Minimum levels and dynamics of carbon reserves in temperate trees at severe carbon limitation

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Contents

	Acknowledgements	6
	General introduction	7
Chapter 1	Living on next to nothing: Tree seedlings can survive weeks with very low carbohydrate concentrations	14
Chapter 2	Shaded trees save their carbon	46
Chapter 3	Long-term shade acclimation in temperate forest trees	74
Chapter 4	Additional experiments	93
4.1	Root respiration and carbon reserves in temperate tree seedlings during lethal carbon starvation	93
4.2	Carbon allocation during drought	103
	General summary and conclusions	117

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General introduction

Approximately 90% of the global biomass carbon (C) pool is stored in forests (Olson et al. 1983). Because the reaction of forests to future climate changes will have significant effects on the world-wide biomass pool, the responses of trees to elevated atmospheric CO_2 concentrations and increasing drought scenarios has been diligently investigated over the last decades. However, despite these previous efforts, it is still not entirely clear, whether these major environmental changes will induce a higher, or a lower frequency of forest dieback events (Körner 2003, McDowell et al. 2018). After all, it can not be excluded that increased C gain due to elevated CO_2 reduces the chance of C starvation during potentially C-limiting stress scenarios like drought, freezing damage, herbivory or under shade (Sala et al. 2010, Wiley and Helliker 2012).

The presence of sufficient C reserve pools has been suggested to be decisive for trees to survive stressful periods, where the net-C balance of trees can be negative for longer periods. For example, it has been suggested that the so called 'isohydric' mechanism of drought tolerance, where trees close stomates early in a dry period in order to reduce water loss, can induce C-limitation during prolonged drought (Lajtha and Barnes 1991, McDowell et al. 2008). Another example are tree species growing at their cold limits at the alpine tree line, which might be able to assimilate and store more C under elevated CO_2, which allows for more re-growth after loss of green tissue by frost of mechanical damage (Handa et al. 2005, Wiley and Helliker 2012). However, despite the important role of C storage compounds in trees for tolerance to drought, frost, herbivory or shade, we still have very limited knowledge about the precise control mechanism for C storage in trees.

C reserves in trees consist mainly of low molecular weight sugars and starch (non-structural carbohydrates, NSCs), but also of storage lipids, and basically all other compounds that primarily serve other functions than storage, but can be re-introduced (recycled) into the primary metabolism under C-limitation, *e.g.* some hemicelluloses, acids, and proteins (Chapin et al. 1990, Brouquisse et al. 1998, Martin et al. 2002,

Hoch 2007, Schädel et al. 2010). In the vast majority of plants, NSCs are the quantitatively most important C reserve group, and NSC concentrations show large seasonal fluctuations in trees of temperate and boreal regions (Martínez-Vilalta et al. 2016). Most prominently, NSC reserves are used to supply spring leaf-flush in temperate trees, although strong reductions of NSCs are generally restricted to terminal branches (Schädel et al. 2009, Klein et al. 2016). Significant decreases in NSC concentrations have been observed in trees in reaction to C-limiting treatments like defoliation, reduced CO_2 concentrations, or shading (Veneklaas and den Ouden 2005, Schmid et al. 2017). As a consequence, NSC tissue concentrations are often used to predict the C supply status of trees (Hoch 2015), and measurements of high NSC concentrations at ambient conditions have led to the conclusion that trees are currently not C-limited (Körner 2003).

For NSCs to serve as indicators of C supply, the formation of C reserves has to largely follow the C source-sink balance in trees, where NSC are only built up when the supply of C assimilates by photosynthesis exceeds the C demand from C sinks, like growth and respiration (Chapin et al. 1990, Hoch 2015). However, Wiley and Helliker (2012) questioned the ubiquitous usability of NSC concentrations as a proxy for the C-balance of trees, suggesting that the importance of C reserves for stress survival can lead to a preferential formation of C reserves, even against prevailing C demand from other C sinks. As a consequence, NSCs might be also 'actively' accumulated in trees at C limiting situations. Under such conditions, increased NSC pools in trees might rather indicate than refute the presence of C-limitation. Wiley and Helliker (2012) suggested closer investigations of the dynamics of NSC storage to determine, if and under which conditions trees might actively increase NSC pools under potential C-limiting stress. Such investigations would certainly improve the usefulness of NSC measurements to predict the C supply status of trees.

Within this thesis, I addressed very basic questions with respect to the control mechanisms for C reserve formation in trees under C limiting growth conditions that have not been systematically addressed so far. The main research goals were (i) the identification of the minimum tissue concentrations of NSC in tree organs at lethal C starvation, (ii) the investigation of the long-term effects of C limitation by shading on growth, gas exchange and C storage of different tree species to identify possible trade-offs between biomass production and storage, (iii) the assessment of the significance of the presence of NSC pools for the survival of trees under environmental stress, like drought.

Chapter 1: Dynamics of NSCs and growth before, during and after C-starvation

C starvation as defined by McDowell (2011) implies a complete depletion of C stores and subsequent death, but such cases are rarely observed under natural conditions (Martínez-Vilalta et al. 2016). At exposure to 1-6% of full sunlight, the description of substantial decreases but no complete depletion of NSC concentrations in tree tissues (Veneklaas and den Ouden 2005, Piper et al. 2009, Maguire and Kobe 2015) raised the question, if a complete depletion of NSC can even occur in trees, or if a substantial proportion of the NSC pools stored in wood are basically sequestered and inaccessible for remobilization (Millard et al. 2007, Sala et al. 2010). Previous exposition of tree seedlings to complete darkness (0% light) has yielded NSC concentrations as low as 2-6% per dry matter (Marshall and Waring 1985, Piper and Fajardo 2016, Wiley et al. 2017). However, we miss detailed information about inter-specific and tissue-specific differences. There is also a data gap for growth and recovery of NSC reserves before, during and after severe C-limitation. In chapter one, I present a complete darkening study with seedlings of two temperate broad-leaved and two conifer tree species, during which we quantified NSC concentrations in all major tissues, and specifically explored the changes of NSC tissue concentrations during and following severe C limitation via harvests of entire tree seedlings at seven different time points.

Chapter 2: Dynamics of NSC and growth in temperate trees during long-term C-limitation

The current use of NSCs to predict the C supply status of trees, assumes that NSCs accumulate only in times of C surplus (Hoch 2015). However, as it has been pointed out by Wiley and Helliker (2012), the importance of C storage for tree survival might lead to an active up-regulation of C reserves on account of other C sinks, like growth, under situations of C-limitation. In a three-year field trial, I exposed saplings from ten temperate tree species (six deciduous broad-leaved and four evergreen conifers) to C limiting conditions by continuously growing them at only 6% of full sunlight. Previous studies on the effect of shading revealed decreasing, but also increasing C reserves in tree seedlings and saplings (Veneklaas and den Ouden 2005, Poorter and Kitajima 2007, Piper et al. 2009). However, no study so far investigated the time course of NSC concentrations in reaction to shade over multiple growing seasons. Within this long-term shading experiment, I aimed to assess the presence or absence of 'active' C storage formation in C limited trees by quantifying photo-assimilation, growth and NSC storage under deep shade conditions. Specifically, I addressed the question, whether the allocation of photo-assimilates to growth or storage at C limitation is species dependent, and if trees show a change in allocation preferences from growth to storage over multiple-seasons under deep shade.

Chapter 3: Shade acclimation in trees: a matter of photosynthesis or C allocation?

Shading studies are frequently used to explore the dynamics of NSCs under C-limitation. Often, seedlings that are acclimated to full sunlight are shaded for one growing season, and results are used to explain species-specific shade tolerances, or to predict the ecological significance of NSCs for tree resilience under environmental stress (Schall et al. 2012, Giertych et al. 2015, Xie et al. 2018). Although there is strong evidence for a fast acclimation of the photosynthetic apparatus of plants to shade (Cui et al. 1991, Fujita et al. 1994, Lenssen et al. 2003), we surprisingly lack systematic long-term data on photosynthetic acclimation of trees to low light. We therefore cannot exclude more pronounced acclimation in perennial plants after multiple growing seasons at low light. Within this chapter, I tried to investigate the

potential of photosynthetic long-term acclimation to 6% of full sunlight over multiple growing seasons in saplings of six temperate broad-leaved tree species. By using the trees from the above-mentioned long-term shading experiment, I measured photosynthetic light response curves in trees adapted to high or low light over three consecutive years. Based on these measurements, I modeled annual net-assimilation rates and compared them to the amount of C annually allocated to growth and C storage. With this study, I aimed to assess the significance of long-term photosynthetic acclimation and its effect on C allocation in broad-leaved trees under continuous deep shade conditions.

Chapter 4: Additional experiments

In chapter four, I present results from two studies (4.1 and 4.2) with tree seedlings that aimed to explore i) the priority of root respiration as a major C sink during severe C-limitation, and ii) the significance of NSC for drought survival.

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Chapter 1

Living on next to nothing: Tree seedlings can survive weeks with very low carbohydrate concentrations

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Summary

1. The usage of non-structural carbohydrates (NSCs) to indicate carbon (C)-limitation in trees requires knowledge of the minimum tissue NSC concentrations at lethal C-starvation, and the NSC dynamics during and after severe C-limitation.
2. We completely darkened and subsequently released seedlings of two deciduous and two evergreen temperate tree species for varying periods. NSCs were measured in all major organs, allowing assessment of whole-seedling NSC balances.
3. NSCs decreased fast in darkness, but seedlings survived species-specific whole-seedling starch concentrations as low as 0.4-0.8% per dry matter (DM) and sugar (sucrose, glucose and fructose) concentrations as low as 0.5-2.0% DM. After re-illumination, the refilling of NSC pools began within three weeks, while the resumption of growth was delayed or restricted. All seedlings had died after 12 weeks darkness, and starch and sugar concentrations in most tissues were lower than 1% DM.
4. We conclude, i) that under the applied conditions, tree seedlings can survive several weeks with very low NSC reserves probably using also alternative C-sources like lipids, proteins or hemicelluloses. ii) Lethal C-starvation cannot be assumed, if NSC concentrations are higher than the minimum concentrations found in surviving seedlings. iii) NSC reformation after re-illumination occurs preferentially over growth.

Introduction

Like all plants, trees depend on the continuous supply of carbon (C) that is fixed from atmospheric CO_2 via photosynthesis. In view of the currently increasing C supply for plants from rising atmospheric CO_2-concentrations, it might be questioned, if tree growth is currently limited by C availability (Körner 2003). However, even at elevated CO_2-concentrations, trees might still face C-limitation as a result of environmental or biological stresses like drought or leaf-loss caused by herbivores (Wiley et al. 2013, Sevanto et al. 2014). C-reserves are hypothesized to play a fundamental role in plant survival under environmental stress, because they help a plant to overcome periods when C-demands exceed the current photosynthetic C-uptake (Chapin et al. 1990, Sala et al. 2011).

Over the last decades, C-reserve concentrations in plant tissues have often been used as a proxy for the C-balance of trees (Hoch 2015). Comparative analyses of tissue non-structural carbohydrates (NSC) concentrations to assess the C-balance of trees during stress have been used in numerous previous studies (Hoch et al. 2003, Körner 2003, Würth et al. 2005, Palacio et al. 2008, Hoch and Körner 2012, Hartmann et al. 2013, Piper et al. 2015). These studies follow largely the storage accumulation concept by Chapin et al. (1990) that considers mobile C-compounds as a buffer for C-source and sink activities, and concentrations are assumed to mirror the net C-balance between C-acquisition by photosynthesis and C-sink activities like growth and respiration (Sala et al. 2011). In this respect, several previous studies suggested that tree growth is not C-limited in the absence of NSC depletion (Körner 2003, Würth et al. 2005, Millard et al. 2007). In contrast, Wiley and Helliker (2012) proposed that growth in trees with high NSC concentrations may be even more C-limited, because storage might occur at the expense of growth in order to survive periods of C-limitation. Consequently, Wiley and Helliker (2012) predicted that C-storage might often occur, in competition with other C-sinks, i.e. following the concept of reserve formation defined by Chapin et al. (1990).

If C-storage is occurring at the expense of other C-sink activities, NSC tissue concentrations might not directly reflect a plant's C-balance, thereby questioning the

usefulness of NSC measurements as a proxy for C-supply (Sala et al. 2012). However, Palacio et al. (2014) pointed out that current evidence in support of such a trade-off is equivocal, because cell growth processes are generally more sensitive to cold stress or drought than to processes related to C-gain (Körner 2015). In the majority of reported cases, increased C-reserve pools in trees under environmental stress are thus likely associated with C-sink-limitation while a trade-off between storage and growth might occur under true C-limitation (e.g. from sustained severe defoliation, persistent deep shade or extremely low CO_2 concentrations, Palacio et al. 2014). Hence, more information about the physiological controls of C-storage is still required to determine whether and to what degree tissue NSC concentrations are adequate as indicators of the net C-balance of plants (Hartmann and Trumbore 2016).

In this study, we aim to approximate minimum NSC concentrations, since such thresholds for NSCs are currently unexplored, hindering our ability to determine when mortality has resulted from C-starvation (Palacio et al. 2014). Low molecular sugars in particular should not decline to zero even at C starvation as it was defined by McDowell (2011) because of their multifunctional nature (e.g. as intermediate metabolites, C-transport compounds, osmolytes, or C-source for growth and respiration; Hoch 2015, Hartmann and Trumbore 2016). In addition, NSC reserves of different tree organs (e.g. leaves, stem wood, roots) and species might show different NSC thresholds and responsiveness to C-source-sink changes (i.e. the speed with which NSC concentrations change with reduced or increased C-supply). Exposing trees to darkness is a straightforward method that allows monitoring NSC concentrations at C-limitation. Previous deep-shading experiments have shown significant decreases in tissue NSC concentrations in seedlings, but often C-limitation was not severe enough to cause mortality (Veneklaas and den Ouden 2005, Fischer et al. 2015, Maguire and Kobe 2015). Several studies that employed complete darkening or extreme deep shade quantified NSC concentrations at tree mortality, but only for a single species and often not for all tissues separately (Marshall and Waring 1985, Piper et al. 2009, Sevanto et al. 2014, Piper and Fajardo 2016, Wiley et al. 2017). In addition, they did not identify the minimum NSC concentrations that still allows seedling to survive, nor did they investigate the NSC dynamics after C-limitation.

Therefore, it remains unclear whether the threshold NSC concentrations below which death becomes inevitable are different to NSC concentrations at death.

Here we present an experimental study, where seedlings of four tree species including deciduous broad-leaved and evergreen conifers were exposed to periods of complete darkness with subsequent re-illumination. The individual analysis of sugar and starch in all major organs allowed us to report a whole seedlings NSC balance, as well as the dynamics of NSCs during and after severe C-limitation. Specifically, we addressed the following hypotheses:

1. Tissue concentrations of C-reserves will decrease in complete darkness, but, if non-lethal, will recover after re-illumination of seedlings.
2. After re-illumination, the rebuilding of C-reserve of seedlings will be prioritized over other C-sink activities such as growth.
3. There is a stronger decline in tissue starch concentrations under C-limitation than in low molecular weight sugar concentrations, due to the multiple physiological functions of the latter.
4. When lethal C-starvation occurs, tree seedlings show species-, organ- and compound-specific non-zero concentrations of non-structural C-reserves.

Materials and Methods

Study site and study species

The study was conducted in 2015 at the Institute of Botany of the University of Basel, Switzerland and at the Max-Planck Institute for Biogeochemistry in Jena, Germany. Two-year-old bare-root seedling stock of *Acer pseudoplatanus* L. and *Quercus petraea* L. was used in Basel, and four-year old bare-root seedling stock of *Picea abies* (L.) H.KARST. and *Pinus sylvestris* L. was used in Jena. Hereafter the species will only be referred to by their genus name. The 30 – 50 cm tall seedlings of each species were purchased from local nurseries (dormant *Acer* and *Quercus* from Forstgarten Lobsingen, Switzerland and *Picea* and *Pinus* from Pflanzenhof Tonndorf, Germany) and stored in darkness at 4°C for a few days until potting. All seedlings were planted before bud-break in 3 litre square pots (14 cm wide and 23 cm deep) in a

commercial plant substrate (Ökohum, Herrenhof, Switzerland) containing bark humus, plant humus, peat, wood fibre and the plant available nutrients nitrogen (N, 260 mg/l), phosphate (P_2O_5, 180 mg/l) and potassium (K_2O, 480 mg/l). The pH-value ($CaCl_2$) of the soil was 5.8, the salinity (KCl) 1.8g/l and 90 % of the substrate consisted of organic matter. In total 130 seedlings each for *Picea* and *Pinus*, respectively, were potted on March 23, 2015, and 108 seedlings each for *Acer* and *Quercus*, respectively, were potted on March 27, 2015. After potting, the 476 seedlings were placed outdoors until the start of the experiment. Before potting, all seedlings of the two broad-leaved species (*Acer* and *Quercus*) were weighed individually to obtain the initial fresh biomass.

Experimental set up

The aim of the experiment was to expose tree seedlings to complete darkness for different periods of time with subsequent re-exposure to light, after which the seedlings were monitored for survival, and the recovery of NSC pools. To maintain similar climatic conditions during light and darkness, the experiments were carried out in climate controlled growth chambers (see below).

The temperature was kept at 22°C during daytime and 15°C during night throughout the duration of the experiment. In Basel, non-darkened (control) growth chambers with an overall mean relative air humidity above 66% were exposed to sunlight in a greenhouse, with a maximum photosynthetic photon flux density (PPFD) of 790 µmol m^{-2} s^{-1}. The day/night duration in these control chambers ranged between 16h/8h and 13h/11h, following the natural outside conditions in Basel between May and September. When PPFD fell below 150 µmol m^{-2} s^{-1}during daytime hours, additional light was provided by sodium-vapour lamps (MT 400 DL/BD). Dark chambers were fully covered by a 0.15 mm thick opaque black poly-foil (Blacho-Tex AG, Hägglingen, Switzerland).

In Jena, we used phytochambers (custom built facility, York Refrigeration, Mannheim, Germany) with artificial light provided at PPFD of 700 µmol m^{-2} s^{-1} by sodium-vapour lamps (MT 400 DL/BD, EYE Lighting International of North America Inc., Mentor, Ohio, US). After six to nine weeks in the dark, conifers needles showed burning symptoms after re-exposure to light at 700 µmol m^{-2} s^{-1}. Hence, re-illuminated seedlings were progressively exposed starting at 75 µmol m^{-2} s^{-1} to

acclimate, later to 300 μmol m^{-2} s^{-1}. Day/night duration was 15h 25min/8h 35 min in control chambers, and 24 h night in dark chambers.

Within each species, seedlings were subjected to one of six darkening treatments, which differed in the length of the darkness period (Table 1.1): 1) LC (no darkening; control): continuous day/night conditions, 2) D1 (1 week of darkness): seedlings were exposed to complete darkness for the first week of the experiment, 3) D3 (3 weeks of darkness): seedlings were completely darkened for the first three weeks of the experiment, 4) D6 (6 weeks of darkness): seedlings were exposed to complete darkness for the first 6 weeks of the experiment, 5) D9 (9 weeks of darkness): seedlings were completely darkened for the first 9 weeks of the experiment, 6) DC (complete darkness): seedlings were continuously kept in complete darkness for 12 weeks (the whole experiment; Table 1.1). Before the start of the experiment, trees were sorted according to their height from the smallest to the tallest seedling and then distributed equally to the different treatments to ensure similar tree height distribution in all treatments groups. The darkening treatments started on May 28, 2015 for *Acer* and *Quercus*, i.e. about one month after bud break. In the case of the two conifers, the darkening treatments started on July 22, 2015 for *Picea* and August 19, 2015 for *Pinus*, i.e. after the current year shoot expansion had terminated. To measure growth and NSC concentrations throughout the experiment, four seedlings per treatment and species were sampled on seven collection (harvest) dates during the experiment (Table 1.1). In *Acer* and *Quercus*, seedling survival after darkening was assumed for harvested seedlings by assessing the ability of the remaining, non-harvested seedlings to re-flush after re-illumination during at least seven weeks (depending on the preceding darkness period). In conifers, trees were considered dead when all needles were brown and had shed.

Table 1.1 Harvest overview of the different treatments. Numbers are placed at specific dates when a sub treatment was harvested, and shaded areas indicate darkness periods. 1 = Harvest of *Acer* and *Quercus*; 2 = Harvest of *Picea* and *Pinus;* n = 4 per harvest.

Treatment	Harvest 1 Week 0	Harvest 2 Week 1	Harvest 3 Week 3	Harvest 4 Week 6	Harvest 5 Week 9	Harvest 6 Week 12	Harvest 7 Week 15
LC	1,2	1,2	1,2	1,2	1,2	1,2	1
D1		1,2	1,2	1,2	1,2	1,2	1
D3			1,2	1,2	1,2	1,2	1
D6				1,2	2	2	1
D9					1,2	1,2	1
DC						1,2	1

Measurements

For *Acer* and *Quercus*, the following tissues were stored separately in paper bags: leaves, re-flushed leaves after darkness, new shoots, stems, coarse roots (> 2 mm diameter) and fine roots (< 2mm diameter). From each seedling, a 3 to 4 cm long piece of the lower stem and the main root were cut, the bark (including all phloem) was peeled off with a knife. The total leaf area of each seedling was determined with a Li-3100 leaf area meter (LI-COR, Lincoln, NE, USA). For *Picea* and *Pinus*, around 500 mg of fresh biomass from each of the following tissues was separately collected at each harvest: current-year and last-year needles from the uppermost two whorls and their corresponding branches (wood, phloem, and bark combined), coarse roots (> 2 mm diameter) and fine roots (< 2mm diameter). Immediately after harvest, all sampled plant material was shock-heated in a microwave at 900 W twice for 15 seconds to stop enzymatic activity (Popp et al. 1996), and then oven-dried at 75 °C for 72 h. After measuring tissue dry biomass, all samples were ground to a fine homogenous powder with a horizontal ball-mill (MM 400, Retsch, Haan, Germany), and the powder was then stored dry until chemical analysis.

NSC analyses (Starch, Sucrose, Glucose and Fructose)

Non-structural carbohydrates (NSCs) were analysed after a modified protocol by Wong (1990) and Hoch et al. (2002) as described in Plavcová et al. (2016). In short, 8-12 mg of the fine plant material powder were weighted into 6 ml glass vials and

extracted with 2 ml distilled water by boiling at 100°C for 30 minutes over steam. An aliquot of 200 µl of the extract was treated with invertase from *bakers yeast* (Sigma-Aldrich, St. Louis, MO, USA) to degrade sucrose into glucose and fructose. 100 µl of this extract was mixed with 100 µl of a glucose-hexokinase assay (glucose-hexokinase assay reagent, Sigma Aldrich, St. Louis, MO, USA) thereby converting glucose to glucose-6-P and fructose to fructose-6-P. Added isomerase (from bakers yeast; Sigma-Aldrich, St. Louis, MO, USA) converted fructose-6-P to glucose-6-P. Due to the presence of NADP in the assay, all glusoe-6-P was converted to gluconate-6-P and NADPH. Finally, the concentration of NADPH (equating the concentration of glucose) was determined photometrically at 340 nm in a 96-well micro plate photometer (HR 7000, Hamilton, Reno, NE, USA). 500 µl of the original extract were treated with a fungal amyloglucosidase from *Aspergillus niger* (Sigma-Aldrich, St. Louis, MO, USA) in a 49°C water bath over night, to break down starch to glucose, and the total glucose (corresponding to NSCs) was determined photometrically as described above. The concentration of starch was calculated as total NSCs minus low-molecular weight sugars. To ensure activity of the enzymes, pure starch and solutions of glucose, fructose and sucrose were used as standards, and to control reproducibility of the extraction, standard plant powder (Orchard leaves, Leco, St. Joseph, MI, USA) was included. All NSC concentrations are given as % dry matter.

