs. TNC 2008–2012, (b) ratio of tree-ring width 2013 : 2008–2012 for low and high TNC 2008–2012 (see Materials and methods for details to the low vs. high TNC classification); the size of the symbols reflect tree-ring width in 2013, the dashed horizontal reference line indicates identical tree-ring width for 2013 and 2008–2012. Each symbol represents data from one tree ($n = 20$ for each treatment). (c) Autocorrelation of ring width for control and irrigated trees. Data from control and irrigated trees were pooled in panel (b).

Sevanto 2014), a critical issue considering that the outer sapwood usually contributes most to overall sap flow (Cermak & Nadezhdina 1998). Equilibrating short-term asynchrony between carbon demand and supply (Sala, Woodruff & Meinzer 2012) leads to an intense exchange of sugars with the phloem (Richardson *et al.* 2013). In the intermediate part of the sapwood, the NSC pools are probably turned over less frequently and mainly serve as longer term reservoir and to maintain metabolic activity of the living (parenchyma) cells (Carbone *et al.* 2013). Such a gradient in NSC concentration and the observed drop in the soluble carbohydrates from the outer to the middle zone are consistent with a ‘last in, first out’ mechanism of NSC dynamics (Richardson *et al.* 2013). The innermost

part of the sapwood is generally less active with respect to water transport, respiration and storage (Cermak & Nadezhdina 1998; Gartner, Baker & Spicer 2000; Pruyn, Gartner & Harmon 2002). Yet, it maintained relatively high %NSC. This raises the question whether these reserves are still mobile or sequestered (van Bel 1990; Palacio *et al.* 2013) and/or used for heartwood formation (Saranpää & Höll 1989). Addressing this question would, however, require a higher spatial and temporal resolution of NSC measurements than performed here.

The NSC pool size (TNC) in the inner sapwood was considerable, and accounted for 43% of all NSC stored in the entire sapwood in both treatments (Fig. 1b). If these carbohydrate reserves were not accessible (Palacio *et al.*

2013), the available NSC pool for growth would be much smaller than suggested by some studies (Ryan 1989; Hoch, Richter & Körner 2003). The significant increase of the TNC and tree-ring width in the outermost two segments (i.e. during the treatment) in the irrigated trees (Fig. 1b,d) indicates that supplemental water improved tree vitality both in terms of long-term resources (TNC) and shorter term competitiveness (higher growth rates).

CARBON ALLOCATION TO GROWTH AND STORAGE DEPENDS ON AVAILABLE RESOURCES

Our results indicate different carbon allocation strategies depending on available resources. First, wider tree-rings showed a lower %NSC, but higher TNC irrespective of treatment (Fig. 3b,c). In other words, more vigorous trees allocated relatively more NSC into ring growth than storage, while still increasing their NSC pool size. Such a carbon allocation pattern that depends on tree vitality and growth conditions could be an evolutionary strategy to balance short- and long-term fitness. If a vigorous tree allocates proportionally and absolutely more carbon to growth, it increases its short-term competitive ability, while concomitantly building up a larger NSC pool that likely increases long-term safety and resilience (Sala, Woodruff & Meinzer 2012), thus fostering future tree performance. On the other hand, if tree vitality is low and/or carbon might become limited, prioritizing carbon allocation to storage over tree-ring growth is consistent with a 'bet-hedging' strategy to reduce short-term risks and improve long-term fitness (Stearns 1976; Chapin, Schulze & Mooney 1990; Wiley & Helliker 2012). An evergreen species as Scots pine with several active sapwood rings certainly has more degrees of freedom in this respect, because it can survive with barely or even without any (ring) growth in a specific year (Rigling *et al.* 2002), as long as the leaf area and therefore the required water-conducting tissue do not change (Zweifel *et al.* 2006), and sufficient carbohydrates are available for vital functions (Pruyn, Gartner & Harmon 2002; Zha *et al.* 2004; Sala, Woodruff & Meinzer 2012). If this pattern would only show in control trees, one could argue that water deficit will limit cell division and enlargement (Hsiao & Acevedo 1974), NSC mobilization and transport in the phloem (Sevanto 2014), but the same relationship between %NSC, TNC and ring width showed in the irrigated trees for which we can reasonably exclude any significant water limitation. Our results thus suggest an active control of carbon storage as opposed to a passive overflow storage of carbon not used for growth (Sala, Woodruff & Meinzer 2012; Saffell *et al.* 2014).

Our second important finding in this context – the positive effect of a larger NSC pool size (TNC) on next year's growth (Fig. 5a,b) – supports the aforementioned view, and is also exciting in another respect: it suggests a physiological role of TNC in explaining the autocorrelation of tree-ring growth. In a year following ample carbon accumulation, rings are wider, and here we found direct and

novel evidence that the concomitant increase in the TNC carries over to the growth of the following year. Consistently, the irrigated trees with a larger NSC pool size also showed a stronger and longer autocorrelation in ring width (Fig. 5c). Although larger cells would also result in wider rings without requiring more carbon, evidence from other conifer species showed a correlation of $r \geq 0.97$ between ring width and number of cells (Olano *et al.* 2012; Gricar *et al.* 2015). Our interpretation is also supported by correlations between NSC levels and secondary growth (Desoto, Olano & Rozas 2016), as well as evidence found by Carbone *et al.* (2013) for a link between NSC pool size (and age) and ring growth using ^{14}C methods in red maple trees.

Conclusions

A reasonable assumption is that ray abundance, NSC reserves and growth rate are all linked and respond to environmental drivers in synchrony to maintain homeostasis in tree functioning. However, our results show that ray abundance in the stem sapwood of Scots pine is a rather constant characteristic, which shows low-frequency responses to long-term changes in external conditions delayed by several years, and which does not seem to be controlled by NSC storage demand. From the tree functional point of view, it is puzzling to observe that ray abundance varies that much among trees, given the pivotal role of rays for tree carbon and water cycle. The high low-frequency variability in ray abundance, coupled with the only weak coherence among individual time-series, suggests a high intrinsic, i.e. genotypic control of ray abundance – a hypothesis that still needs to be tested. From a methodological point of view, our results highlight the different perspectives obtained by performing NSC analyses on tree-ring compared to metric levels. We also found it was relevant to consider both NSC concentration and NSC pool size. While the NSC concentration seems to be the relevant metrics for processes on tissue level such as respiration, transport and maintenance of hydraulic integrity, the NSC pool size likely reflects the carbon allocation to growth vs. storage. From a life-history strategy point of view, the carbon allocation patterns observed here rather support a prioritization of storage over growth ('active storage') than passive overflow storage in the isohydric Scots pine. Overall, we encourage to perform more research on – long-term – ray variability within and among individuals, including the internal and environmental drivers of this variability, and how rays vary in other tree organs, notably branches and roots, in order to improve the mechanistic understanding of the role that this vital tissue plays for tree functioning and performance.

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Data accessibility

All data used in this manuscript are present in the supporting information (Tables S1–S5).

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Supporting Information

Details of electronic Supporting Information are provided below.

Table S1. Annual mean tree-ring width and basal segment area of all study trees.

Table S2. % Ray parenchyma and basal segment ray parenchyma area of all study trees.

Table S3. % NSC, insoluble NSC and soluble NSC per sapwood segment of all study trees.

Table S4. Annual tree-ring width [mm] of all study trees.

Table S5. Annual % ray parenchyma of all study trees.

Appendix S1. Details on statistical model selection.