Calculations and statistics

In order to compare the whole-seedling NSC concentration among treatments and dates, a weighted mean concentration of NSC was calculated for each seedling by integrating tissue mass and NSC concentrations as described in Hoch et al. (2002), using the following formula:

$$\sum_{org=1}^{n} \frac{conc_{org} \text{x } biom_{org}}{100}$$

where n is the number of organs, $conc_{org}$ is the organ specific NSC concentration (% dry matter) and $biom_{org}$ the organ specific fraction of the total biomass.

Because the initial biomass of the seedlings was rather variable already at the beginning of the experiment, we calculated the relative biomass change by dividing the final dry biomass of an individual by its initial total dry biomass before planting. Initial total dry biomass was calculated by multiplying the initial fresh biomass by 0.5 (assuming a mean water content of winter dormant stem and roots of 50%, as confirmed by separate measurements). For the relative biomass changes, values higher than 1 indicate a net increase, while values lower than 1 indicate a seasonal net-decrease. Because sequestered C (growth) and NSCs (storage) are both included in the dry biomass of a seedling, NSC mass (NSC concentration * biomass) was subtracted from the dry biomass (g) of each individual before growth analysis. For relative biomass change calculations, NSC mass also was subtracted from initial dry biomass, using averaged NSC measurements (n=6) of seedlings before leaf flush in March.

To test the effect of the length of the darkness treatment on NSC concentrations at the end of the experiment (week 12 in *Picea* and *Pinus*, week 15 in *Acer* and *Quercus*), a full-factorial two-way ANOVA (type III) was performed with darkening length and tissue type as fixed factors, and individual as a random factor. Differences among treatments and dates within a species and tissue type were tested for significance with a Tukey-Kramer HSD test at a significance level of $P < 0.05$. Whenever only two groups were compared, we used Student's t-test. To test the influence of the preceding darkness length on the speed of increase in NSC concentration (% per dry matter) following re-illumination, we performed a two-way ANOVA with the interaction of light re-exposure length (zero and three weeks) and the length of the preceding darkness period (three, six or nine weeks) as explanatory variables.
A potential trade-off between growth and NSC storage was analysed for *Acer* and *Quercus* by correlating NSC concentrations at the end of the experiment (week 15) with the net biomass change between week 0 and 15 for each darkening treatment. To test whether there was a linear or an exponential relationship between NSCs and biomass increment at week 15 (y-axis) in each darkness treatment of *Acer* and *Quercus*, a linear model was applied to *1)* the un-transformed dry biomass and to *2)* the logarithmized dry biomass. For all statistical analysis, the software R 3.0.2 was used.

Results

Survival and growth response to various periods of darkness

In *Acer* and *Quercus*, one week of darkness had no effect on the visual appearance of seedlings, whereas after 3 weeks of continuous darkness, some leaves started to furl, turned blackish and finally dried out. In both broad-leaved species, seedlings started to lose their old leaves after 3 and 6 weeks of continuous darkness, but produced new long, etiolated shoots with small, whitish leaves (Fig. 1.1, 1.2). No etiolated shoots were observed in conifers, where a loss of turgor (bending of new shoots) and paling of mainly current-year needles appeared after 3 weeks of darkness (Fig. 1.1). However, all four *Picea* seedlings that were in darkness until week 12 kept green needles and turgescent new shoots for 9 to 10 weeks of darkness.

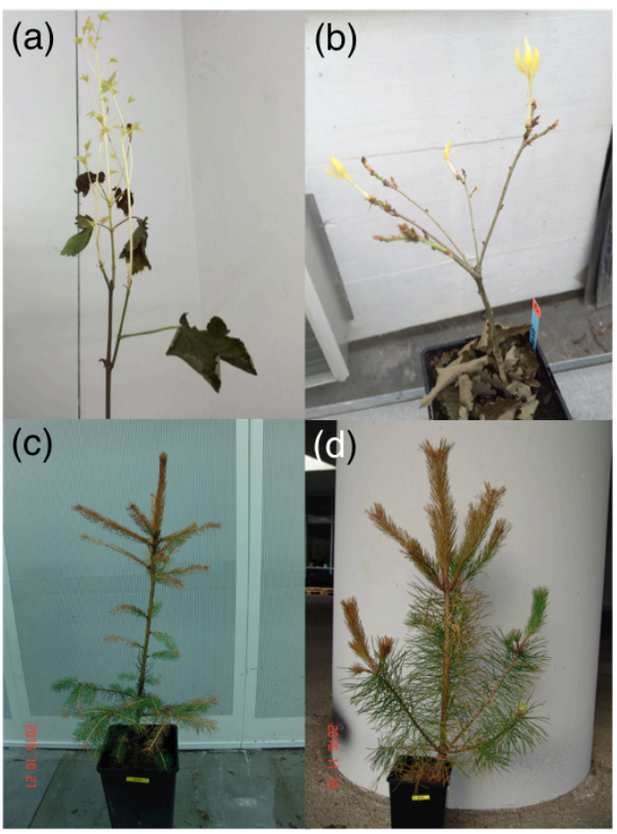

Fig. 1.1 Etiolated shoots with small, whitish leaflets in *Acer* (a) and *Quercus* (b), and brown needles in *Picea* (c) and *Pinus* (d) after 9 weeks of continuous darkness.

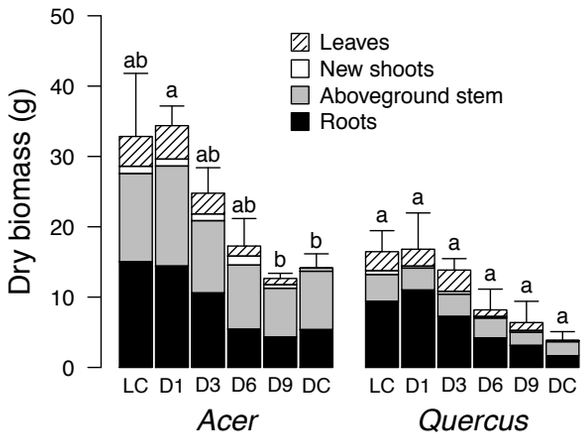

Fig. 1.2 Total dry weight in response to different treatments at the end of the experiment (week 15). Columns are means (+SE) total dry biomass separated for leaf mass (shaded), new shoots (white), stem mass fraction (grey) and root mass fraction (black). Different letters indicate significant differences in biomass between treatments in one species at $p < 0.05$ by Tukey-Kramer HSD test. Light control, LC; one week darkness, D1; 3 weeks darkness D3; 6 weeks darkness, D6; 9 weeks darkness, D9; dark control, DC. $n = 4$.

Almost all seedlings in darkness died between week 9 and 12. Broadleaved seedlings that were re-illuminated after 9 weeks produced normal-sized green leaves from the pre-formed etiolated leaves within a few days. In contrast, after twelve weeks of continuous darkness, all seedlings that were re-illuminated were unable to flush, or the previously flushed, etiolated shoots had already wilted. However, one *Acer* seedling that had been re-illuminated after 12 weeks of darkness still had two living re-flushed leaves at week 19. In both conifer species, seedlings that were re-illuminated after 9 weeks of darkness kept their remaining green needles, while after 12 weeks of darkness no green needles were present and no re-flushing was observed within 6 weeks after re-illumination, so they were presumed dead.

Seasonal growth was followed in detail only in *Acer* and *Quercus*, which showed similar growth dynamics. Total seedling biomass increased significantly between week 0 and 12 in the LC, D1 and D3 treatments, whereas seedlings of treatment D6, D9 and DC tended to decrease their biomass due to leaf shedding, but no loss of etiolated shoots occurred. However, darkened *Acer* seedlings showed a decrease in belowground biomass (standardized by individual pre-treatment fresh weight) over the time course of the experiment (Student's *t*-test, $df = 5.81$, $P = 0.02$), while no root dieback was observed in darkened *Quercus* seedlings. Surprisingly, the overall biomass increment of *Acer* seedlings from D1 and D3 stopped at week 9 (8 and 6 weeks after re-illumination, respectively), while seedlings from LC continued to grow through week 15 (Fig. 1.3).

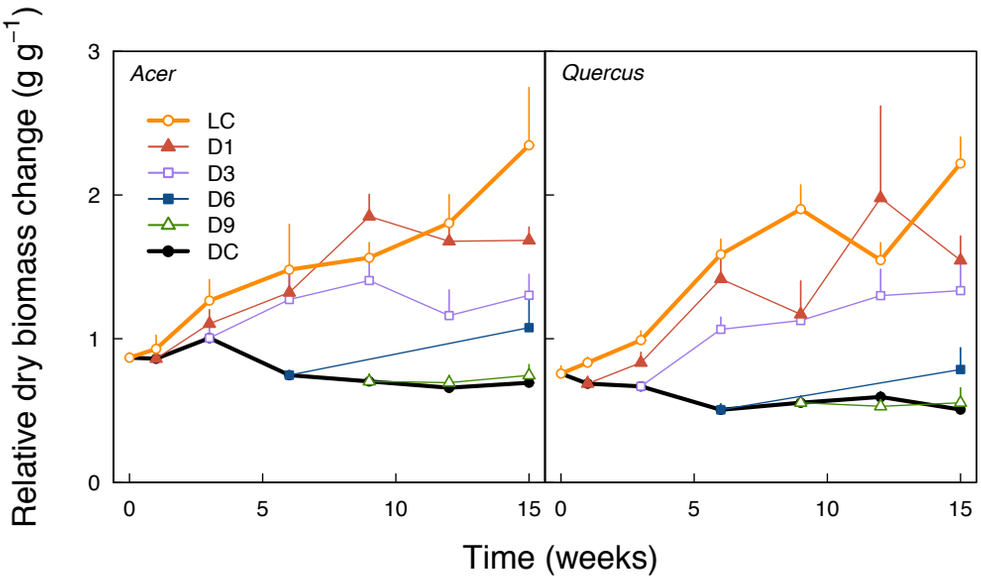

Fig. 1.3 Growth development of broad-leaved deciduous tree saplings in six different darkness treatments. Biomass (means + SE) has been standardized by total dry weight (g) divided by 0.5* initial fresh weight (g). The six different treatments are indicated in different colours as followed: light control (LC) = orange, one week darkness (D1) = red, 3 weeks darkness (D3) = violet, 6 weeks darkness (D6) = blue, 9 weeks darkness (D9) = green, dark control (DC) = black. n = 4.

Non-structural carbohydrates in darkness

For *Acer* and *Quercus*, the experiment started four weeks after bud break in spring, when NSC reserves are generally low in deciduous trees because C is allocated to leaf production (Fig, 2, 3). Therefore, starch concentrations in broad-leaved species were still low at the beginning of darkening in most tissues (below 2% in stem xylem, stem bark and young shoots, and between 6 and 12% in leaves and root xylem) at week 0 of the experiment, but increased quickly in LC and reached a maximum around week 6 (Fig. 1.4, 1.6, 1.7). These "optimum trajectories" were also observed in darkened seedlings (D1 and D3) after re-illumination. In both evergreen conifers, starch concentrations at the start of the experiment were substantially higher, ranging from 4% in *Picea* branches to 25% in *Pinus* needles (Fig. 1.4, 1.8, 1.9). Sugar concentrations were more similar among species and tissues in LC seedlings, ranging from 3 to 8% in *Picea*, *Pinus* and *Acer*, and from 4 to 12% in *Quercus* (Fig. 1.5, 1.10, 1.11, 1.12, 1.13).

One week of darkness led to significant decreases of the weighted whole-seedling mean NSC concentrations (as the sum of sugars and starch) by 43, 51 and 37% in

Quercus, *Picea* and *Pinus*, but not in *Acer*, where starch continued to decline in LC but not in darkened seedlings (Fig 2). Beyond one week, starch and sugar concentrations declined quickly at an exponential rate under continuous darkness in all investigated species and tissues (Fig. 1.4, 1.5). Three weeks after darkening, starch concentrations in most tissues of DC seedlings except *Acer* root wood, *Picea* needles and most *Quercus* tissues had already declined to starch concentrations found in dead DC seedlings at week 12 ($p > 0.05$, Fig. 1.6, 1.7, 1.8, 1.9). Sugar and starch in last-year needles of *Picea* and *Pinus* decreased less quickly than in current-year needles (Fig. 1.8, 1.9, 1.12, 1.13), and last-year needles maintained a higher NSC concentration after 12 weeks darkness compared to other tissues (Fig. 1.14).

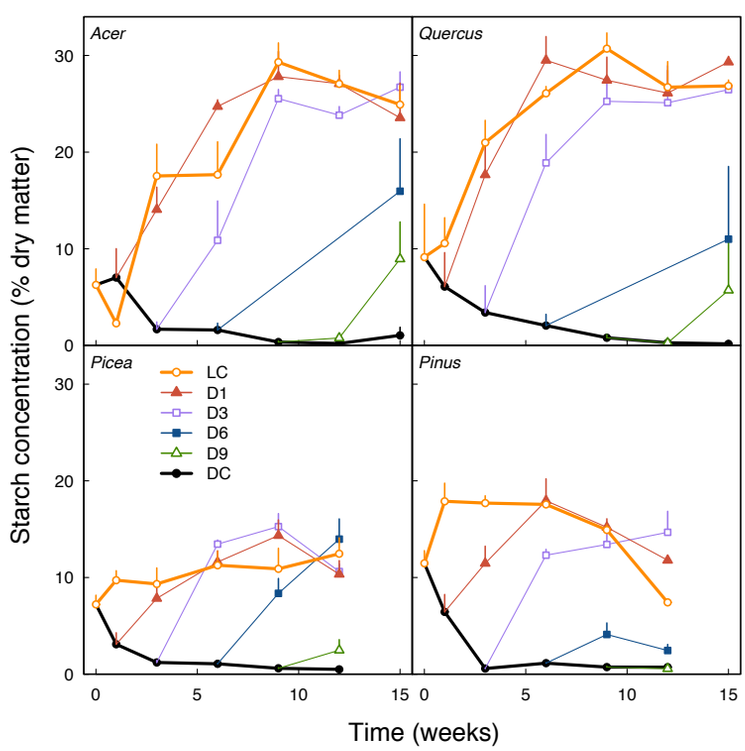

Fig. 1.4 Time course of starch in four different species at different darkness treatments and subsequent re-illumination. Starch is given as the mean (+SE) of the weighted concentration over entire individuals as described in the methods. Light control (LC) = orange, one week darkness (D1) = red, 3 weeks darkness (D3) = violet, 6 weeks darkness (D6) = blue, 9 weeks darkness (D9) = green, dark control (DC) = black. n = 4.

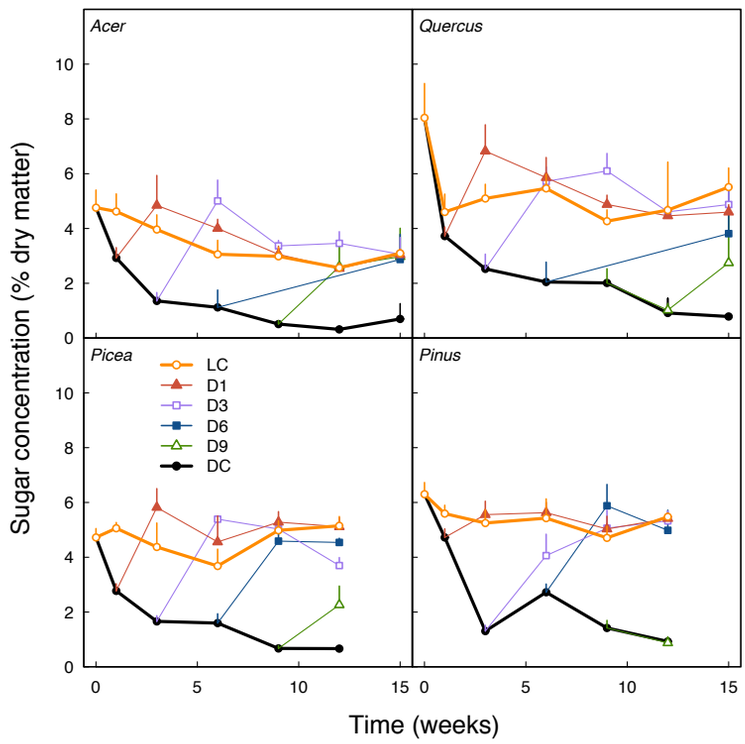

Fig. 1.5 Time course of low molecular weight sugars (sucrose, glucose and fructose) in four different species in different darkness treatments and subsequent re-illumination. Sugar is given as the mean (+SE) of the weighted concentration over entire individuals as described in the methods. Light control (LC) = orange, one week darkness (D1) = red, 3 weeks darkness (D3) = violet, 6 weeks darkness (D6) = blue, 9 weeks darkness (D9) = green, dark control (DC) = black. n = 4.

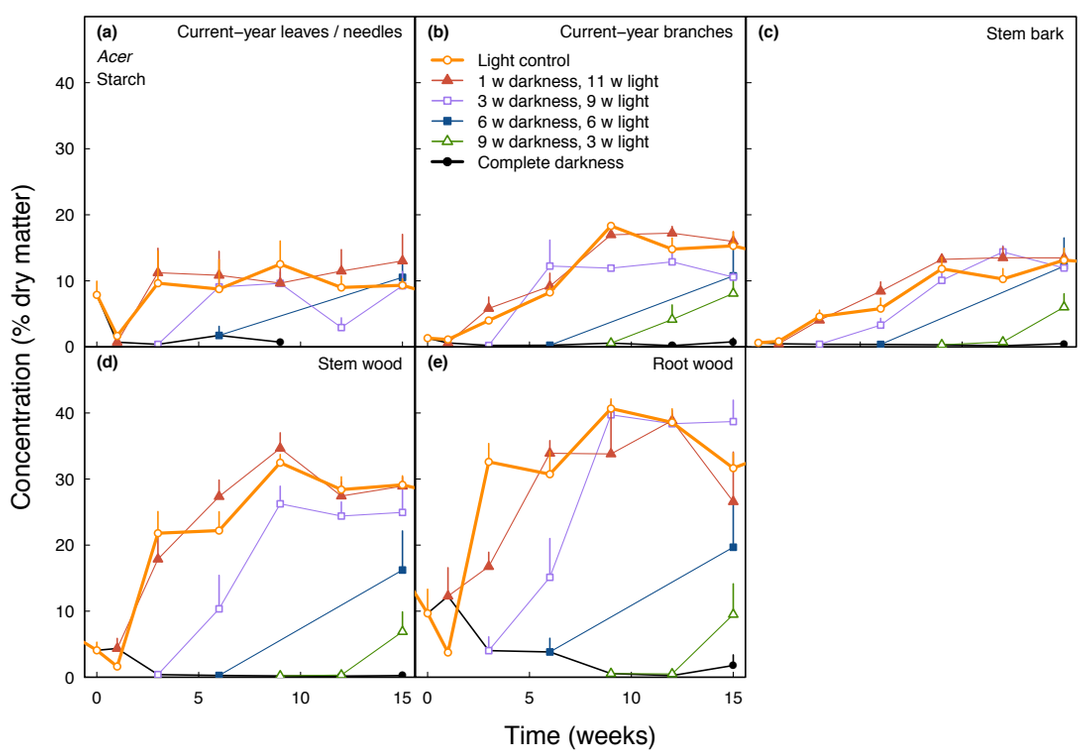

Fig. 1.6 *Acer pseudoplatanus:* Starch (mean + SE) in five different tissues at six different darkness treatments and subsequent re-illumination. n = 4.

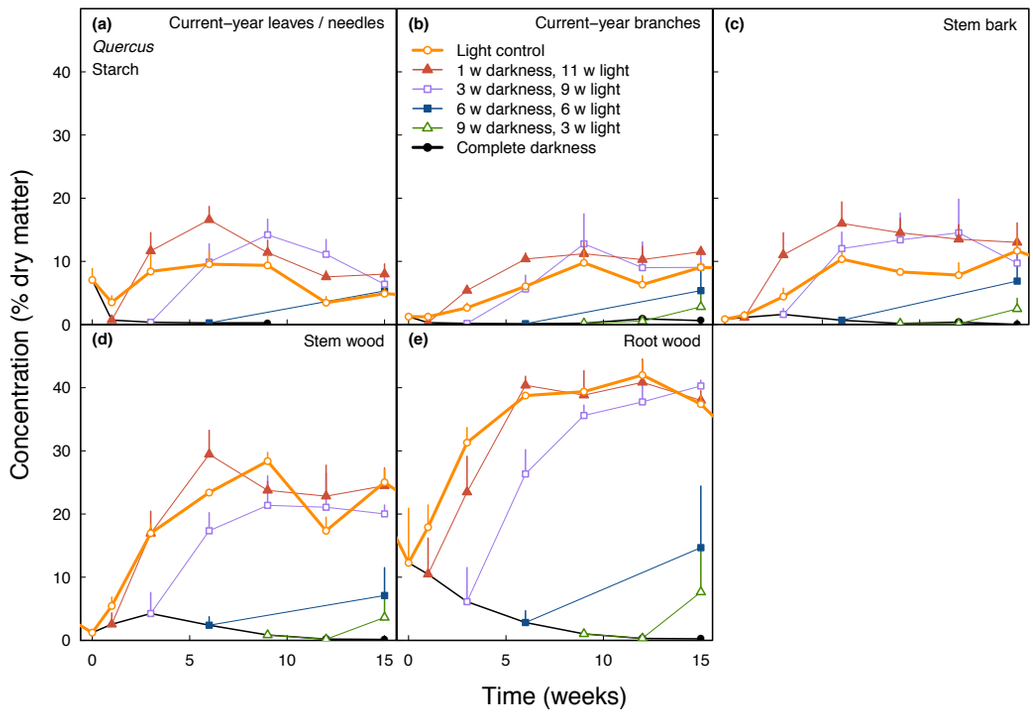

Fig. 1.7 *Quercus petraea:* Starch (mean + SE) in five different tissues at six different darkness treatments and subsequent re-illumination. n = 4.

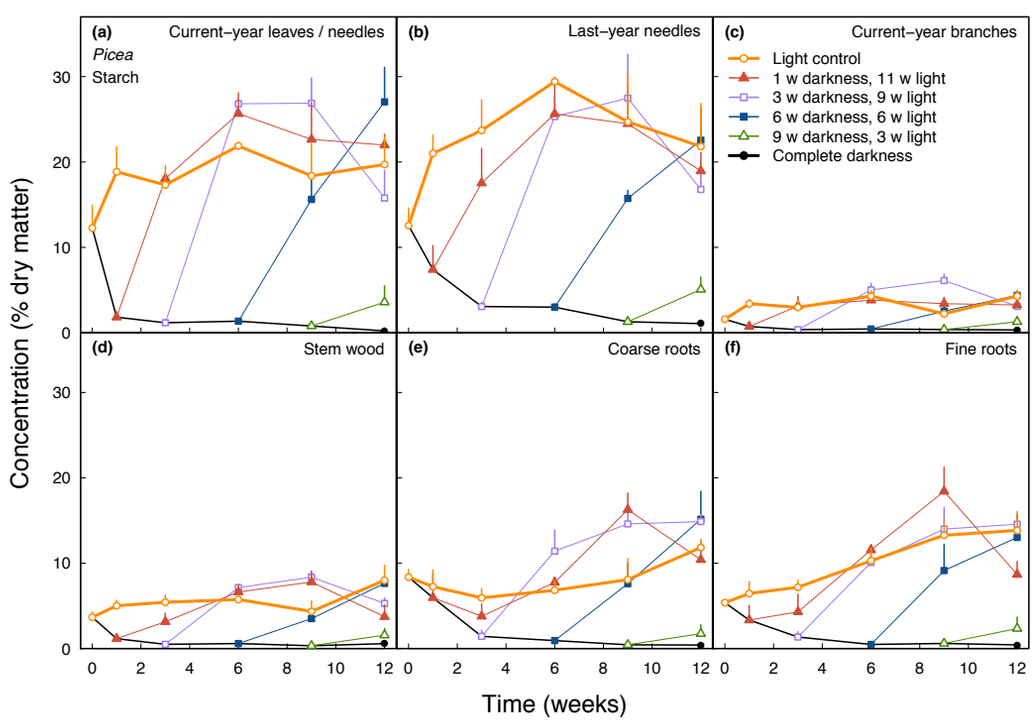

Fig. 1.8 *Picea abies:* Starch (mean + SE) in six different tissues at six different darkness treatments and subsequent re-illumination. n = 4.

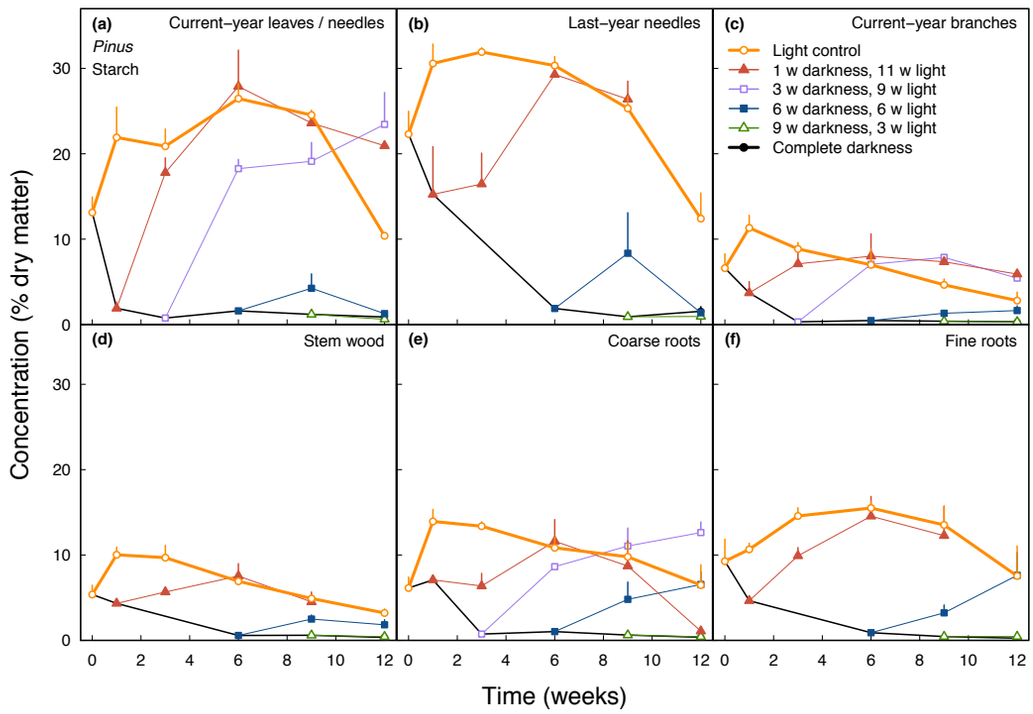

Fig. 1.9 *Pinus sylvestris:* Starch (mean + SE) in six different tissues at six different darkness treatments and subsequent re-illumination. n = 4.

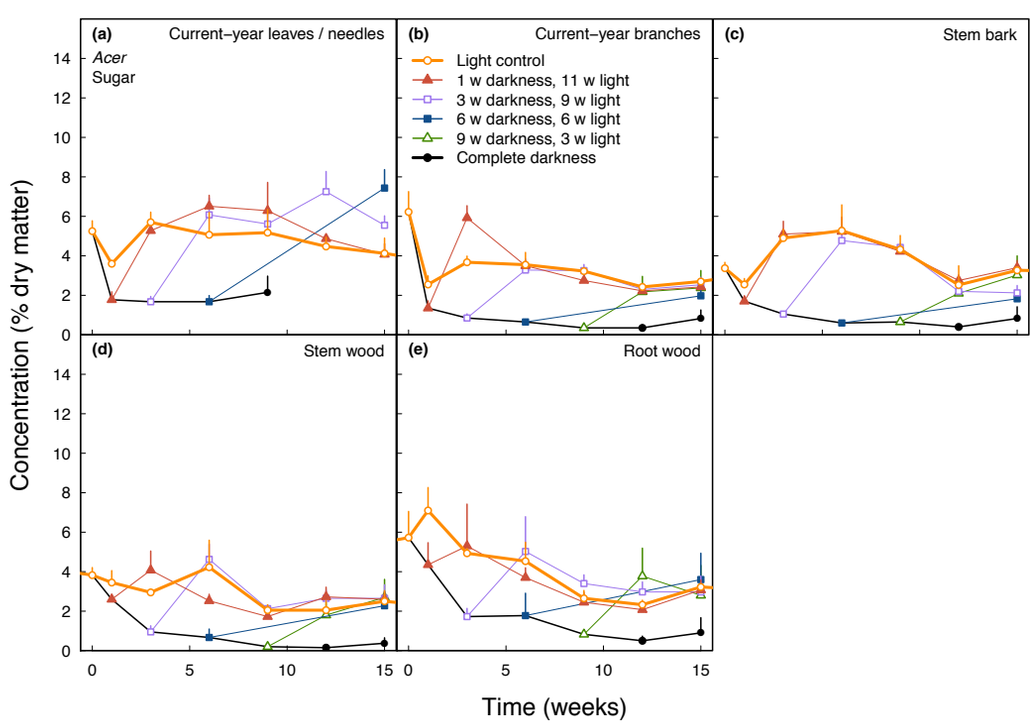

Fig. 1.10 *Acer pseudoplatanus:* Sugar (mean + SE) in five different tissues at six different darkness treatments and subsequent re-illumination. n = 4.

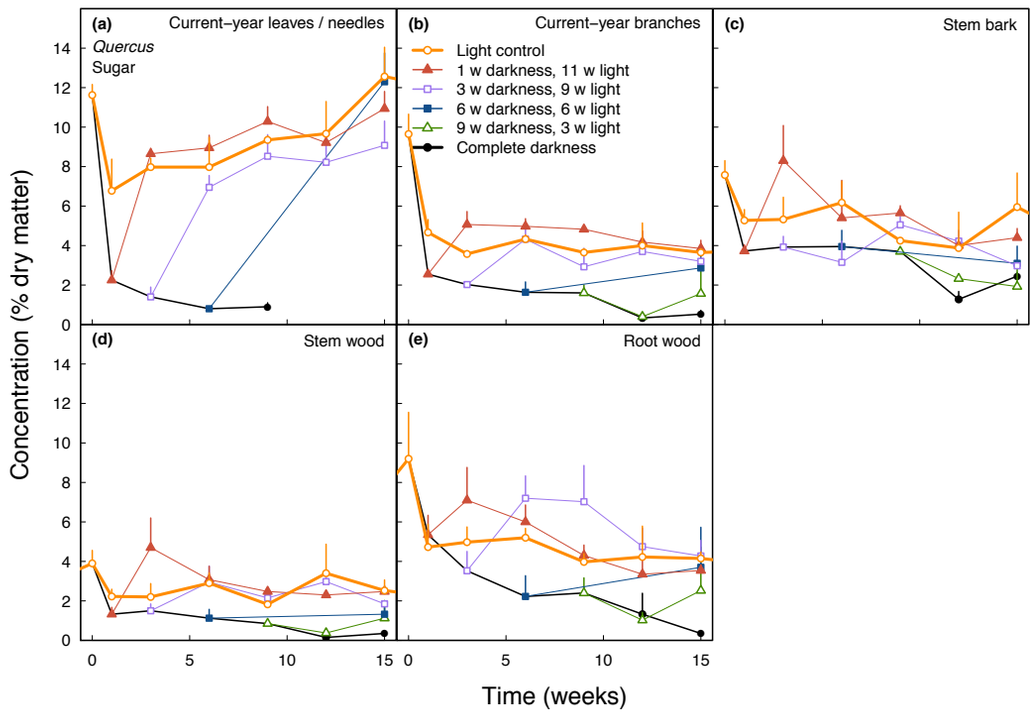

Fig. 1.11 *Quercus petraea:* Sugar (mean + SE) in five different tissues at six different darkness treatments and subsequent re-illumination. n = 4.

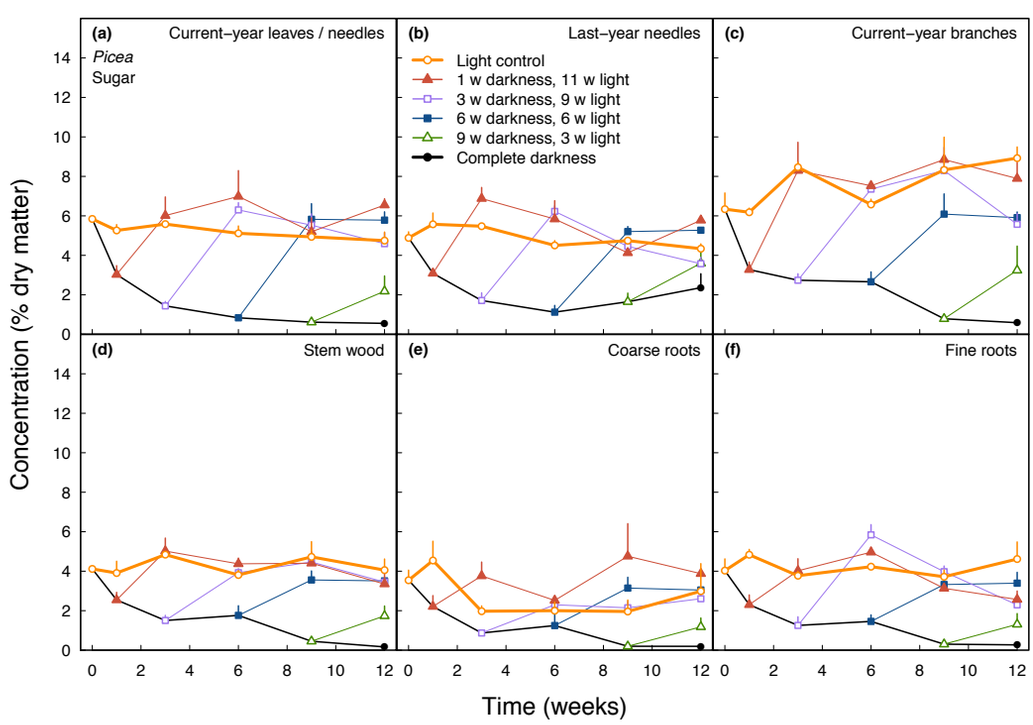

Fig. 1.12 *Picea abies:* Sugar (mean + SE) in six different tissues at six different darkness treatments and subsequent re-illumination. n = 4.

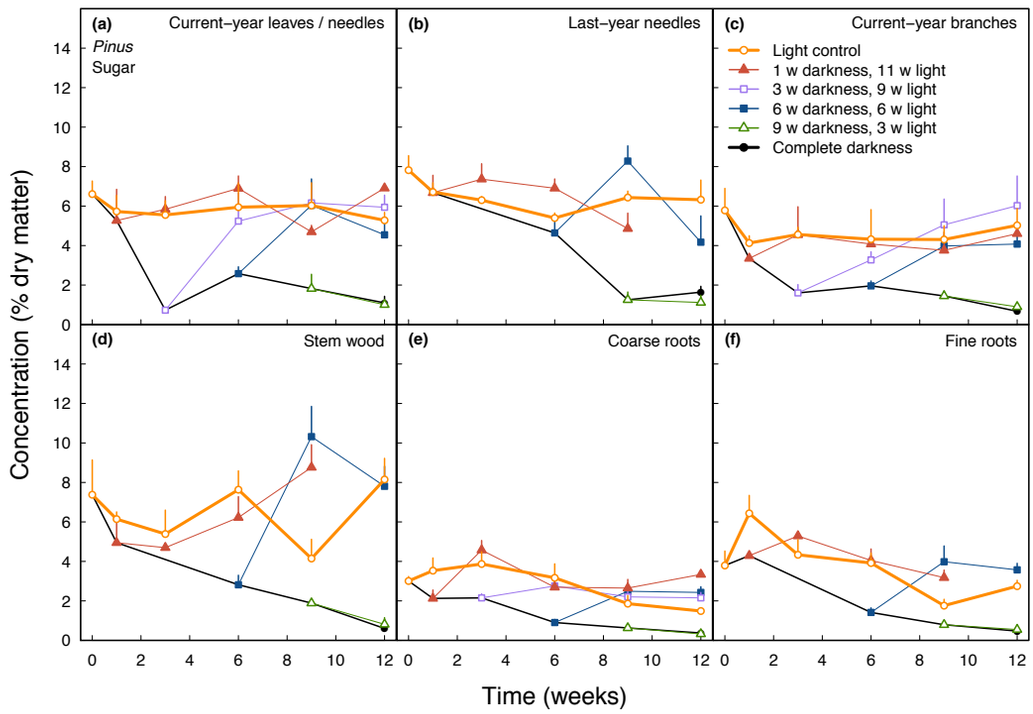

Fig. 1.13 *Pinus sylvestris:* Sugar (mean + SE) in six different tissues at six different darkness treatments and subsequent re-illumination. n = 4.

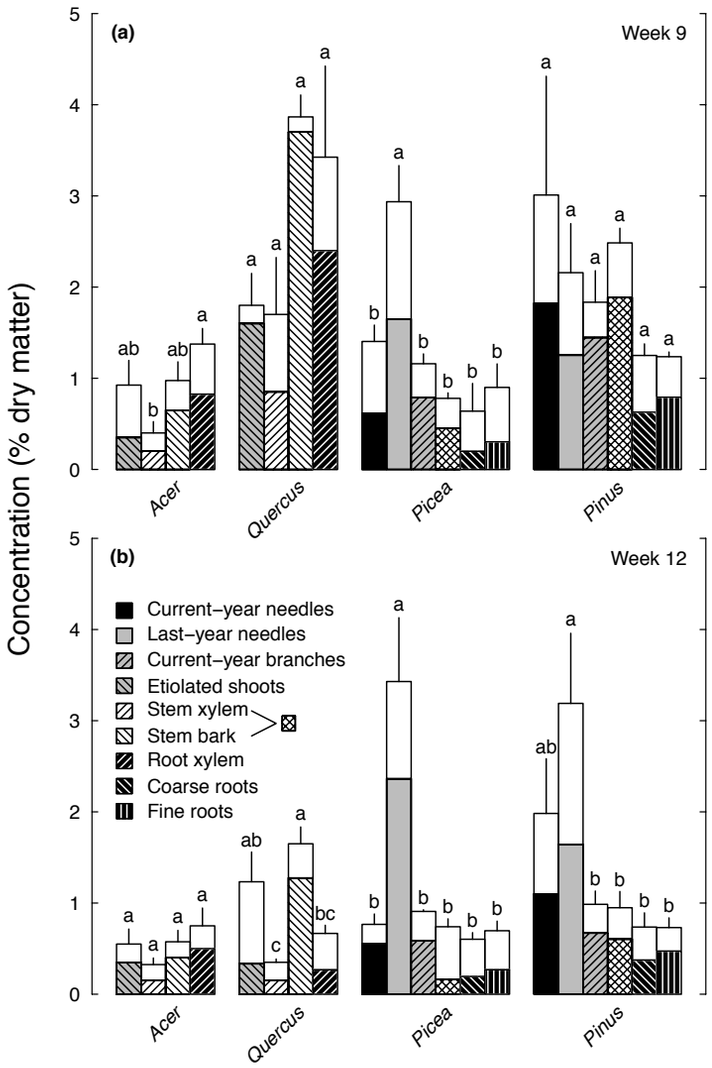

Fig. 1.14 (a) Minimum non-lethal and (b) lethal starch (open bars on top) and sugar (closed bars at the bottom) concentrations (+SE) in different tissues in (a) 9 weeks darkened seedlings, whose relatives from the same treatment have re-flushed after re-illumination, and (b) of C-starved seedlings, which have either not re-flushed after re-illumination following 12 weeks darkness (*Acer* and *Quercus*), or had no green needles remaining (*Picea* and *Pinus*). Letters above rectangles are statistical measures (Tukey's HSD) within each species.

At week nine in darkness, whole-seedling starch concentrations were between 0.4% (Acer*)* and 0.8% (*Quercus*), and starch concentrations in every tissue of all species were similar to those in dead seedlings at week 12 (*t*-test, $P > 0.05$, Fig. 1.14) and had decreased by more than 90% compared to initial concentrations (Fig. 1.4). Starch concentrations in etiolated shoots of *Acer* and *Quercus* did generally not exceed 0.5%, although one *Acer* seedling of the DC treatment showed remarkably high starch concentrations of 2.5 % in new shoots at the final harvest at week 15 under continuous darkness (data not shown). In contrast to starch, weighted whole-seedling sugar concentrations had only declined by 74 to 89% compared to initial concentrations after nine weeks of darkness. Whole-seedling sugar concentrations were between 0.5% (*Acer*) and 2% (*Quercus*). Sugar concentrations in etiolated

shoots ranged from 0.1% to 2.5%, and were twice as high in *Quercus* compared to *Acer*. At death, most tissues showed NSC concentrations below 1%, with similar sugar and starch concentrations for most tissues (Fig. 1.14). However, last-year-needles in dead *Pinus* and *Picea* individuals retained significantly higher sugar (1.6% and 2.4%) and starch (1.6% and 1.1%) concentrations (Fig. 1.14). In addition, in dead *Quercus* seedlings, stem bark retained higher sugar, and etiolated *Quercus* shoots retained higher starch concentrations compared to all other non-green tissues of *Quercus* and the three other species (Fig. 1.14). After 12 weeks of darkness, NSCs were reduced by at least 94% compared to maximum concentrations in all tissues, except in previous-year needles of *Picea,* where NSCs decreased by 91%. Between week 9 and 12 in darkness, whole-seedling NSC concentrations were non-significantly reduced by 42, 75, 9 and 23% (*t*-test, $P > 0.05$) in *Acer, Quercus, Picea* and *Pinus*.

Dynamics of non-structural carbohydrates after re-illumination

Following re-illumination of seedlings after one or three weeks of complete darkness, NSC reserves recovered quickly to control concentrations. Sugar and starch concentrations of D1 and D3 seedlings recovered to at least 80% of the control (LC) values within two to three weeks in *Picea*, and within about 6 weeks in *Acer, Quercus* and *Pinus* (Fig. 1.4, 1.5). Also the D6 seedlings showed NSC concentrations of about 80% of the LC by the end of the experiment in most tissues (Fig 1.10-1.9), but starch concentrations in stem xylem and root xylem of *Acer* (Fig. 1.6) and *Quercus* (Fig. 1.7). Starch in last-year needles of *Pinus* was still significantly lower compared to the respective LC seedlings by the final harvests at week 12 and 15 (Fig. 1.9). Consequently, by the end of the experiment, the weighted mean starch (but not sugar) concentrations of D6 seedlings were significantly lower than in LC seedlings of *Acer*, *Quercus* and *Pinus* (Fig. 1.4, 1.5). When trees had been exposed to nine weeks of darkness, NSC concentrations did not recover in any of the species until week 12, although NSCs increased significantly in *Acer* and *Picea* (Fig. 1.4, 1.5). After week 12, starch and sugars in broad-leaved species increased in all tissues except *Quercus* stem bark in the D9 treatment (Fig. 1.7, 1.11). Unlike in *Acer* and *Quercus*, the last harvest in *Picea* and *Pinus* was performed at week 12; thus it is unclear whether NSCs in the *Pinus* D9 treatment could have increased after more than three weeks of

re-illumination, similar to *Acer* and *Quercus* (Fig. 1.4, 1.5). In all four species, starch concentrations in seedlings recovered slower the longer they had been exposed to darkness (significant interaction in a two-way ANOVA, Fig. 1.15). In broad-leaved species, the starch interaction was strongest in stem wood while in conifers, the strongest effect was observed in needles.

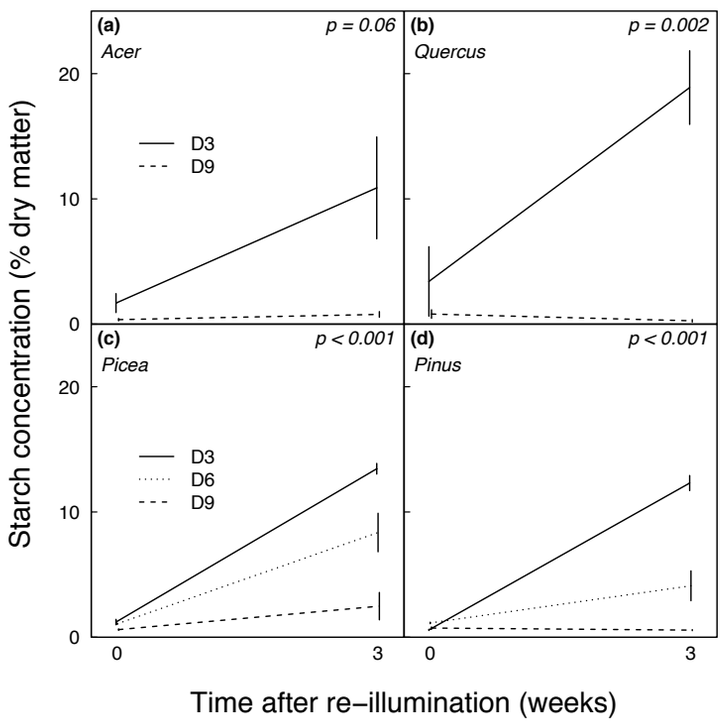

Fig. 1.15 Results of a full-factorial two-way ANOVA, showing the recovery of starch concentration (±SE) after different darkness periods in four species (a-d). Starch concentrations weighted by seedling size were compared between re-illumination and three weeks after re- re-illumination among different lengths of the darkening treatment: D3 (3 weeks darkness), D6 (6 weeks darkness) and D9 (9 weeks darkness). The increase in starch concentration showed significantly different slopes after different darkness periods in *Quercus* (b), *Picea* (c) and *Pinus* (d) (i.e. a significant interaction; $P < 0.05$, 3 and 18 *df*). Starch concentrations recovered faster in shorter darkening treatments following re-illumination.

NSC storage vs. growth in broad-leaved species

The potential trade-off between growth and NSC storage was investigated in more detail in the two broad-leaved species. Among the LC, D1 and D3 seedlings, there was a strong negative effect on final biomass with increasing duration of darkness (significant if initial biomass was included as a covariate), while NSCs increased by similar amounts from week 0 to week 15 among LC, D1 and D3 (Fig. 1.16). By

contrast, for seedlings that had spent six or more weeks in darkness and which did not show any net biomass gain during the 15 weeks experiments, the final NSC concentration was significantly higher the shorter the darkness treatment (i.e. highest in D6 and lowest in DC; Fig. 1.16), indicating a preferential investment of newly assimilated C into storage rather than growth after darkening.

There was virtually no growth below a final mean NSC concentration of approximately 25% per dry matter in *Acer* and below approximately 30% per dry matter in *Quercus*. For both species, exponential models best explained the relationship between final NSCs and biomass increase (Fig. 1.16).

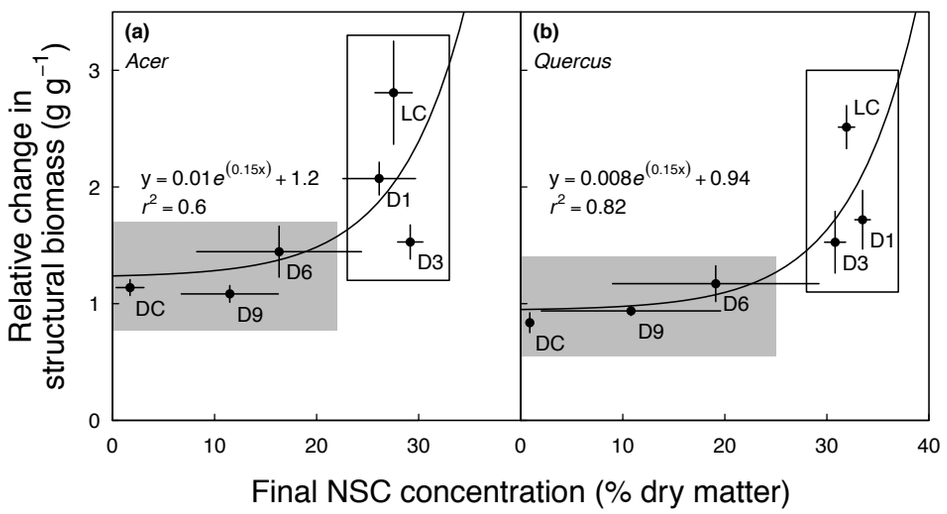

Fig. 1.16 Relative biomass change of individual seedlings in different treatments during the experiment, related to their non-structural carbohydrate (NSC) content after 12 weeks in *Acer* (a) and *Quercus* (b). Relative biomass change equals the dry biomass at week 12 minus the dry mass percentage of NSCs at week 12, divided by the estimated initial dry biomass (the initial fresh biomass*0.5) minus the dry mass percentage of NSCs in seedlings harvested before potting (±SE; y-axis). Final NSC concentration is the weighted NSC concentration after 12 weeks (±SE; x-axis) among all tissues in the six different light treatments. Treatments with already refilled storage are highlighted with an open rectangle, those with not yet refilled storage with a shaded rectangle. A linear model was applied to the logarithmized relative biomass increase, equal to an exponential model. *P* means the slope of the linear fit. LC, light control; D1, one week dark; D3, three weeks dark; D6, six weeks dark; D9, nine weeks dark; DC, Complete darkness; n=4. $df_{Quercus}$ = 16.6, $P_{Quercus}$ = 0.02, df_{Acer} = 6.49, P_{Acer} = 0.06.

Discussion

Main findings

Our results show that seedlings were able to survive several weeks with extremely low starch and sugar (sucrose, glucose and fructose) concentrations in all tissues. Thus, mortality by C-starvation in tree seedlings should only be assumed if these NSC reserves are lower than the organ-specific concentrations we observed a few days before death after nine weeks in darkness. The observed concentrations were reduced by at least 82% of maximum concentrations in all tissues. Considering that up to three weeks of darkness during the peak growing season did not show any sustained effect on tissue NSC concentrations by the end of the season, it can be concluded that NSC pools in seedlings respond very quickly to C-source-sink imbalances, and re-filling of depleted C-stores after re-illumination might be the prioritized allocation pathway for new photo-assimilates. As the seedlings were able to survive several weeks with NSC reserves below 2%, which in addition did not decrease during that time, future experiments should investigate, which alternative C-reserves can be additionally used to sustain respiratory demands under severe C-limitation. Finally, it remains to be tested to what extent these findings on young seedlings will apply to mature trees and for other environmental conditions.

Responsiveness of NSC to C-source-sink imbalances

Under complete darkness, NSC concentrations declined strongly in all tissues but were able to recover very quickly after re-illumination of seedlings. The fast recovery of NSCs following darkness suggest that the NSC pool of tree seedlings is very sensitive to short-term C-source-sink imbalances, but these imbalances only have a minor impact on NSC pools later in the season. Consequently, situations of short-term C-limitation, with negative effects on productivity, can probably not be deduced from NSC analyses several weeks after the event.

Surprisingly, not only starch but also low molecular weight sugar concentrations were lower than 10% of maximum tissue concentrations (except sugars in last-year needles of *Picea* and *Pinus*, which declined by 85%). Overall, sugars fell below 1% dry matter after six weeks under darkness in all investigated tissues except needles. These

reductions are substantially higher than reported seasonal fluctuations across different functional plant types, which showed a maximum reduction by 40-70% in sugars and by 65-85% in starch (seasonal minimum concentrations compared to maxima; Martínez-Vilalta et al. 2016). Piper and Fajardo (2016) found starch concentrations of about 1.5% after 11 weeks of darkness in *Acer pseudoplatanus* seedlings, clearly exceeding the 0.2% starch in *Acer* after 12 weeks darkness in our study, while Wiley et al. (2017) found 0.3% starch similar to our study in C-starved *Populus tremuloides* seedlings. However, sugar concentrations in *Acer* and *Populus* in these studies by Piper and Fajardo (2016) and Wiley et al. (2017) were substantially higher than in our darkened *Acer* seedlings at week 12 (2-6% vs. 0.3%). Also, Marshall and Waring (1985) found sugar concentrations of 2% after 17 weeks of darkness in *Pseudotsuga menziesii* roots, which was again significantly higher than the root sugar concentrations of around 0.3% after 12 weeks in our study. These apparent discrepancies could be a result of the different methods of sugar analysis, which additionally measured several higher molecular weight sugars along with those quantified in this study (i.e. sucrose, glucose and fructose), but these differences might also result from methodological biases among methods (Quentin et al. 2015).

Previous studies found that the variation in NSC concentrations in response to environmental stresses like low temperature, drought or different atmospheric CO_2-supplies, was largely due to changes in starch concentrations, whereas low molecular weight sugar concentrations stayed relatively constant (Körner 2003, Schädel et al. 2010b, Hoch and Körner 2012, Hartmann et al. 2013, Puri et al. 2015). In contrast to starch, low molecular weight sugars are used as intermediate metabolites, C-transport compounds as well as osmotic regulators beside their function as C-reserves (Pallardy 2008). Thus, a complete depletion of low molecular weight sugars in living plant cell should not occur (Hoch 2007). Our analytical approach does not allow to identify whether the observed low sugar concentrations occurred homogeneously in all living cells or are a result of C-starvation in only a part of the cells while others were not affected (e.g. partial die-back of parenchyma cells within wood). However, considering the fast and total recovery of sugar and starch concentrations in surviving trees after re-illumination, we assume that the first explanation is correct. If true, this

implies that, counter previous assumptions, living plant tissue can survive and stay physiologically active with very low concentrations of sucrose, glucose and fructose.

NSC thresholds at lethal C-starvation

With this study, we explicitly aimed to quantify lethal concentration thresholds for different tree tissues. We identified a narrow range between the observed lower NSCs boundary in dead seedlings at week 12 and the upper boundary in living seedlings at week 9. We found the lowest starch and sugar thresholds in stem wood of *Acer,* which were between 0.18% DM (lower boundary) and 0.2% DM (upper boundary) in starch and between 0.15% DM and 0.2% DM in sugar. Significantly higher threshold concentrations were found in photosynthetic tissue (0.9-1.6% DM for starch in *Pinus* needles and 1.7-2.4% DM for sugars in *Picea* needles).

Considering that the seedlings in our study could survive with very low tissue NSC concentrations for several weeks, it seems likely that they were using alternative C-reserve sources to maintain physiological functions like respiration, which might have been curtailed by programmed leaf and needle death. In darkened *Arabidopsis*, reduced demands due to shedding of reproductive organs has been demonstrated (Lauxmann et al. 2016). While the tree seedlings investigated in this study seemed to have sacrificed young needles and leaves, only broad-leaved species developed new etiolated shoots, indicating different strategies to dark-stress between broad-leaved trees and conifers. Compounds that can act as C-reserves beside sugars and starch are basically all organic compounds that can be recycled and reintroduced into the primary metabolism (Chapin et al. 1990). For example, some tree species are able to accumulate neutral lipids (triacylglycerol) in their woody tissues, which are considered exclusive C-storage compounds (Hoch et al. 2002). In *Pinus sylvestris*, shading resulted in a decline of carbohydrates with a simultaneous shift to more lipid-dominated respiration (Fischer and Höll 1991, Fischer et al. 2015). However, in *Acer, Picea* and *Quercus*, triacylglycerol concentrations have been found to be very low in woody tissue (Hoch et al. 2003), thus lipids should not account for a significant portion of the storage pool in these two species. In some tree families, there are other oligo– or polysaccharides like raffinose and stachyose, as well as polysaccharides of the fructan family, which can be used as C-storage compounds (Pallardy 2008).

However, the species in the current study or related species from the *Fagaceae*, *Sapindaceae* and *Pinaceae* families do not accumulate high concentrations of those compounds (Hoch et al. 2003). After cellulose, the second most abundant polysaccharide in nature are hemicelluloses that generally make up 10 to 30% of dry biomass in plants (Schädel et al. 2010a). Although hemicelluloses are generally functionally bound as structural elements of cell walls, some parts of hemicelluloses, especially glucose from side branches, might also act as C-reserves (Hoch 2007). In addition, amino acid degradation is known under starvation conditions in *Zea* and *Arabidopsis* (Brouquisse et al. 1998, Izumi et al. 2013, Ishihara et al. 2015). Since the current study focused on starch, sucrose, glucose and fructose, it remains uncertain whether and which alternative C-compounds the tree seedlings may have used to survive several weeks with almost zero NSC reserves. However, our results revealed extremely low starch and sugar levels as early starvation markers. Potential late starvation markers like low levels of other soluble sugars, hemicelluloses, lipids, and proteins might provide a more precise insight into C-starvation.

Trade-off between C-storage and growth

There is an ongoing debate on the possibility of a trade-off between growth and storage, where trees invest preferentially in C-storage at the expense of growth (Palacio et al. 2008, Sala et al. 2012). Palacio et al. (2014) questioned the validity of such a trade-off scenario under most natural conditions, but assumed that under environmental conditions leading to true C-limitation (e.g. sustained severe defoliation, deep shade) a competition for C between storage and growth might occur. Impaired growth and NSC recovery (Fig. 1.15) after longer darkness periods was likely caused by reduced C-uptake due to a degradation of the photosynthetic machinery and leaf loss. However, the limited amount of C gain in all seedlings with presumably damaged photosynthetic machineries (D6, D9, DC) was rather invested into storage refilling, than into growth (Fig. 1.16).

After re-illumination, growth does not necessarily stop until storage is refilled, but our results suggest that growth rates are modified to ensure a target NSC concentration (25% in *Acer*, 30% in *Quercus*). Therefore, growth might not occur when the target NSC threshold cannot be met. Such a threshold suggests that a trade-off between growth and storage might have occurred in this experiment, and points towards a

mechanism allowing trees to 'sense' its total C reserve pool size. Similar mechanisms have been demonstrated for *Arabidopsis* on a diurnal scale (Gibon et al. 2004, Rolland et al. 2006, Smith and Stitt 2007, Gibon et al. 2009, Sulpice et al. 2009). However, it is uncertain whether results from herbs, where C-storage is largely restricted to the leaf mesophyll, hold true also for trees, in which most C is stored in living parenchyma of woody tissues. A priority of storage against growth was shown at branch level in young walnut trees by Lacointe et al. (2004), who found similar NSC concentrations in sun or shade but less growth in shaded branches. In drought-stressed scots pines, sugar accumulation was also shown to occur independently of growth trends (Galiano et al. 2017).

While final NSC reserve concentrations were less sensitive to C-limitation than growth in broadleaved species in the current experiment, it remains uncertain whether C-allocation to storage occurred directly at the expense of growth. Both allocation to storage and growth reductions may have occurred independently and in response to phenological signals. Note that the darkening treatment was applied during the peak growth period and that seedlings may not have been able to compensate growth reductions later in the season, when growth already started to slow down in the temperate trees species used for this experiment. To unequivocally determine if C-storage in C-limited seedlings happens against C-demands for growth or not, future studies should thus focus on a seasonally unbiased study, either by investigating species in a-seasonal (tropical) regions, or apply moderate C-limitation over several seasons in subtropical, temperate or boreal regions.

NSC reserves under environmental stress

C-reserves are hypothesized to play a fundamental role in the tolerance of trees against environmental stress like drought. Over the last years, several studies have analysed changes in NSC tissue concentrations in trees during drought (Würth et al. 2005, Sala and Hoch 2009, Galvez et al. 2011, Anderegg et al. 2012, Hartmann et al. 2013, Sevanto et al. 2014). Many of these investigations documented no decrease and often even an increase of C-reserves in trees exposed to drought. An explanation for such increases is that growth and respiration demand for C is reduced faster than photosynthetic activity; a response known as sink limitation (Körner 2003). By

contrast, Wiley and Helliker (2012) proposed that increasing storage could have evolved as an active response to an increased threat of C-starvation, assuming that higher C-storage can help species to better survive environmental stress like drought, and increased NSC levels might thus be even exacerbating C-limitation to growth. In the current experiment seedlings did experience very severe C-limitation due to absence of photosynthesis, which led to a significant decrease in their C-reserves, not an increase. Nevertheless, we found indications for a preferential investment of C into storage over growth in seedlings that were released from severe C-limitation.

As discussed earlier, the limitation to assess C-starvation via NSC concentrations so far has been that the minimum concentrations of NSC, below which death becomes inevitable, were not known. It has been hypothesized that persistently high concentrations of NSC in trees may be required for maintaining xylem hydraulic conductivity (Sala et al. 2012, Dietze et al. 2014), but in our study, all seedlings were able to survive several weeks with very low starch and sugar concentrations. At least for young seedlings and the absence of water stress, the presence of high NSC concentrations in the xylem thus seems not to be necessary for the integrity of the water transport system. It is unclear however, if low NSC concentrations can damage the transport system in tall, mature trees and seedlings that are exposed to stress such as drought or freezing temperatures (Galvez et al. 2011, 2013). However, the results of the current experiment suggest that, at least for tree seedlings, C-starvation may occur only once starch, sucrose, glucose and fructose pools are below 1% in all woody tissues.

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Chapter 2

Shaded trees save their carbon

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Summary

1. A possible up-regulation of nonstructural carbohydrates (NSCs) at periods of carbon (C)-limitation challenges the usage of NSCs to determine a plant's C supply. Still, a trade-off between C storage and growth has not yet been experimentally investigated under long-term C-limitation.
2. We exposed ten temperate tree species differing in shade tolerance to 6% of ambient sunlight for three years to induce C-limitation, and additionally defoliated *Carpinus betulus*. To determine C allocation under shade, we repeatedly measured biomass increment and NSC concentrations.
3. Shade strongly reduced growth. After an initial two-fold decrease, NSC concentrations of shaded saplings recovered to 71-126% of un-shaded saplings in the third season. However, NSCs were generally more depleted under shade after leaf flush and during herbivore attacks. While 25% of defoliated and shaded *Carpinus* died, surviving saplings re-flushed and showed wood NSC concentrations of 1% DM in fall, compared to 13-28% DM in un-defoliated, shaded *Carpinus*.
4. We conclude that, irrespective of shade tolerance, NSC storage is favored over growth under prolonged shade. Thus, high NSC concentrations do not exclude C-limitation. Yet, our results suggest that decreased NSC concentrations indicate C-limitation, and that NSC concentrations lower than *c.* 20% of controls indicate a life-threatening C-shortage.

Introduction

Trees contribute about 90% to the global biomass carbon (C) pool (Olson et al. 1983). Trees store freely available C-reserves, so-called non-structural carbohydrates (NSCs, i.e. free sugars and starch), which can exceed 6 tons per ha in forests (Hoch et al. 2003, Würth et al. 2005). These carbohydrates are often quantified to estimate a plant's C-balance, assuming that NSCs are controlled by the net-balance between photo-assimilation and C-usage (respiration, growth and other sinks; Körner 2003). The understanding of the C-balance of a single tree, which cannot be easily assessed by conventional methods (e.g. gas-exchange measurements) might answer fundamental questions regarding tree performance at elevated CO_2 levels or under environmental stress, like drought (Hoch 2015).

Currently, it is still highly debated, how NSCs in trees are influenced by the C source-sink balance of a tree (Lacointe et al. 2004, Palacio et al. 2008, Sala et al. 2011, Galiano et al. 2017), and as a result, whether they can be used to predict the latter (Wiley and Helliker 2012, Palacio et al. 2014). It is further not well understood, to which extent they can be formed and built-up against prevailing C-sink demands (growth) under C-limitation, like in deep shade. Sulpice et al. (2009) found a negative correlation between the end-of-day leaf starch concentration and plant growth in *Arabidopsis*, pointing to a possible trade-off between storage and sinks. Smith and Stitt (2007) demonstrated, that the amount of starch stored during daytime and the speed of starch degradation over night is tightly controlled by day-length in *Arabidopsis*, showing a close coordination between growth and storage. Even more than annual herbs, perennial species like trees have to rely on stored C to survive longer periods of negative C-balance (e.g. dormant season, re-growth after damage). In temperate tree species, NSC pools are re-built and accumulated towards the end of the season, even after different C-limiting treatments like defoliation, low CO_2 concentrations, darkness, or a combination of those (Palacio et al. 2012, Wiley et al. 2013, Schmid et al. 2017, Weber et al. 2018). Still, it remains unclear if such strong up-regulation of NSCs occurs directly at the expense of growth. For example, Schmid et al. (2017) showed that stem diameter growth in defoliated and CO_2-deprived saplings rather depends on the amount of active foliage (the need for vessels) than on

the available C (NSCs), which argues against a reduction of growth in favor of end-of-season NSC re-filling.

Exposing plants to shade not only induces C-source limitation, but also increases etiolation (shoot length per shoot biomass, Berkowitz et al. 1995), and exposition of broad-leaved tree species to complete darkness induces production of white etiolated shoots, accompanied by a strong decrease of NSC reserves (Weber et al. 2018). Such reaction reveals the need to grow higher in order to reach more favorable light conditions. In contrast to defoliation, which might cause C-sink limitation (Wiley et al. 2013, Schmid et al. 2017), etiolation in shade suggests rather an absence of C-sink limitation. Thus, any growth decrease in shade is likely caused directly by limitation of C-assimilation. Shading is a typical situation for seedlings and saplings growing in the understory. Thus, it is a well-suited manipulation approach to induce C-limitation.

Recent shading studies showed 30-60% decreases of NSC concentrations in temperate tree saplings but also decreasing growth and survival at 1-6% light (Veneklaas and den Ouden 2005, Piper et al. 2009, Maguire and Kobe 2015). However, none or low temporal sampling resolution in many shading studies makes it difficult to tell whether storage or growth decreased first in shade, and high mortality often came into play already in an early phase of the experiment, further complicating functional investigations on a possible growth vs. storage trade-off.

If there is a trade-off between storage and growth, it might be found most prominently in species that are used to low light environments. Maguire and Kobe (2015) suggested a correlation between shade tolerance (survival) and stored NSCs in tree seedlings under shade. However, Piper et al. (2009) contradicted such a correlation, questioning the connection between NSC storage at low light and shade tolerance. Yet, the prioritization of growth in shade seems to depend on the species' shade tolerance, given that growth in shade intolerant species is generally reduced less strongly at low light compared to shade tolerant species (Chen et al. 1996, Poorter 1999, Myers and Kitajima 2007, Imaji and Seiwa 2010). If the above-mentioned decreases of NSC concentrations at low light are quantified and compared to growth reductions, differences in the low-light C allocation patterns between shade tolerant

and intolerant species might become quantifiable (Lusk and Piper 2007, Piper et al. 2009). However, studies that investigated such allocation patterns so far, did not standardize growth by initial biomass, and thus could not provide relative reductions of growth in shade (i.e. a reduction of the relative growth rate).

Up to date, we are lacking detailed insights into the dynamics of C reserves in non-lethally C-limited trees over longer time periods. Such analyses could reveal if, and to which extent, C storage is prioritized over growth at C-limitation. Especially, they would show, if C-limited trees under shade aim to reach NSC tissue concentrations of non-C-limited trees after an initial decrease of NSCs, or, if new and lower steady state NSC concentrations are reached, which would enable trees to invest a relative higher fraction of C-assimilates into growth than in the former scenario. To our knowledge, there are no studies available that have investigated the effect of deep shade on tree growth and NSC concentrations with sequential harvests over several seasons. Within a field experiment, we thus investigated the effect of permanent strong shading on growth and C-reserves of tree saplings over the course of three growing seasons. To cover a large spectrum of taxa, ten temperate tree species were used. These species include evergreen conifers and deciduous tree species, differing in growth and their adaptation to different light environments (Landolt et al. 2010).

Based on existing knowledge and the above mentioned research goals, the following hypotheses were addressed:

1. Shade intolerant, early successional species like *Betula pendula* or *Pinus sylvestris* are "C-spenders" in shade. These species allocate significant amounts of C from reserves to growth even after years of severe shading, resulting in constantly and strongly reduced NSC reserves. Such reduced NSC concentrations are likely to make them prone to other C-limiting events like defoliation or herbivory, eventually causing mortality.
2. Shade tolerant, late successional species such as *Fagus sylvatica or Abies alba* are "C-savers" in shade. These species maintain a high minimum C-reserve level, while growth remains low over time. NSC concentrations of such species might be less useful to infer a tree's C supply status, because they are largely influenced by a trade-off between storage and growth.

Materials and Methods

Study site and experimental set-up

The study was conducted between March 2015 and November 2017 in an open field *c.* 10 km south-west of Basel, Switzerland (47°29'40"N, 7°31'15"E). In March and April 2015, a total of 1020 three-year-old saplings from 10 common European forest tree species were purchased from a local nursery (Forstgarten Lobsingen, Switzerland): *Abies alba* MILL., *Betula pendula* ROTH, *Carpinus betulus* L., *Fagus sylvatica* L., *Picea abies* (L.) H.KARST, *Pinus sylvestris* L., *Prunus avium* L., *Pseudotsuga menziesii* (MIRBEL) FRANCO, *Quercus petraea* (MATTUSCHKA) LIEBL. and *Tilia platyphyllos* SCOP (Table 2.1). Hereafter the species will be referred to by their genus name only.

Table 2.1 List of investigated species, separated by functional groups and light levels, to which seedlings of the respective species are adapted to (1 = strong shade, 2 = intermediate shade, 3 = weak shade, 4 = bright light, Landolt et al. 2010).

Species	Functional group	Light adaptation
Abies alba	evergreen conifer	1
Picea abies	evergreen conifer	1
Pinus sylvestris	evergreen conifer	4
Pseudotsuga menziesii	evergreen conifer	2
Betula pendula	deciduous broad-leaved	4
Carpinus betulus	deciduous broad-leaved	2
Fagus sylvatica	deciduous broad-leaved	1
Prunus avium	deciduous broad-leaved	3
Quercus petraea	deciduous broad-leaved	3
Tilia platyphyllos	deciduous broad-leaved	2

Directly after delivery, 960 saplings were stored in a darkened 4 °C storage room before planting. 6 saplings of each species were harvested before planting, and processed as described below to obtain initial biomass and NSC reserves. The stored broad-leaved saplings were planted in the field on March 30, 2015, and conifers were planted on April 20, 2015. On an area of ca. 120 m^2, saplings were planted on 4 plots per species (40 plots total), with each plot including 24 saplings. The initial distance between saplings within one plot was 20 cm, and the distance between plots was 60

cm. Because saplings were continuously removed from the plots over the duration of the experiment, the space between the individual stems increased over time.

To induce C-limitation, a 3 m tall shading tunnel tent covered with a green four-layer plastic fiber shading net (Hortima AG, Hausen, Switzerland) was placed above 20 of the plots on a total area of 60 m^2 (12 m length and 5 m width). To prevent lethal stress during the initial phase after transplantation, the shading was put in place about six weeks after planting on June 2, 2015. During the subsequent 30 months until November 20, 2017, shaded saplings were exposed permanently to 6% of ambient sun-light (overall mean PPFD = 13 µmol m^{-2} s^{-1} during daytime hours, Fig. S1). The un-shaded half of the trees (control) received full sunlight (overall daytime mean PPFD = 201 µmol m^{-2} s^{-1}, Fig. S1). The mean daytime radiation during the growing season (April to October) was 29 µmol m^{-2} s^{-1} for shaded and 500 µmol m^{-2} s^{-1} for unshaded saplings. During the entire growing season of 2016, broad-leaved species assimilated 84-97% less C (g $sapling^{-1}$) under shade, based on calculations using PPFD plus temperature loggings combined with photosynthetic light response curves, specific leaf area and total leaf mass measurements (data not shown). In addition to the natural rainfall (645, 997 and 765 mm a^{-1} during 2015-2017, 7 km north-east from the study site; data provided by MeteoSwiss), plots were watered in dry periods to ensure sufficient water supply. Mean soil water potential over the last two seasons at 20 cm soil depth was -0.07±0.04 MPa in shade vs. -0.08±0.02 MPa in the control treatment (n = 5, MPS-2 sensors, Decagon, Pullman WA, USA). Mean annual soil temperature was 9.8°C vs. 11.7°C (MPS-2 sensors, Decagon, Pullman WA, USA) in shade and controls, respectively, while mean annual air temperature was 10.3°C in shade vs. 10.8°C in the control plots (HOBO pro V2 sensors, Onset, Cape Cod MA, USA, Fig. S2).

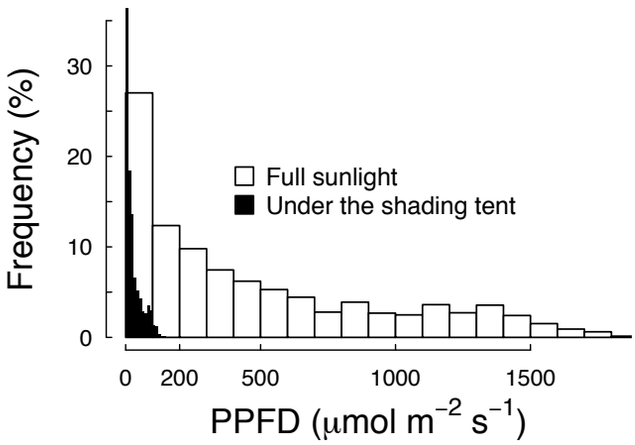

Fig. 2.1 Histogram of daylight (photosynthetic photon flux density) measurements taken (HOBO pendant, Onset, Cape Cod, MA, USA) at the field site over the course of 29 months, with a temporal resolution of 15 min. Light intensity values were converted from Lux to μmol m^{-2} s^{-1} with a calibration factor retrieved from PPFD measurements during the same time at the field site, measured with a *LI-6400xt* (LI-COR, Lincoln, NE, USA).

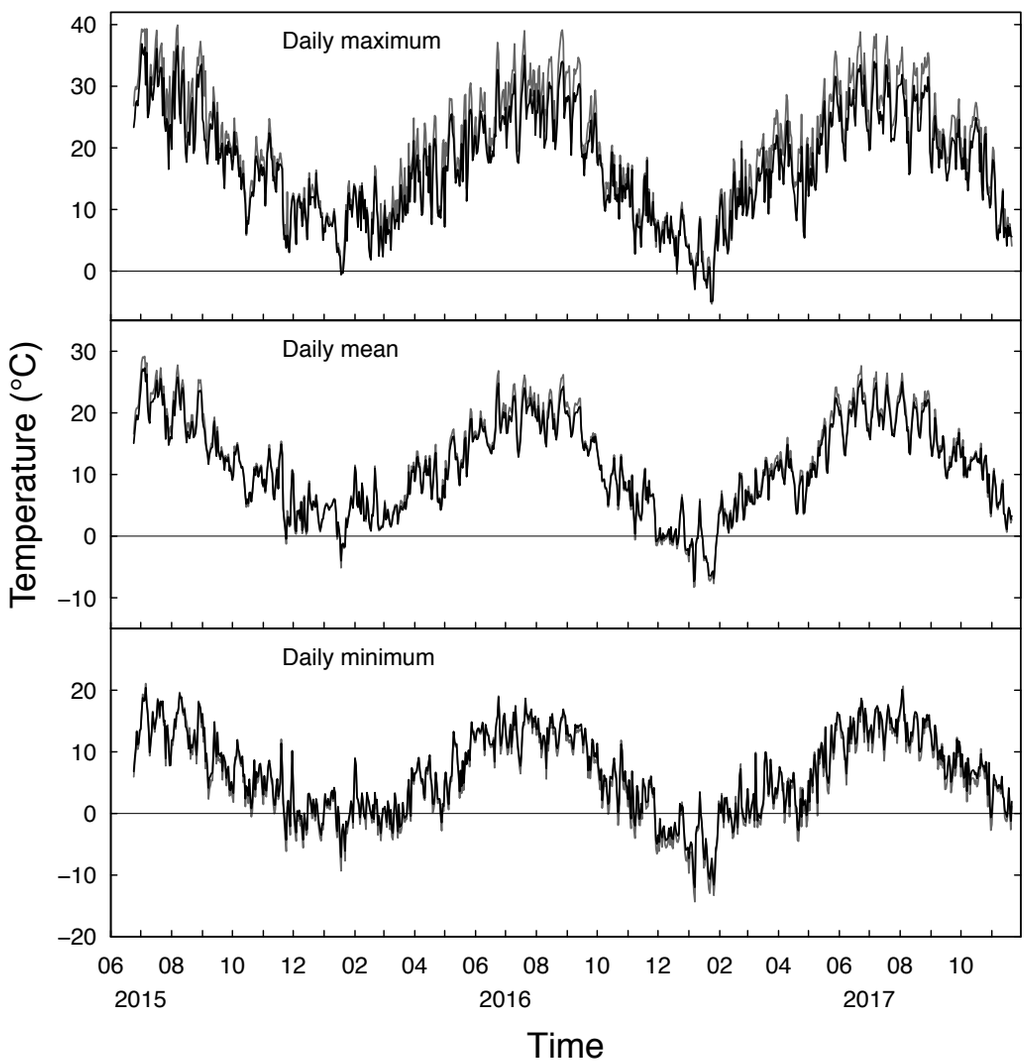

Fig. 2.2 Fluctuations of daily air temperatures in the open at full sunlight (grey) and inside the shading tent at 6% light (black) during the study time over three seasons.

Sampling and field measurements

To quantify biomass increment and collect tissue samples for NSC analyses, 6 saplings per treatment and species were harvested each year in March (before bud break in spring), July (mid-season) and November (after leaf fall in autumn) except in 2017, when no saplings were harvested in July, due to the limited number of surviving trees. After harvest, all sampled saplings were immediately separated into the following organs: belowground tissue, stems, and leaves. All samples were oven-dried at 80°C for 72 h, and aboveground dry biomass (without leaves in deciduous species) was weighed to quantify growth. For NSC analysis, a small subsample (~1 g dry mass) of stem wood (without bark), root wood (without bark) and leaves of each sapling was shock-heated in a microwave at 900 W twice for 15 s to stop enzymatic activity (Popp et al. 1996), and then oven-dried at 80°C for 72 h. After measuring tissue dry biomass, all samples were ground to a fine homogenous powder with a horizontal ball-mill (MM 400, Retsch, Haan, Germany), and the powder was then stored dry until chemical analysis.

Because shoots tended to growth rather horizontally under shade, the shoot length was measured as the distance between the root collar and the tip of the longest branch.

In August during all three growing seasons, the specific leaf area (SLA, m^2 kg^{-1}) was measured in four saplings from both light treatments in the six broad-leaved species. Round leaf stamps with 5 mm diameter were taken from three different leaves of each sapling, and immediately oven-dried at 80°C for 72 h. Leaf stamps from one sapling were weighed together and related to the initial stamp area to calculate the mean sapling SLA as m^2 kg^{-1}.

NSC analyses

Non-structural carbohydrates (NSCs) were analysed after a modified protocol by Wong (1990) and Hoch et al. (2002) as described in Weber et al. (2018). In detail, 8 – 12 mg of the plant powder were weighted into 6 ml glass vials and extracted with 2 ml distilled water by boiling at 100°C for 30 minutes over steam. An aliquot of 200 µl of the extract was treated with invertase from *bakers yeast* (Sigma-Aldrich, St. Louis, MO, USA) to degrade sucrose into glucose and fructose. 100 µl of this extract was mixed with 100 µl of a glucose-hexokinase assay (glucose-hexokinase assay reagent,

Sigma Aldrich, St. Louis, MO, USA) thereby converting glucose to glucose-6-P and fructose to fructose-6-P. Added isomerase (from bakers yeast; Sigma-Aldrich, St. Louis, MO, USA) converted fructose-6-P to glucose-6-P. Due to the presence of NADP in the assay, all glucose-6-P was converted to gluconate-6-P and NADPH. Finally, the concentration of NADPH (equating to the concentration of glucose) was determined photometrically at 340 nm in a 96-well micro plate photometer (HR 7000, Hamilton, Reno, NE, USA). 500 µl of the original extract were treated with a fungal amyloglucosidase from *Aspergillus niger* (Sigma-Aldrich, St. Louis, MO, USA) in a 49°C water bath over night, to break down starch to glucose, and the total glucose (corresponding to NSCs) was determined photometrically as described above. The concentration of starch was calculated as total NSCs minus low-molecular weight sugars. To ensure activity of the enzymes, pure starch and solutions of glucose, fructose and sucrose were used as standards, and to control reproducibility of the extraction, standard plant powder (Orchard leaves, Leco, St. Joseph, MI, USA) was included. All NSC concentrations are given as % dry matter.

Additional defoliation treatment and mortality monitoring

To investigate the effect of an additional C-limitation stress, $n = 4$ control and shaded *Carpinus* were completely defoliated on June 5, 2017. The regrowth was monitored throughout the season and all defoliated trees were harvested in November 2017 as given above for growth and NSC analyses. Saplings were regularly visually monitored for mortality during each growing season. Ignoring the mortality following transplantation that was mainly observed in *Pinus*, mortality rates were calculated starting from March 2016 in each species and treatment, giving the fraction of dead saplings among all planted saplings per species and treatment. In Summer 2016, especially high mortality occurred in shaded *Fagus*, most probably due to strong infestation by aphids (*Phyllaphis fagi* L.) mainly on shaded individuals. As a counteraction, we treated all *Fagus* saplings with insecticide on (Pyrethrine; Gesal, Compo, Allschwil, Switzerland) in the early season of 2017.

Calculations and statistics

To compare the whole-sapling NSC concentrations among treatments and dates, a weighted mean concentration of NSCs was calculated for each sapling by integrating tissue mass and NSC concentrations as described in Hoch et al. (2002), using the following formula:

$$\sum_{org=1}^{n} \frac{conc_{org} \text{x } biom_{org}}{100}$$

where n is the number of organs, $conc_{org}$ is the organ specific NSC concentration (% dry matter) and $biom_{org}$ the organ specific fraction of the total biomass.

Because the strongest shoot growth occurred in May and June, we pooled aboveground biomass measurements from July, November from one year and subsequent March of the following year to calculate the absolute biomass after each growing season. This reduced the variation caused by random selection of individuals. The annual relative growth rate (RGR, g g^{-1} a^{-1}) was then calculated, dividing the mean biomass after each growing season by the mean biomass before the growing season. The standard error (SE) of the RGR in each growing season (as shown in Fig. 2.7) was propagated from the SE of absolute dry biomass before and after each growing season, using a general formula for error propagation in ratios (Geary 1930).

To investigate the trajectory of growth versus NSCs in reaction to shade, we compared the annual RGR with the November mean sapling NSC concentrations among each species and season. NSC from March and July was omitted for this trajectory, since NSCs likely show maximum variation during summer in deciduous broad-leaved species, and during spring in evergreen conifers.

Significant differences between light treatments on growth and NSC concentrations at specific harvest dates were determined by Student's t-tests. To test the effect of shading on growth, we performed a full-factorial ANOVA with harvest season and species as fixed factors. To test NSC concentrations changes over winter, we performed individual two-factorial ANOVA tests with sampling season (autumn or spring) and shading year (2015 or 2016) as fixed factors. The influence of the light treatment on the seasonal variation of NSCs, was tested for significance for every

species with a two-factorial ANOVA with treatment, harvest date and their interaction as factors. Biomass and NSC concentrations of *Carpinus* were tested for significant differences ($\alpha = 0.05$) among defoliation and light treatments with Tukey's Honest Significant Difference (HSD) test.

Results

Phenology and survival

Leaves and needles of shaded saplings showed a darker green, and were more horizontally oriented compared to unshaded controls of all species (Fig. 2.3). In most species, leaf bud break in spring 2016 and 2017 was advanced in shade by two to three weeks (Fig. 2.4). However, bud break in shaded *Betula* was delayed by *c.* three weeks compared to controls (Fig. 2.4).

Fig. 2.3 Photographs taken at 100% light in the open (a-b) and at 6% light under the shading tent (c-d) from two species: *Abies alba* (a,c) and *Prunus avium* (b,d). Photographs were taken shortly after leaf flush on May 9, 2017, which was after 24 months of shading in c-d.

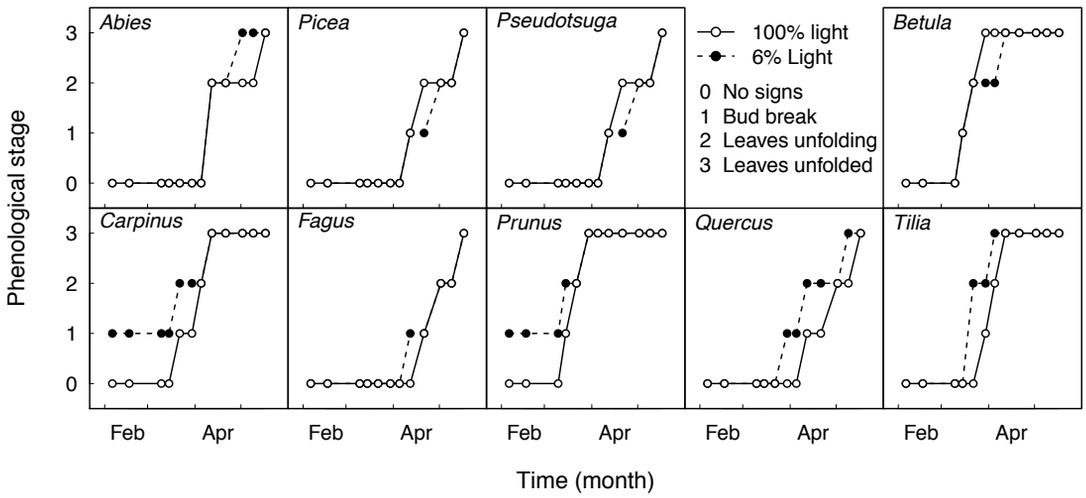

Fig. 2.4 Leaf flush observations in spring 2017. The rise from one phenological stage to the other was marked when the next stage was observed in >50% of all seedlings per species and treatment.

In June 2015 after transplantation, conifers (shaded and controls) flushed new needles *c.* one month after the deciduous species. In spring 2016, most shaded *Picea* still flushed new leaves with a delay of two months compared to controls in 2016, but flushed early again in April 2017. Leaf fall of deciduous species in autumn was delayed by about one week under shade compared to controls in most species, and over four weeks in shaded *Prunus* and *Tilia.* SLA in broadleaved species increased by 134-198% (depending on the species) in shade across all three seasons (t-test, $p < 0.001$). Mortality occurred in shade during the first shading season (2015) in *Pinus* and *Pseudotsuga,* and no *Pinus* sapling in shade survived Winter 2015/2016 (Fig. 2.5). In 2016, mortality was high in *Pseudotsuga*, *Fagus, Tilia* and *Betula* (25-61%; Fig. 2.5).

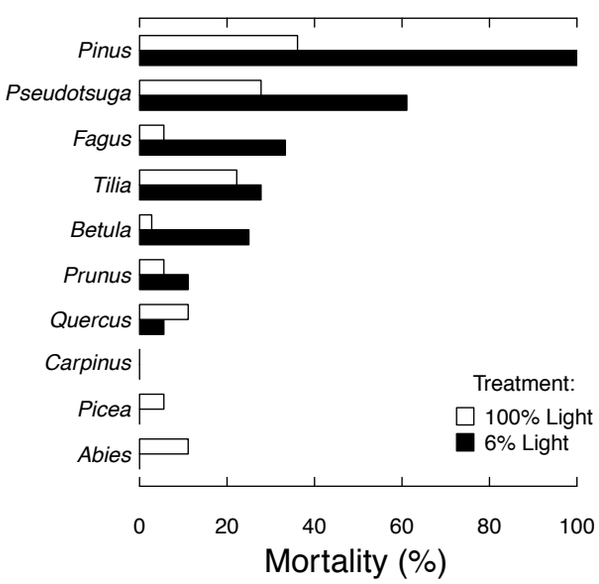

Fig. 2.5 Seedling mortality among 10 different species between death of the first seedling in December 2015 and the end of the experiment in November 2017. Percentage of mortality equals the number of dead seedlings per planted seedlings.

Aboveground growth

Likely also due to the later start of shading in 2015, the aboveground biomass increment over the entire season was not significantly different between controls and shaded saplings (Fig. 2.6). In 2016 and 2017, aboveground biomass increased exponentially in control saplings. After June 2016, biomass in controls was significantly different from shaded individuals in most species, except in *Pseudotsuga* and *Tilia* (Fig. 2.6). After June 2017, biomass in controls was significantly different from shaded saplings in all species (Fig. 2.6). During 2017, biomass increased moderately but significantly in all species in shade (two-factorial ANOVA, $p < 0.01$).

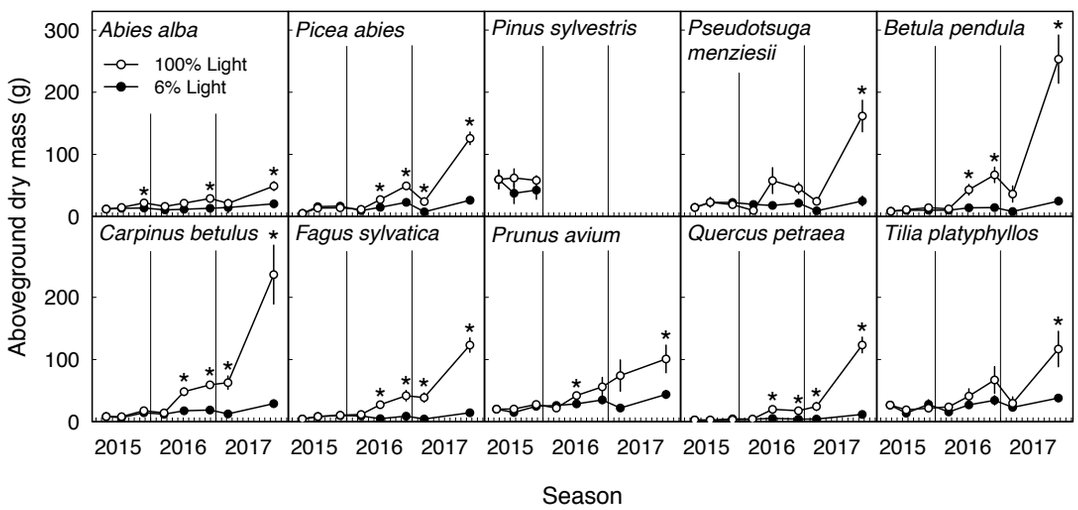

Fig. 2.6 Aboveground dry biomass in 10 species (without leaves in deciduous species) at 100% sunlight (open circles) and at 6% light (closed circles) during three years (means ± SE, $n = 6$). Asterisks above open circles indicate significant differences between controls and shaded individuals at a given harvest date (*t*-test, $p < 0.05$).

In 2015, the aboveground RGR in controls and shaded saplings was around 1 g g^{-1} a^{-1}, but increased in 2016 and 2017 to values between 1 and 4 g g^{-1} a^{-1} in controls of most species, except in *Abies*, where RGR in controls remained very low around 0.5 g g^{-1} a^{-1} (Fig. 2.7). In shaded saplings, RGR remained at or below 1 g g^{-1} a^{-1} in all species throughout the three seasons. Broad-leaved species had 0-38% less leaf dry mass per individual in shade compared to controls in July 2015, and 57-93% less in July 2016 (data not shown). Shoot length did not differ between light treatments in 2015, but was significantly shorter in shaded compared to controls in *Fagus* and *Betula* saplings by the end of the second growing season (Fig. 2.8). At the end of the experiment in autumn 2017, only *Prunus* and *Tilia* had similar shoot length in both light treatments

(*t*-test, $p > 0.05$), while in the remainder species shoot length was 22-50% shorter in shaded compared to control saplings (Fig. 2.8).

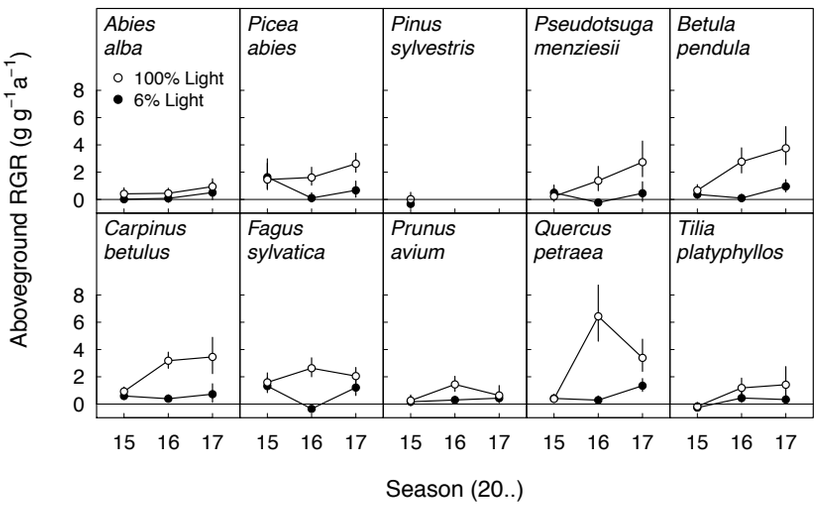

Fig. 2.7 Aboveground relative growth rate (RGR) as described in the methods section. Data from July, November and subsequent March after each annual growth in spring were pooled (propagated means ± SE, $n = 18$).

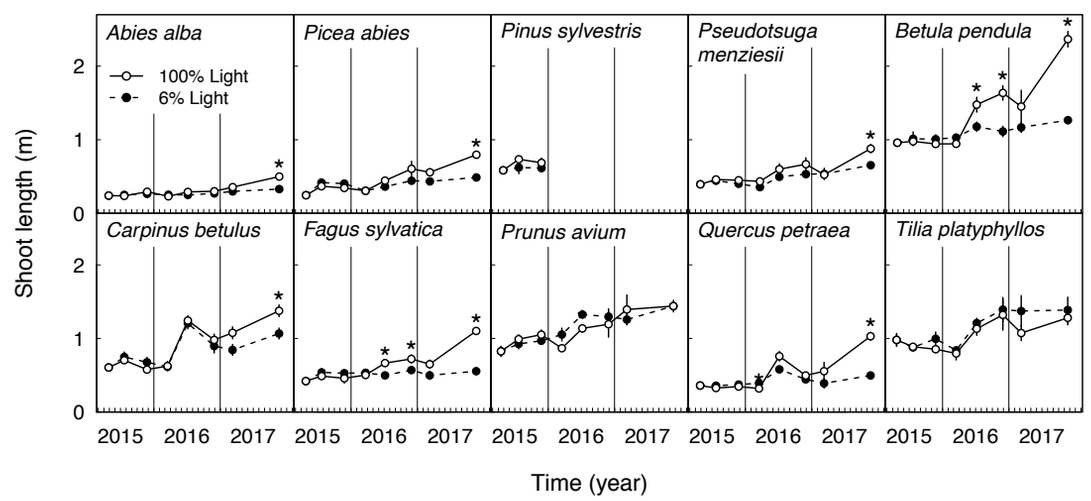

Fig. 2.8 Aboveground shoot length in 10 species at 100% sunlight (open circles) and at 6% light (closed circles) during three years (means ± SE, $n = 6$). Asterisks above open circles indicate significant differences between controls and shaded individuals at a given harvest date (*t*-test, $p < 0.05$).

Non-structural carbohydrates

Across all species, NSC concentrations of shaded saplings decreased by about 50% in the first year, but approached values of unshaded control saplings during the second year, and completely recovered to control values by the end of third season (Fig. 2.9). However, beside this overall trend among all species, we observed species- and functional type specific responses of NSC concentrations to shade.

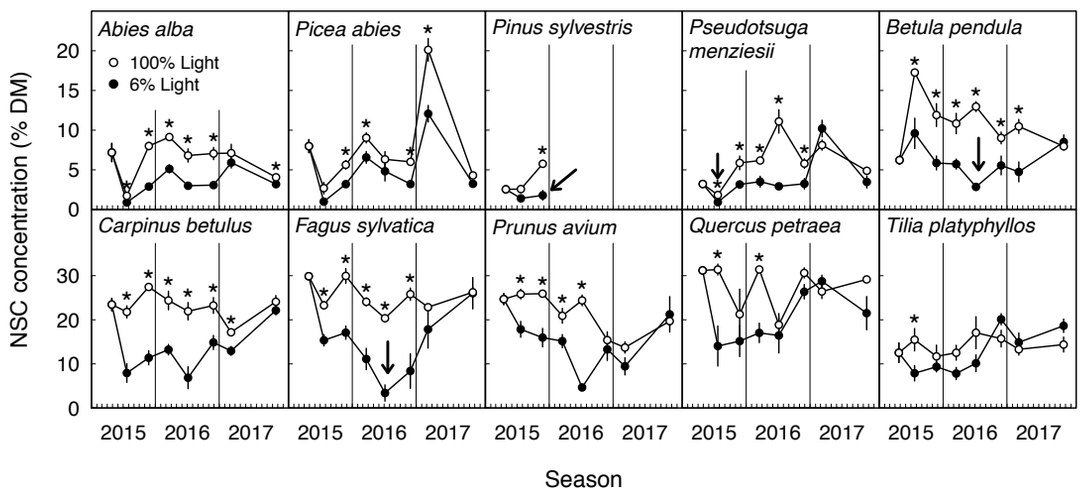

Fig. 2.9 Time course of non-structural carbohydrates (NSCs) concentrations in ten different species at 100% light (open circles) and 6% light (closed circles) between March 2015 and November 2017. NSCs are given as the mean (± SE) of the weighted concentrations per dry matter over entire individuals as described in the Methods section (n = 6). Leaves were excluded in deciduous species. Mortality events with over six fatalities per treatment are marked with black arrows. Asterisks above open circles indicate significant differences between controls and shaded individuals at a given harvest date (t-test, $p < 0.05$).

In deciduous broad-leaved species, the pre-treatment NSC concentrations (weighted among organs) were between 6 % (*Betula*) and 31 % DM (*Quercus;* Fig. 2.9). In most deciduous species, weighted whole-sapling NSC concentrations remained unchanged between March and July 2015 in controls, but declined to 8-18 % DM in shade. One exception was *Betula*, where NSCs increased during the first half of the season 2015 in shaded saplings, and even stronger, in controls (Fig. 2.9). Weighted whole-sapling NSC concentrations did not recover in shade by November of the first season, resulting in 20 % (*Tilia*) to 60 % (*Carpinus*) lower NSC concentrations compared to controls (Fig. 2.9). From November 2015 until the end of the experiment, whole-sapling NSC concentrations remained above 7 % DM in unshaded control saplings of all broad-leaved species. Between March and July 2016, whole-sapling NSCs

declined strongly in shaded *Fagus, Prunus, Betula* and *Carpinus* to 18 %, 19 %, 22 %, and 32 % of control concentrations, respectively, and less strongly in *Tilia* (59 % of control) and *Quercus* (80 % of control, Fig. 2.9). By November 2016, weighted whole-sapling NSC concentrations in *Tilia*, *Quercus* and *Prunus* did not differ significantly between shaded and control saplings (Fig. 2.9), while concentrations in shade *Betula* and *Carpinus* were around 60 % of control concentrations, and shaded *Fagus* (most likely weakened by a strong aphid infestation in summer) showed only 32% of control NSC concentrations by November 2016. By the end of the experiment in November 2017, weighted whole-sapling NSC concentrations were not significantly different anymore between shaded and controls in all broad-leaved species (Fig. 2.9).

In all four investigated conifer species, NSCs declined between March and July in shaded and control saplings during the first season (2015), but the whole-sapling mean concentrations increased again until November in both treatments (Fig. 2.9). While the NSC concentrations in November 2015 were similar or higher than those measured in March 2015 in controls, they were significantly lower in shaded saplings (Fig. 2.9). Especially low NSC concentrations were found in shaded *Pinus*, which also showed complete mortality in shade by the end of the first season.
While NSCs were consequently not measured anymore after 2015 in *Pinus*, the remaining three conifers showed all markedly higher NSC concentrations in March compared to November in 2016 and 2017, indicating substantial photosynthesis over winter (Fig. 2.9). By November 2016, weighted whole-sapling NSC concentrations in shaded conifers were still significantly lower from controls, ranging from 43% *(Abies)* to 56% *(Pseudotsuga)* of controls (Fig. 2.9). By the end of the experiment in November 2017, weighted whole-sapling NSC concentrations in shade saplings had reached control levels in *Picea* and *Pseudotsuga*, but not in *Abies* (78% of control levels, Fig. 2.9).

In contrast to the broad-leaved species, the seasonal variation of NSC concentrations was more similar between shaded and control saplings in conifers. Over all three seasons, the light treatment did not interact with the sampling date in *Abies*, *Picea*, and *Pseudotsuga* (two-factorial ANOVA with interaction, $p > 0.05$).

In all investigated broad-leaved and conifer species, NSC concentrations in shoot wood were 23-62% lower than in root wood (Fig. S7, S8). NSCs in leaves of broad-leaved species contributed 6-16%, and in conifer needles 60-78 % to the total sapling NSC pool (Fig. S9). The above-mentioned increase of conifer NSC reserves during winter was entirely caused by increases in needle NSC concentrations in these evergreen trees.

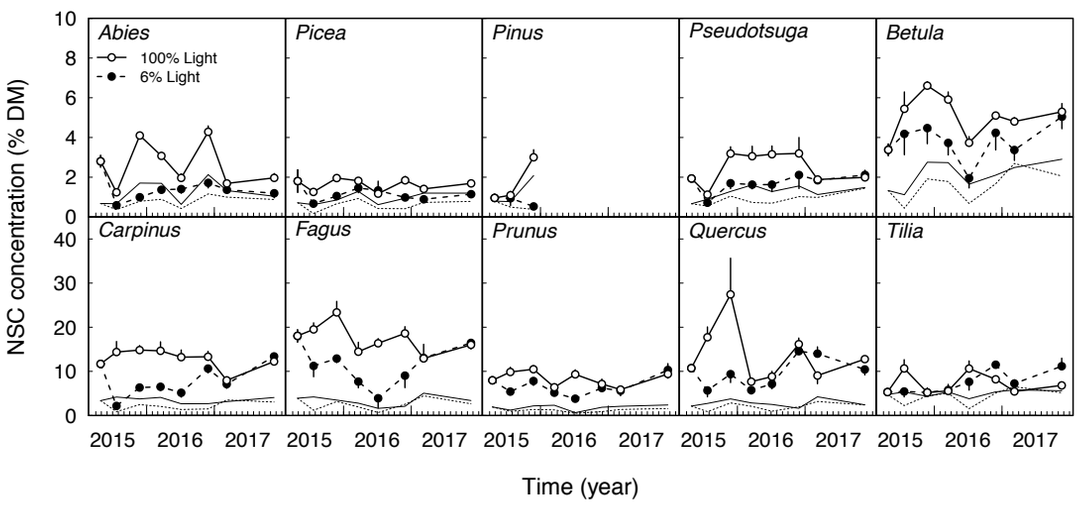

Fig. 2.10 Stem wood: Time course of non-structural carbohydrates (NSC = starch and sugars; circles; means ± SE) and sugars (lines without circles; means) in ten species and two different light treatments over three years. $n = 6$.

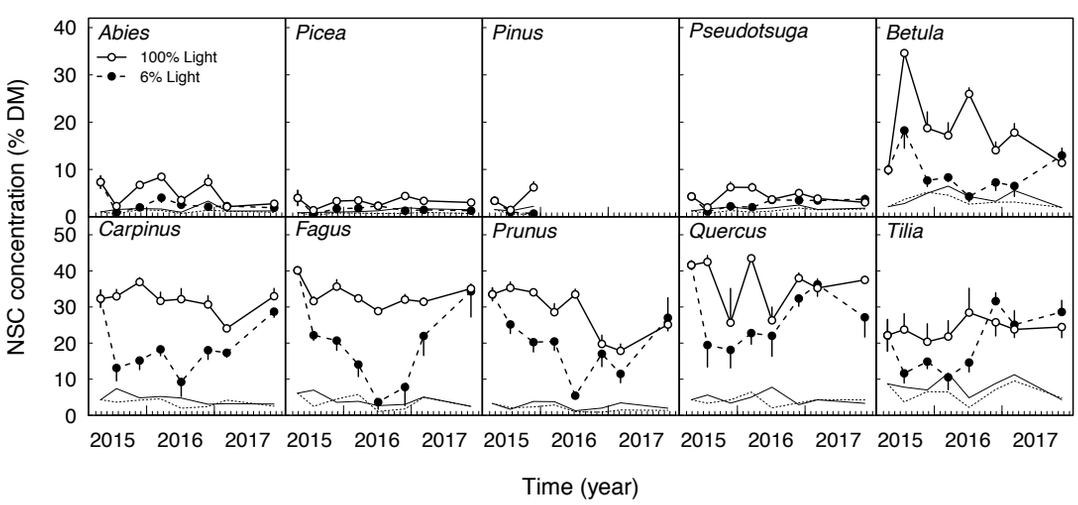

Fig. 2.11 Root wood: Time course of non-structural carbohydrates (NSC = starch and sugars; circles; means ± SE) and sugars (lines without circles; means) in ten species and two different light treatments over three years. $n = 6$.

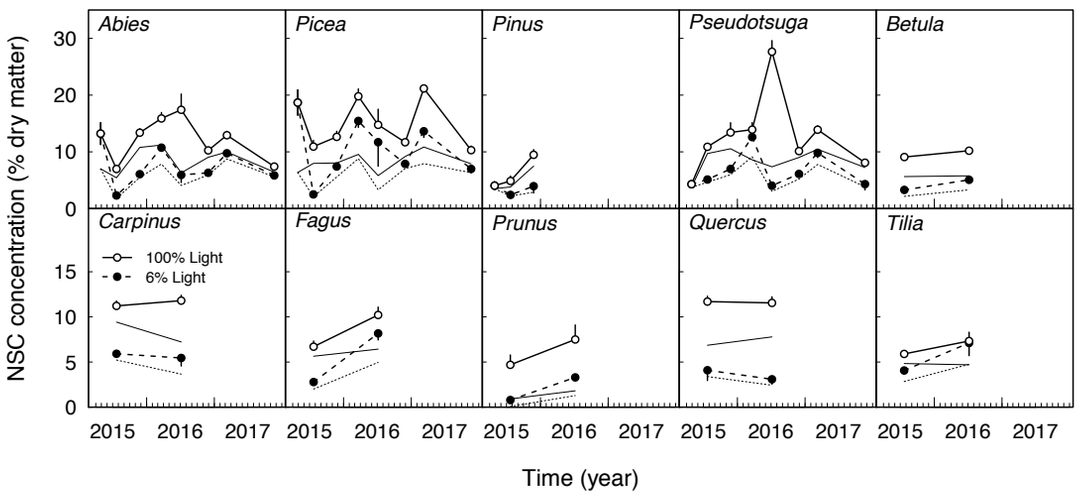

Fig. 2.12 Leaves: Time course of non-structural carbohydrates (NSC = starch and sugars; circles; means ± SE) and sugars (lines without circles; means) in ten species and two different light treatments over three years. Leaves in deciduous species were only harvested in summer 2015 and 2016. $n = 6$.

Additional defoliation treatment and herbivory

In 2017, we performed an additional defoliation treatment on *Carpinus* saplings. All defoliated saplings produced new leaves within two weeks after defoliation, except one defoliated, shaded sapling, which died after defoliation, without having flushed new leaves. NSC concentrations in defoliated *Carpinus* saplings recovered completely to the levels of undefoliated saplings until November in unshaded saplings. In contrast, NSC concentrations in November were severely depleted in defoliated saplings from the shading treatment (0.8% and 1.0% DM in stem and root wood, respectively; Fig. 2.13). In stem wood of defoliated, shaded saplings, starch concentrations were decreased by almost 100% and sugars by 80%, compared to non-defoliated saplings at full sunlight. Starch and sugars in root wood were decreased by 98% and 87%, respectively.

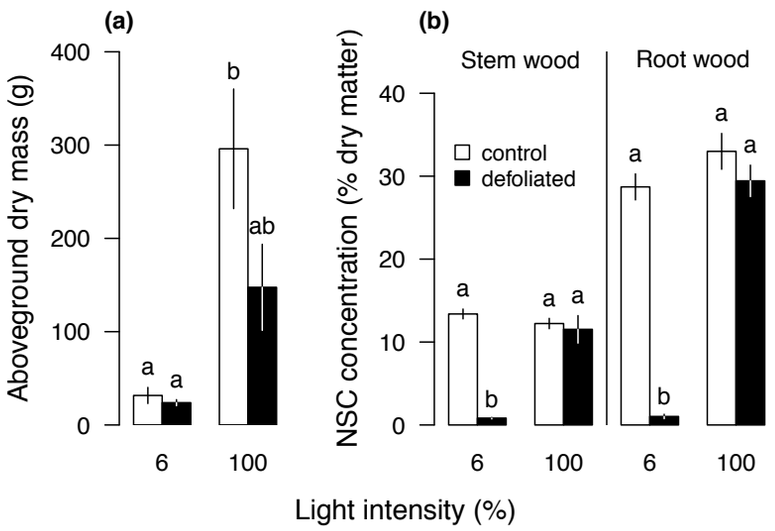

Fig. 2.13 Aboveground dry biomass (a, means ± SE) and non-structural carbohydrates (NSCs, b, means ± SE) in *Carpinus betulus* in November 2017, five months after defoliation (black) and in non-defoliated seedlings (white) at different light levels in stem wood (b, left) and root wood (b, right). Statistical significance letters above bars are comparable within biomass data only, and in NSC data within one organ only (Tukey's HSD, $p < 0.05$, $n = 4.8$).

Although NSCs were not measured in dead individuals, mortality was mostly observed when NSC concentrations of the surviving individuals from the same treatment and species were also low, like in shaded *Pinus* in November 2015 (weighted mean NSCs of 1.8% DM, Fig. 2.9), or in shaded and herbivore-stressed *Fagus* in July 2016 (4.0% DM, Fig. 2.9). We observed an overall minimum stem wood NSC concentration of 0.5% DM in shaded *Pinus* in November 2015 (Fig. 2.11), a minimum root wood NSC concentration of 0.6% DM in shaded *Picea* in July 2015 (Fig. 2.10). However, we observed no mortality in shaded *Abies* and *Picea*, despite very low weighted whole-sapling NSC concentrations of 0.9% DM and 1.0% DM, respectively, in July 2015 (Fig. 2.9).

Growth vs. NSCs under long-term C-limitation

A comparison of November NSC concentrations with the RGR in each growing season yielded a similar trajectory of growth versus storage in all species (Fig. 2.14). We observed a stronger reduction of RGR in shade than the reduction of end of season NSC concentrations compared to controls, in line with a "C-saver" strategy in both full shading seasons 2016 and 2017 (number "1" and "2" in Fig. 2.14). No

species showed a higher reduction of NSC concentrations compared to the reduction in RGR, (as indicative of a "C-spender" strategy). No trajectory could be observed in *Pinus*, due to the complete mortality of shaded saplings in 2015. The relatively late start of shading in the middle of the "acclimation season" 2015 (marked as "0" in Fig. 2.14), caused annual RGR in shade to be as high as in controls, which possibly hindered recovery of NSC concentrations by fall after the start of shading. Generally, the change in RGR versus the change in NSC concentration did not correspond to the species' light adaptations (Table 2.1), since light adapted and early successional species did not show more growth or less NSCs than shade adapted, late successional species (Fig. 2.14).

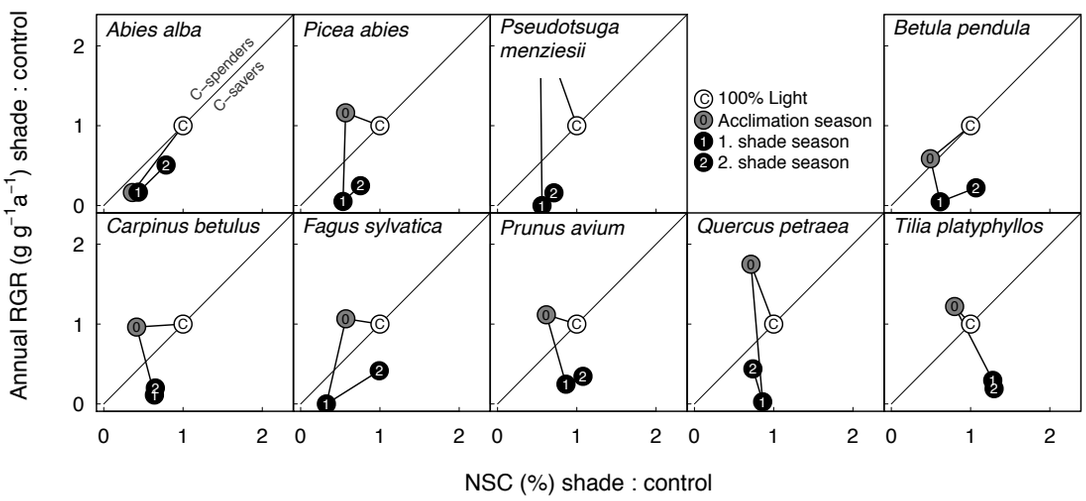

Fig. 2.14 Comparison of NSC storage and growth in nine species: The shade : control ratio of weighted whole-sapling NSC concentrations in November of three different seasons (x-axis, propagated means, $n = 6$) versus the shade : control ratio of the annual aboveground dry matter RGR in each season (y-axis, propagated means, $n = 18$, see methods section). Note that the point '0' for *Pseudotsuga* is higher than 2 (2.3) and thus not visible.

Discussion

Main findings

Within this study, we showed that in tree saplings that have been exposed to long-term C-limitation by shading, growth rates were modified to ensure a species-specific NSC concentration, and that after two full seasons, this 'target' concentration was almost equal between both applied light treatments. Future studies should thus be aware of such an up-regulation when using NSC concentrations of temperate species as an indicator for C limitation. However, we demonstrated that a recent onset of C-

limitation is reflected in decreased NSCs, and life-threatening C-limitation induces a depletion of C reserves to less than *c.* 20% of controls, eventually after a re-flush of leaves. This highlights that the observed priority of NSC storage is lost below a certain C availability. Furthermore, C allocation preferences (storage vs. growth) under shade did not correlate with species-specific shade tolerance, which suggests more complex mechanisms behind shade tolerance.

Seasonal NSC fluctuations

Seasonal fluctuations of NSC concentrations in conifers in this study correspond to those in other studies (Hoch et al. 2003, Bansal and Germino 2008). However, the inconsistent fluctuations of NSC concentrations during summer between both light treatments in broad-leaved species indicate that 6% light not only caused an initial decrease in NSC concentrations, but also altered the seasonal NSC dynamics. The advanced leaf flush in *Carpinus*, *Prunus*, *Quercus* and *Tilia* in shade, which was likely caused by slightly milder minimum temperatures in winter under the shading net, probably caused an advance in NSC fluctuations. However, since leaf flush was delayed in shaded *Betula*, we need to assume that seasonal NSC fluctuations were delayed in this species. The lack of interaction between harvest date and light treatment in *Fagus*, suggests that phenology in *Fagus* was not influenced by the slightly warmer winter minima temperatures under shade, which corresponds to the well-known photoperiodic control of bud break in this species (Heide 1993, Basler and Körner 2012).

Significance of Non-structural carbohydrates in shade

Consistent with previous deep-shade experiments, we found a two-fold decrease of NSC concentrations after one shading season (Veneklaas and den Ouden 2005, Piper et al. 2009, Maguire and Kobe 2015). The complete recovery of NSC concentrations to control levels after three seasons corresponds to findings in a tropical forest, where NSC concentrations even decreased with light availability in naturally grown saplings (Poorter and Kitajima 2007). The same study clearly highlighted the importance of NSC reserves in shade. But in contrast to the mentioned study, we did not find increased NSCs in shade, and NSC concentrations as such did not correlate with the species' shade tolerance (Table 1), which was also not the case in other shading

studies (Lusk and Piper 2007, Piper et al. 2009). For example, we found the lowest overall NSC concentrations at low light in shade-tolerant *Abies*, despite a complete absence of mortality in shade, and at the end of the experiment, *Abies* was the only species with significantly lower NSC concentrations in shade compared to controls. In contrast to Kobe (1997), our data suggest that survival in shade depends on more factors than stored NSCs. Unlike Kobe et al. (1995), we also found no correlation between low-light survivorship and growth rate at high light, due to the high survival at low light in fast-growing species like *Carpinus* and *Picea*. The applied 6% of full sunlight environment in this experiment compares well to the 5-7% of above-canopy radiation that is on average found in the understory of a mixed conifer-deciduous forest (Messier et al. 1998), and it is slightly lower than the 7-13% reported for a boreal birch forest (Messier et al. 1998). Therefore, the close to natural light levels in our "low light" treatment did not induce enough mortality in most investigated species to draw general conclusions about shade tolerance.

We quantified NSC concentrations related to the sapling dry matter. Thus, NSC concentrations might be influenced by structural tissue density (Würth et al. 2005). However, the observed strong up-regulation of NSC concentrations before autumn can be hardly explained alone by a decrease in tissue density under shade.

Mortality

Lethal C-limitation seems to always occur after a depletion of NSC below *c.* 1% DM, as previously described in darkened saplings (Marshall and Waring 1985, Sevanto et al. 2014, Wiley et al. 2017, Weber et al. 2018) and in defoliated *Carpinus* during this experiment. Such low concentrations indicate that the plant internal prioritization of NSC storage under C-limitation gets lost, when the C availability becomes too low. We therefore suggest a species-specific threshold percentage level of full sunlight, at which C storage is abandoned to supply vital C-sinks like respiration or the production of etiolated shoots before C-starvation (Wiley et al. 2017, Weber et al. 2018). Based on the observed low NSC concentrations and the complete mortality in *Pinus*, such a threshold is likely over 6% of full sunlight in *Pinus sylvestris*, and probably below 6% in the remaining investigated species. Such a threshold might correspond well to inter-specific shade tolerance.

Did NSC up-regulation cause a decrease in growth?

The annual NSC and RGR reduction in shade across all species results in an apparent optimum trajectory (Fig. 6). In contrast to our initial hypothesis, all investigated species tended to "save" their C at low light. While the complete refilling of NSC to 100% of control concentrations was achieved by most species by the end of the third season, the annual RGR remained at around 30% of controls among all species (Fig. 6). Still, additional root biomass measurements could have substantially changed the total biomass RGR in shade. But it seems unlikely that shaded saplings allocated more of the scarcely available C into roots than into shoots, given the wet and nutrient-rich soil conditions at the study site (Dias-Filho 2000, Schall et al. 2012).

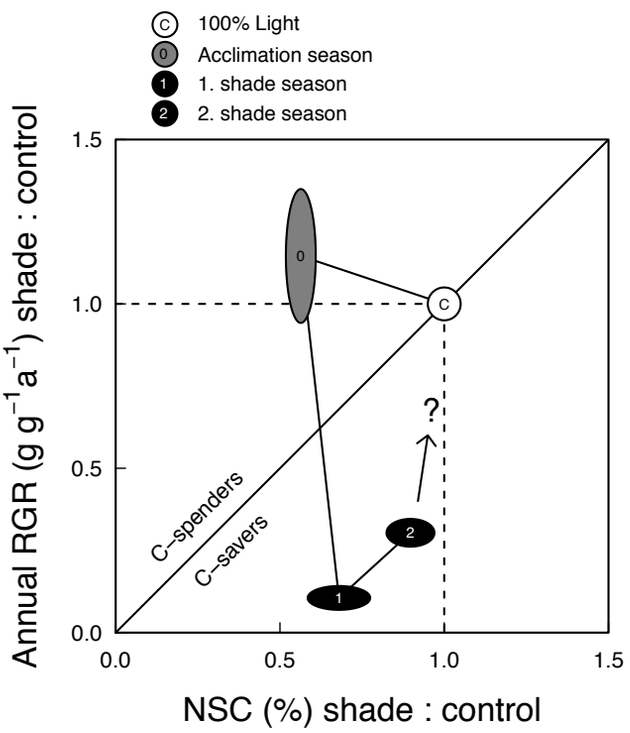

Fig. 2.15 Comparison of NSC storage and growth (relative growth rate, RGR) in three different seasons at 100% light and 6% light. X-axis; the shade : control ratio of weighted whole-sapling NSC concentrations after leaf fall in November (means across species ± SE, n = 9). Y-axis; the shade : control ratio of the annual aboveground dry matter RGR in each season (mean across species ± SE, n = 9).

Thus, even shade intolerant species tended to save their C during strong C-limitation, revealing a high risk of C investment into growth when C is scarce. The partial recovery of RGR after the second shading season implies that saplings in deep shade follow a "wait-and-see" strategy, where growth rates are modified in order to ensure a target NSC concentration in fall. So far, an up-regulation of C storage at direct expense of growth could not yet be shown in trees, because growth might also have

declined in response to leaf loss (Palacio et al. 2012, Wiley et al. 2013, Schmid et al. 2017, Weber et al. 2018). In this study, C-limitation caused no leaf loss and was constantly present over three years. The presence of C-limitation in shaded lianas was previously confirmed by Granados and Körner (2002) who showed a higher relative growth effect of elevated CO_2 at low compared to high light. The most plausible explanation for the high NSC concentrations under shade found in our study is therefore an up-regulation of NSC storage activities, presumably at the expense of growth. These findings support the assumption of Wiley and Helliker (2012) that trees might favor C reserve formation over growth at C-limitation. However, our results do not necessarily support the second assumption by Wiley and Helliker (2012), that C-limitation can actually lead to higher NSC pools compared with sufficiently C supplied trees. Additionally, we explain these observations by a lower cost-benefit ratio of reserve formation compared to reserve depletion at the favor of growth. For example, the maintenance of 15% DM NSCs as an important buffer against C-limiting events, obviously corresponds to a reduction of structural growth by only about 15% (which are allocated to storage).

In contrast to the priority of NSC storage in shaded *Carpinus*, we observed a reversal of C allocation priorities in shaded and defoliated *Carpinus*. Defoliation caused saplings to invest most of the available NSC into leaf production, although C assimilation for the rest of the season in shaded saplings seems to have gained only 4% of the concentrations in unshaded saplings (Fig. 4). Leaf production thus seems to be of higher priority than NSC up-regulation under lethal C-limiting conditions, which again confirms that below a certain C availability in saplings, NSC storage loses its priority above growth, as observed recently under complete darkness (Wiley et al. 2017, Weber et al. 2018). In all investigated species and tissues, it seems that NSC concentrations lower than 20% compared to those in controls indicated a life-threatening C-limitation.

In deciduous species, NSCs tended to be more depleted during summer under shade compared to controls. For example, we observed a strong depletion of NSC in shaded *Prunus* in July 2016, despite similar NSC reserves in shade and controls in the precedent and subsequent winter (Fig. 3). Such depletion indicates that leaf flush in

spring induced a longer-lasting depletion of NSCs under shade, than at ambient sunlight, and additional C-limiting factors then might have intensified such depletion. We thus suggest, that the likelihood of reduced NSC pools in summer is higher in deciduous species. Detection of present C-limitation in deciduous species is therefore more probable when NSCs are assessed a few weeks after leaf flush. In the investigated evergreen species, such "detection time-frame" was not observed, probably because evergreens have the ability to assimilate C during winter before the next leaf flush and thus do not exclusively rely on stored NSC reserves on a periodic basis. However, decreasing NSCs during leaf flush in evergreen oaks, suggest that a time frame for the detection C-limitation might also exist in some evergreen species (Palacio et al. 2018).

In conclusion, our findings emphasize that measurements of tissue NSC concentrations should be used very cautiously in order to assess the C supply status of trees. Certainly, such assessments always require the comparative measurements of NSC concentrations in potentially C-limited and not C-limited control trees. Our findings further highlight the necessity to account for the seasonal dynamics of NSC pools, instead of comparing a single date in order to predict the absence or presence of C-limitation in trees. It remains to be tested, if these results gained from young tree saplings, also apply to mature forest trees.

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Chapter 3

Long-term shade acclimation in temperate forest trees

Raphael Weber, Arthur Gessler, Günter Hoch

Summary

1. The photosynthetic acclimation in trees after multiple seasons in shade is currently unclear, eventually causing short-term shading studies to significantly underestimate the real C assimilation potential of trees under continuous shade. The lack of long-term shading experiments reduces the predictability of the importance of C uptake versus C allocation under C-limitation.
2. We exposed saplings of six temperate deciduous broad-leaved tree species to 6% light for three growing seasons. We sequentially measured photosynthetic light response curves, aboveground biomass production and aboveground non-structural carbohydrate (NSC) pools, to assign shade acclimation to either C uptake or C allocation adjustments.
3. Under shade, photosynthetic parameters like A_{max} changed strongly, but these differences did not change after multiple seasons. Under shade, on average only 38% less C was assimilated per C invested into leaf production. The partitioning of the available C to either leaves or stem was not changed under shade, although less C might have been allocated to roots.
4. We conclude that the acclimation of the photosynthetic apparatus to shade is completed within one growing season. The efficiency of C uptake seems to be more important for acclimation to shade, than the relative distribution of the available C to leaves or stem.

Introduction

The fate of fixed carbon (C) in forests under potential C-limiting situations is still unclear (Hartmann et al. 2018, McDowell et al. 2018). For example, observed decreases of mobile C stores like non-structural carbohydrates (NSC) during long-lasting drought indicate, that drought can cause C-limitation (Klein et al. 2014) or even C-starvation (Sevanto et al. 2014). Such findings raise the question, which plant communities are most sensitive to different types of stress. For example, any C-limiting stress like for example herbivory, frost, or drought might have a stronger impact in already C-limited environments, as it is assumed for forest understories where light is limited (Abrams and Mostoller 1995). Due to high costs of stress simulations in forests, most experiments are performed with potted tree seedlings, which is complicating temporal and spatial predictions on future forest ecosystems. For example, Schall et al. (2012) exposed tree seedlings to drought and shade in one growing season, and observed a higher plasticity in *Fagus sylvatica* compared to *Picea abies*. The inclusion of individual seedling size in statistics was used to interpolate responses from the investigated small seedlings to tall trees. However, interspecific comparison of plasticity in such experiments also requires temporal interpolation, since naturally growing forest understory trees are acclimated to low light starting from their germination. Yet, it is assumed that C gain and allocation under shade in short term-acclimated tree seedlings are similar to long-term acclimated seedlings or naturally growing trees (Giertych et al. 2015, Xie et al. 2018). However, if there is a substantial long-term acclimatization to shade in tree seedlings, C gain in shaded plant communities might be systematically underestimated if assessed from short-term experiments. For example, short-term experiments might suggest C-starvation in understory trees under drought-induced stomatal closure, although plants that are well-acclimated to shade might still be well supplied with C. At least in reaction to elevated atmospheric CO_2, photosynthetic long-term acclimation in trees has been shown to require more than one growing season (Wang et al. 1995, Rey and Jarvis 1998, Turnbull et al. 1998, Rogers and Ellsworth 2002).

Besides photosynthesis, also the C allocation to different plant organs changes at low light conditions. A well-known short-term reaction to deep shade or darkness is the production of etiolated shoots (i.e. the fast production of elongated shoots, Wiley et al. 2017, Weber et al. 2018) to outgrow unfavorable shade conditions. However, this

reaction is futile at persisting shade conditions like in the forest understory, and plants adapted to understory low light conditions do not show etiolation. With increasing forest stand age, C allocation to roots increased in a *Pinus strobus* stand, which might have been either a consequence of tree size, or acclimation to changing light conditions (Peichl and Arain 2007). Such effects need to be disentangled to improve predictions from short-term experiments on forest ecosystems. In addition to the adjustment of the C-allocation to tree organs, perennial plants like trees might also show a long-term acclimation to shade with respect to the allocation of photo assimilates to C-storage. A possible up-regulation of storage against growth has been investigated recently, but could not yet be shown under shade conditions and for more than one growing season (Wiley et al. 2016, Schmid et al. 2017, Weber et al. 2018). Short-term shading (one year) significantly decreased NSC reserves in tree seedlings (Veneklaas and den Ouden 2005, Maguire and Kobe 2015), but Poorter and Kitajima (2007) found increased NSC concentrations in tree seedlings at low light in a tropical forest understory, compared to seedlings at high light conditions. This suggests that NSC concentrations in shaded seedlings might increase with time, which could be interpreted as an acclimation to shade.

So far, it has not been investigated experimentally, how long it takes trees to fully acclimate their C relations to low light conditions. In contrast, the acclimation of the photosynthetic apparatus of leaves to varying light intensity has been studied intensively since decades, revealing fundamental insights (Lichtenthaler et al. 1981, Givnish 1988). For example, significant reactions of leaf nitrogen or chlorophyll concentrations in response to shade can be observed after a few weeks of shading (Cui et al. 1991, Lenssen et al. 2003). At least in cyanobacteria, it is believed that acclimation of the photosynthetic apparatus to shade is completed between hours or a few days after the start of shading (Fujita et al. 1994).

So far, we are missing long-term studies that investigate the acclimation of the C-relations of trees in response to low light over multiple seasons. Precise modeling of the assimilated C over multiple seasons, and the assessment of C pools in different organs can give additional insights into the partitioning of the available C.

To address the lack of long-term data, we exposed temperate deciduous forest tree species to shade over three consecutive growing seasons and measured long-term acclimation of leaf traits, C assimilation and C allocation. Because shade tolerance

was expected to interact with the speed of light acclimation, we investigated six species differing in their shade tolerance (Landolt et al. 2010). Especially, we addressed the following hypotheses:

1. Acclimation of leaves and C assimilation to strong shade (6% of full sunlight) is not completed after one growing season, but continues and may still not be completed even after multiple seasons. Hence, A_{max}, the photosynthetic light compensation point (LCP) and the dark respiration (R_{dark}) continue to decrease with time under shade.
2. Under shade, not only acclimation of photosynthesis is changed, but also the relative distribution of C compounds to different C-sinks like structural growth, leaf production or the formation of C reserves.

Materials and Methods

Study site and experimental set-up

The study was conducted between March 2015 and November 2017 in an open field *c.* 10 km south-west of Basel, Switzerland (47°29'40"N, 7°31'15"E). In March 2015, a total of 576 three-year-old saplings from 6 common European deciduous forest tree species were purchased from a local nursery (Forstgarten Lobsingen, Switzerland): *Betula pendula* ROTH*, Carpinus betulus* L.*, Fagus sylvatica* L.*, Prunus avium* L.*, Quercus petraea* (MATTUSCHKA) LIEBL. and *Tilia platyphyllos* SCOP. Hereafter the species will be referred to by their genus name only. The saplings were planted in the field on March 30, 2015, on an area of ca. 120 m^2. The planting scheme included 4 plots per species, with each plot including 24 saplings. The initial distance between saplings within one plot was 20 cm, and the distance between plots was 60 cm. Because saplings were continuously removed from the plots over the duration of the experiment, the space between the individual sapling stems increased over time.

Saplings were shaded using a 3 m tall tent covered with a green four-layer plastic fiber shading net (Hortima AG, Hausen, Switzerland), which was placed above half of the plots on a total area of 60 m^2 (12 m length and 5 m width). To prevent lethal stress during the initial phase after transplantation, the shading was put in place about eight weeks after planting on June 2, 2015. During the subsequent 30 months until November 20, 2017, shaded saplings were exposed continuously to 6% of ambient

full sunlight (overall daytime mean PPFD of 29 μmol m^{-2} s^{-1} during the growing season). The un-shaded half of the trees (control) received full sunlight (overall growing season daytime mean PPFD = 500 μmol m^{-2} s^{-1}). In addition to the natural rainfall (~850 mm a^{-1}), plots were watered in dry periods to ensure sufficient water supply. Mean soil water potential over the last two seasons at 20 cm soil depth was -0.07 ± 0.04 MPa in shade vs. -0.08 ± 0.02 MPa in control treatment (*n* = 5, MPS-2 sensors, Decagon, Pullman WA, USA). Mean annual soil temperature was 9.8°C vs. 11.7°C (MPS-2 sensors, Decagon, Pullman WA, USA) in shade and controls, while mean annual air temperature was 10.3°C vs. 10.8°C (HOBO pro V2 sensors, Onset, Cape Cod, MA, USA).

Harvest of plant material

Aboveground biomass increase was measured by destructive harvests of n = 6 saplings per species and treatment three times every year, namely in March before leaf flush, in July after the strongest shoot growth, and in November after leaf fall. Due to the limited number of saplings, no biomass was harvested in July 2017. Immediately after harvest, the leaves (if present) and aboveground wood were collected separately in paper bags and dried at 80 °C in a ventilated drying oven. For NSC analyses, a subsample of ca. 1 g dry matter from stem wood *c.* 5 cm above the root collar) and was converted to powder in a horizontal ball-mill (MM 400, Retsch, Haan, Germany).

Specific leaf area

In August during all three growing seasons, the specific leaf area (SLA, m^2 kg^{-1}) was measured in four saplings per species and light treatment. Round leaf stamps with an area of 0.2 cm^2 were taken from three different leaves of each sapling, and immediately oven-dried at 80°C for 72 h. Leaf stamps from one sapling were weighed together and related to the initial stamp area to calculate the mean SLA of each sapling. Total leaf area was calculated by multiplication of SLA with the total leaf dry mass. Due to extremely low variation of SLA within species and treatments (median SE = 0.9 m^2 kg^{-1}), variation of SLA was not included in the calculation of leaf area (Fig. 3.1a).

Non-structural carbohydrates (starch, sucrose, glucose, and fructose)

Stem wood powder was extracted with distilled water by boiling at 100°C for 30 minutes over steam. The extract was then treated with invertase from *bakers yeast* (Sigma-Aldrich, St. Louis, MO, USA) to degrade sucrose into glucose and fructose. This extract was mixed with a glucose-hexokinase assay (glucose-hexokinase assay reagent, Sigma Aldrich, St. Louis, MO, USA) thereby converting glucose to glucose-6-P and fructose to fructose-6-P. Added isomerase (from bakers yeast; Sigma-Aldrich, St. Louis, MO, USA) converted fructose-6-P to glucose-6-P. Due to the presence of NADP in the assay, all glucose-6-P was converted to gluconate-6-P and NADPH. Finally, the concentration of NADPH (equating to the concentration of glucose) was determined photometrically at 340 nm in a 96-well micro plate photometer (HR 7000, Hamilton, Reno, NE, USA). 500 µl of the original extract were treated with a fungal amyloglucosidase from *Aspergillus niger* (Sigma-Aldrich, St. Louis, MO, USA) in a 49°C water bath over night, to break down starch to glucose, and the total glucose (corresponding to NSC) was determined photometrically as described above. The concentration of starch was calculated as total NSC minus low-molecular weight sugars. To ensure activity of the enzymes, pure starch and solutions of glucose, fructose and sucrose were used as standards, and to control reproducibility of the extraction, standard plant powder (Orchard leaves, Leco, St. Joseph, MI, USA) was included. NSC concentrations are given as % dry matter.

C assimilation

To quantify the C assimilation, photosynthetic light response curves (LCs) were measured in situ on leaves during August 2015, June 2016, August 2016 and May 2017, using a portable LI-6400*xt* system (LI-COR, Lincoln, NE, USA). LCs were measured on one randomly selected leaf of $n = 4$ saplings per species and treatment, with seven light set points between 0 and 1500 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). Leaf temperature was set to 20°C.

The observed LCs were mathematically described using an adapted Michaelis-Menten Model:

$$A = A_{max} - \frac{1}{K + a * I}$$

Where A is the C assimilation rate, A_{max} is the maximum C assimilation rate, I is the radiation (PPFD), and K and a are specific parameters describing the shape of the curve. Hereafter, PPFD at an assimilation rate of 0 for each LC will be referred to as the light compensation point (LCP). The assimilation rate measured at zero radiation will be referred to as the leaf dark respiration (R_{dark}). During C assimilation measurements in August 2015, the necessary matching between sample and reference CO_2 concentration before every LC was omitted, which caused an overestimation of A by around 0.5 µmol m^{-2} s^{-1} in all saplings. Therefore, we observed non-plausible, positive R_{dark} and negative LCP values in many saplings. However, these difficulties did not affect differences between both light treatments (Fig. 3.1).

To model the C uptake per season during 2016 and 2017, LCs for each species and treatment from spring of the respective seasons were applied to in situ radiation data logged in 15 minute intervals at the open field site and under the shading net throughout the three growing seasons (HOBO pendant, Onset, Cape Cod, MA, USA). The measured radiation in Lux was converted to PPFD (µmol m^{-2} s^{-1}) using a calibration factor deriving from simultaneous measurements of Lux (using HOBO pendant loggers) and PPFD (using a LI-COR 190R quantum flux sensor) at the field site. To correct C assimilation rates for temperature, a temperature-photosynthesis reaction curve from Bernacchi et al. (2001) was standardized for 20°C leaf temperature and applied to logged temperature data from the field site (30 min intervals, HOBO pro V2 sensors). To model the real net C-assimilation, the net-photosynthesis calculated form the in situ light measurements were multiplied with the respective temperature correction factor. Negative C flux rates (i.e. respiration) were corrected with the same procedure, using a temperature-respiration curve from Hikosaka et al. (1999).

the seasonally assimilated C per total C invested into leaf production (hereafter referred to as the leaf C uptake efficiency) during 2016 and 2017 was calculated by multiplication of the modeled C assimilation (m^{-2} $season^{-2}$) with the species- and

treatment-specific SLA/0.43, assuming a leaf C content of 44% (Poorter et al. 1992). In 2016, total leaf biomass was measured, allowing an approximate calculation of the total assimilated C per tree (g C $tree^{-1}$ $season^{-1}$).

Calculations and statistics

Because wood growth was found to be very low at the second half of the growing season in all species, sapling biomass for each season was determined across the three harvests in July, November and March of the following year. The increase of biomass in each treatment and species was calculated by subtracting the biomass measured for one season with that of the previous season. For the calculations of C pools, we assumed a C dry mass fraction of 44% for leaves (Poorter et al. 1992) and 49% in wood, which corresponds to reported data in temperate angiosperms (Thomas and Martin 2012). The C allocation to aboveground NSC was calculated by multiplication of the measured NSC concentration with the aboveground biomass (with a C mass fraction of 44% in NSC).

The effect of light treatment and sampling dates on the specific leaf area (SLA), maximum C-assimilation *(A_{max})*, light compensation point (LCP) and leaf dark respiration *(R_{dark})* were tested with a full-factorial ANOVA ($p < 0.05$). All statistic tests were peformed with the software R (R 3.3.2).

Results

Leaf traits and C assimilation

Two months after the start of shading, in August 2015, SLA in shade compared to controls was increased by 46% (*Quercus*) to 171% (*Betula*, Fig. 3.1). This difference increased until August 2016, when SLA was increased by 160% *(Quercus)* to 230% *(Carpinus)* in shade compared to the control trees, and did not change further until the third shading season in August 2017. Throughout the experiment, A_{max}, LCP and R_{dark} were significantly different in the shade (full-factorial ANOVA, Fig. 3.1) compared to 100% light. In July 2016, the calculated total leaf area per sapling in the shade was significantly lower in *Betula*, *Fagus* and *Quercus* and ranged between 17% *(Quercus)* and 132% *(Prunus)* of controls (Fig. 3.2).

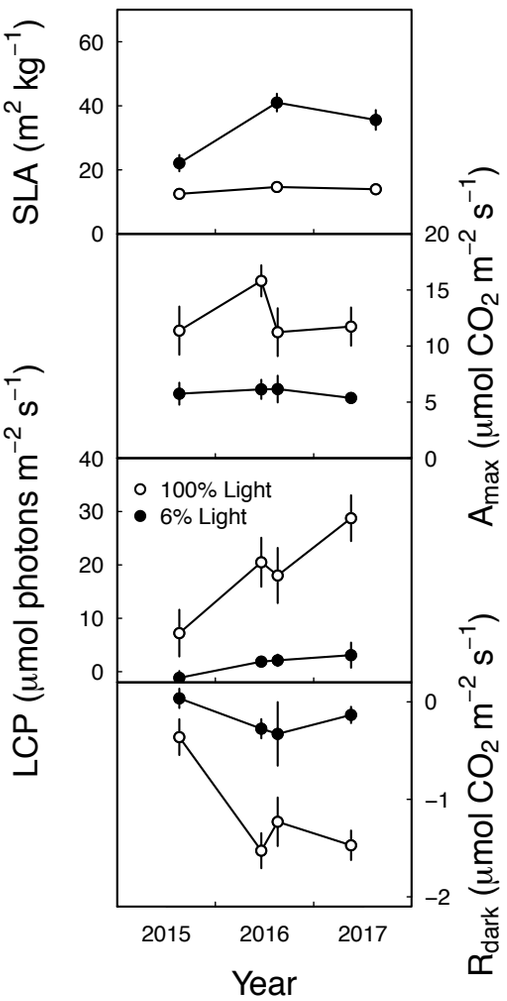

Fig. 3.1 Change of leaf traits and C assimilation in response to shading over three seasons: The points are means across all six species (± SE, n = 6) for specific leaf area (SLA), maximum C-assimilation (A_{max}), light compensation point (LCP) and leaf dark respiration (R_{dark}). SLA is shown for August in each year, while the other parameters are shown August 2015, June 2016, August 2016 and May 2017. For A_{max}, LCP and R_{dark} during August 2015, only differences between treatments are reliable but not absolute numbers, because of the technical difficulties mentioned in the methods section.

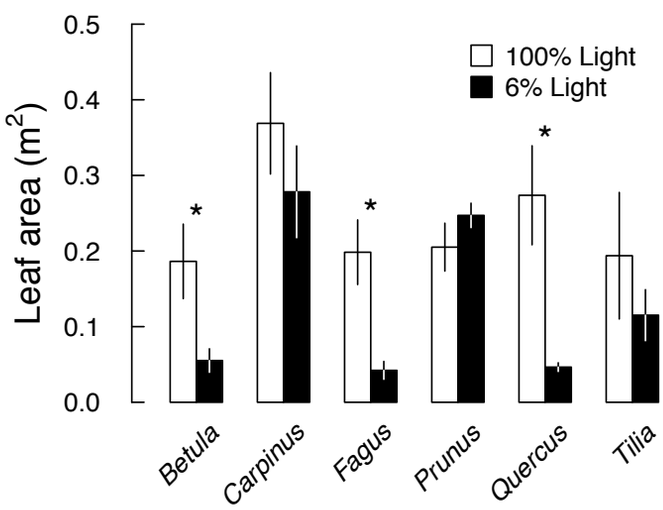

Fig. 3.2 Calculated total sapling leaf area (leaf dry mass*SLA, means ± SE, n = 6) in six species during summer 2016. Asterisks between bars indicate significant differences between treatments (t-test, $p < 0.05$). For leaf area calculation, only variation of leaf mass but not of SLA was included.

Modeled carbon assimilation and allocation

The modeled C assimilation per leaf area strongly differed between controls and shaded individuals (Fig. 3.3). In shaded individuals, the total daily net leaf C assimilation was negative during days with extremely low light (Fig. 3.3). The modeled amount of assimilated C (g sapling^{-1}) during the entire season of 2016 in shaded saplings summed up to 3% *(Quercus)* to 16% *(Tilia)* of C of the respective controls (Table 3.1).

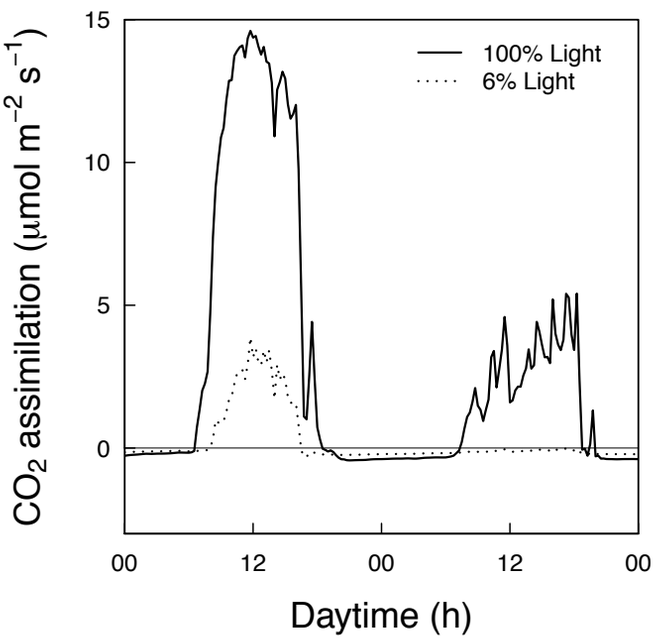

Fig. 3.3 Example of the modeled net leaf CO_2 assimilation rate over two consecutive days in *Prunus avium* during a sunny day (April 30, 2016, left) and during a rainy and dark day (May 1, 2016, right). Calculations are based on the means of four photosynthetic light response curves from June 20, 2016 in each treatment.

Table 3.1 The modeled total amount of assimilated C in g per sapling during the entire growing season of 2016.

Species	Assimilated C (g sapling^{-1})	
	100% Light	6% Light
Betula pendula	119	7
Carpinus betulus	222	27
Fagus sylvatica	139	5
Prunus avium	148	16
Quercus petraea	216	6
Tilia platyphyllos	99	16

The C allocation patterns were investigated in more detail for the second growing season 2016. The modeled C assimilation per leaf area correlated with the total amount of C allocated to aboveground biomass including stem growth, leaf production and NSC storage (ΔC_{above}; Fig. 3.4). According to this correlation, in all species, around 15 % of the net-assimilated C was allocated to aboveground biomass (including wood, leaves or storage), independent of the light treatment (Fig. 3.4 and 3.5).

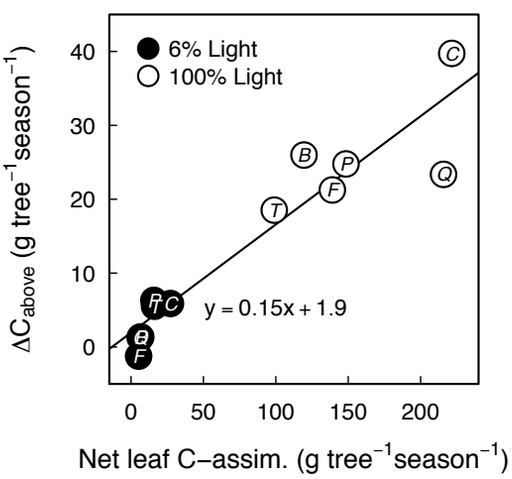

Fig. 3.4 The modeled net leaf C-assimilation per tree and season (x-axis) in six species and two different light treatments in 2016, compared to the total amount of C invested into aboveground biomass (y-axis, all aboveground mass including leaves and storage). The line represents a linear fitting curve (one-way ANOVA, $p < 0.001$, $r^2 = 0.86$). *B, Betula; C, Carpinus; F, Fagus; P, Prunus; Q, Quercus; T, Tilia.*

From the *c.* 15% of the assimilated C that was allocated to aboveground biomass, around 52-74% went to structural growth at full sunlight (Fig. 3.5). This fraction was considerably lower (between 34-67%) under shade, except for shaded *Fagus*, which showed even a negative net-biomass change during 2016 apart from leaf production (Fig. 3.5) that was likely caused by an intense aphid infestation in all shaded *Fagus* individuals. In both light treatments and across all species except shaded *Fagus*, on average 34% of the C allocated aboveground was allocated to leaves, and 0-20% of the allocated C was used to increase the saplings' aboveground NSC pool during the growing season, with high variation between species (Fig. 3.5). These fractions did not differ between shaded and control saplings (full-factorial ANOVA with interaction, Fig. 3.6).

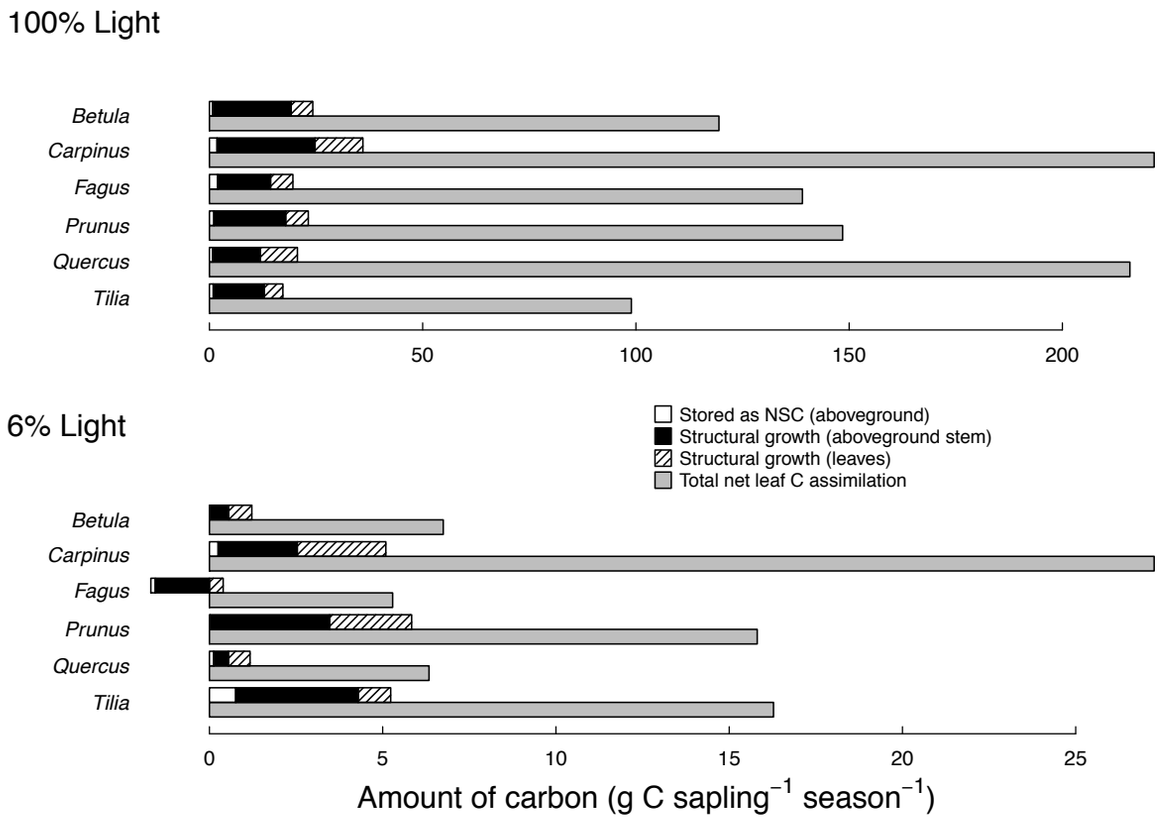

Fig. 3.5 Total net C assimilation per tree and the change of different total aboveground C pools during 2016 in six deciduous tree species, at high light (above) and low light (below).

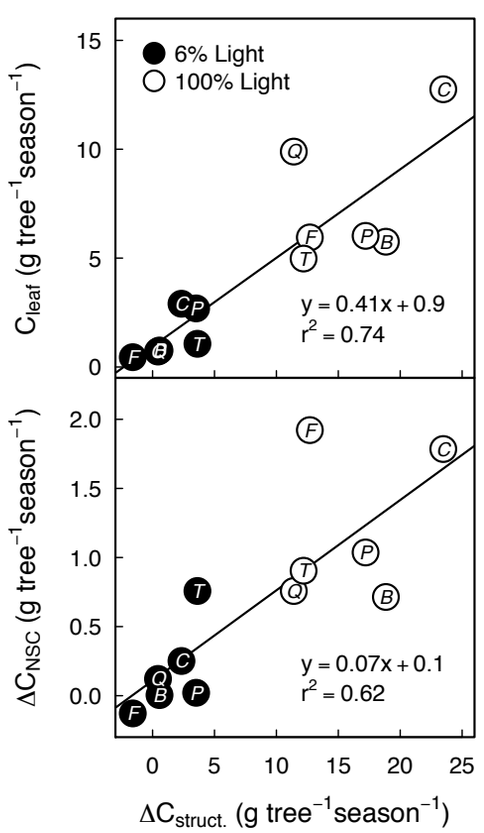

Fig. 3.6 The change in aboveground structural C during 2016 (x-axis) versus the C allocated to leaves (above), and versus the change in C stored in the total aboveground NSC pool (below). The fit line, r^2 and the formula are given for a linear correlation fit. *B, Betula; C, Carpinus; F, Fagus; P, Prunus; Q, Quercus; T, Tilia.*

Leaf carbon uptake efficiency

During the last two growing seasons, leaves on average assimilated 15 times more C than the amount of C required for their production, which hereafter will be referred to as the leaf C uptake efficiency. Across all species, the modeled total amount of assimilated C per C allocated to leaf production (leaf dry mass * 0.44, g g^{-1}, see methods) in shaded saplings was significantly lower compared to controls (full-factorial ANOVA, $p < 0.05$), with a leaf C uptake efficiency of shaded saplings being on average 50% of controls during 2016, and 62% of controls during 2017 (Fig. 3.7). We observed a maximum leaf C uptake efficiency of 32 g g^{-1} in *Betula* leaves at full sunlight, and a minimum leaf C uptake efficiency of 1.4 g g^{-1} in *Fagus* leaves under shade (Fig. 3.7). The low leaf C uptake efficiency in *Fagus* was strongly influenced by a low A_{max} of 3.4 µmol m^{-2} s^{-1} compared to other species in shade (mean of 5.3 µmol m^{-2} s^{-1}).

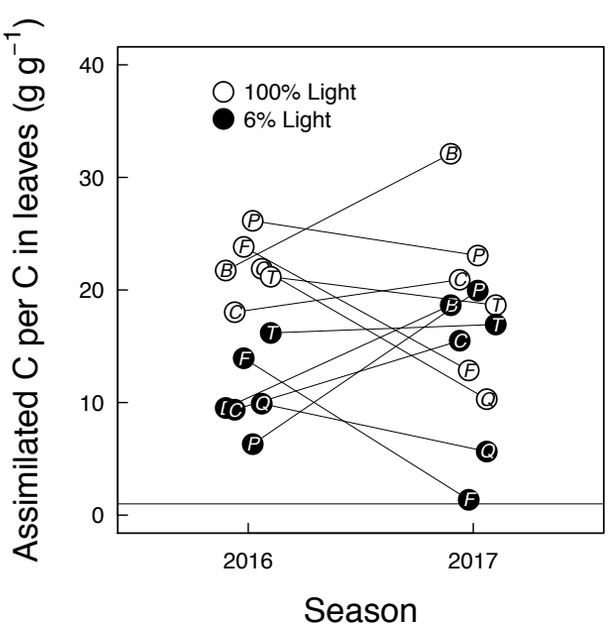

Fig. 3.7 Differences in leaf C uptake efficiency during two growing seasons in six different species. *B, Betula; C, Carpinus; F, Fagus; P, Prunus; Q, Quercus; T, Tilia.*

Discussion

Main findings

In contrast to our first hypothesis, we showed that the largest part of leaf acclimation to low light occurs within the first weeks after the start of shading, and that leaf traits and physiology do probably not acclimatize further in subsequent growing seasons under continuous shade. Also in contrast to our second hypothesis, an increased efficiency of light trapping and C-assimilation at the leaf level seems to be more

important for the acclimation to low light than a change in C allocation; at least for aboveground organs.

Acclimation of leaf traits to shade

The highest SLA was reached in shaded leaves during the second shading season, possibly because most leaves during the first shading season had been produced under high light conditions. Clearly, once built, leaves cannot increase their SLA (*i.e.* become less thick) in response to shading (Tardieu et al. 1999). The strong reactions of C assimilation to shade (the significant decline of A_{max} and R_{dark}, and the strong decrease of LCP in shaded compared to control saplings) already during the first shading season did not become stronger during the two subsequent shading seasons. This suggests that the photosynthetic apparatus acclimatizes quickly to low light, possibly within a few days as suggested by Fujita et al. (1994), and that there are probably no major long-term changes in C assimilation and respiration. Therefore that measurements within a few weeks after the begin of shading are probably sufficient to investigate the physiological acclimation potential of gas exchange at the leaf level in trees (Cui et al. 1991, Lenssen et al. 2003). The fast reactions of SLA, A_{max}, LCP or R_{dark} found in the current study for all investigated tree species contradict our initial assumption that species-specific shade tolerance interacts with the speed of acclimation to low light. It is important to note, that our gas-exchange measurements were performed at different dates during the growing season for the three investigated years. Hence, the observed variation in A_{max}, LCP or R_{dark} between the seasons might have been also caused by differences in the leaf developmental stage or in the climatic conditions preceding the measurements. To elucidate more subtle long-term adjustments of photosynthesis to shading, multiple gas-exchange measurement campaigns across the season would have been necessary.

Total sapling C-assimilation

The model used in this study to calculate C-assimilation over entire seasons assumes that leaves experienced the same amount of light that was received by the light logger. Therefore, self-shading of saplings was ignored, which likely caused an over-estimation of assimilation (Ackerly and Bazzaz 1995). This might be especially the case in saplings at high light, where self-shading can be assumed to occur more

frequently due to differences in canopy architecture (Fig. 3.8). These differences suggest, that avoidance of self-shading is more important under already low light conditions. In contrast, the self-shading effect by leaves of the upper canopy should be stronger under the more unidirectional radiation experienced by the un-shaded control trees, than under the shading net that produces a higher proportion of diffuse radiation. Hence, to finally assess whether self-shading caused a stronger decline in C assimilation in shaded or un-shaded saplings, further measurements of self-shading would be necessary.

Fig. 3.8 Photographs of saplings at 100% light in the open (left) and at 6% light under the shading tent (right): *Betula pendula* (above) and *Tilia platyphyllos* (below). Photographs were taken shortly after leaf flush on May 9, 2017, which was 24 months after the start of shading.

C allocation

In this study, no total C allocation to roots was assessed because the saplings were planted directly in the field, which prevented excavation of the total rooting system. Previously, decreasing C allocation to roots under shade has been shown numerous times (Givnish 1988, Naidu and DeLucia 1997, Dias-Filho 2000, Schall et al. 2012), and was associated with decreased water and nutrient demand at low light. We therefore assume that such a response also occurred in this study. While there is a

consensus that less C is allocated to roots under shade, literature findings seem to be inconsistent regarding the partitioning between the allocation of C to leaves and stem C under shade. For example, Messier and Puttonen (1995) showed a decrease in C allocation to leaves compared to branches in shaded *Betula pendula.* Naidu and DeLucia (1997) observed higher leaf mass investments in naturally growing *Quercus rubra* when artificially exposed to canopy gaps. On the other hand, Lusk (2002) showed an increase of the aboveground leaf mass ratio with decreasing light availability in temperate rainforest species, which was mainly observed in shade intolerant species. No effect was observed by Reich et al. (1998), who concluded that the variation in leaf and root structure has a stronger influence on the inter-specific shade tolerance than biomass partitioning, which supports our findings regarding aboveground biomass. However, considering the strong preference of C allocated to leaves in aphid-infested *Fagus* in shade, we suggest that C-limiting conditions additional to shade can fundamentally decrease C allocation to storage and structural biomass. Weber et al. (unpublished, see chapter 2 of this thesis) reported that NSC concentrations in shaded tree seedlings recovered slowly to control concentrations after an initial two-fold decrease. This suggests that, although the average aboveground C allocation patterns among all species did not fundamentally change under shade, slight adaptations over three years of shading can occur, thereby revealing a slow acclimation of aboveground C allocation to low light conditions.

In conclusion, this study indicated a very quick acclimation of photosynthesis to deep shade conditions in temperate tree species. Saplings that faced long-term shading exhibited surprisingly high C efficiencies in terms of C gained by photo-assimilation in relation to C invested in leaves, that were close to the C efficiencies of un-shaded saplings. Together with the observed constancy of C allocation patters between wood and leaf production under deep shade conditions, our study showed the remarkably high potential of young trees to acclimatize to low light in order to persist under severely limited C supply.

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Chapter 4: Additional experiments

4.1

Root respiration and C reserves in temperate tree seedlings during lethal C-starvation

Introduction

While the dynamics of NSCs and growth during and after C-starvation was described in detail in the first chapter of this thesis, it is still unclear how other C-sinks like respiration react to C shortage. For example, leaf shedding during darkening could be a strategy to minimize plant respiration. Besides reductions in aboveground respiration following leaf shedding, there might be also significant reductions in belowground respiration during C-starvation. Such a reduction of root respiration might be due to the limited availability of C, but also because of an active down-regulation of respiration in order to save C. For example, soil respiration has been shown to rely strongly on current photo-assimilates, and much of this response was accounted to autotrophic respiration (Ekblad and Högberg 2001, Högberg et al. 2001). Although these previous findings suggest a strong decline of respiration in response to C-starvation, there are few direct measurements of root respiration available, and changes in soil respiration after changes in humidity or temperature can not fully be accounted to root respiration (Davidson et al. 1998, Hanson et al. 2000, Zang et al. 2014). Therefore, we are still lacking detailed information on how root respiration changes in response to C-starvation.

Within this experiment, I wanted to test the reaction of root respiration in temperate tree seedlings to C-starvation induced by complete darkening. I grew tree seedlings in hydro culture, which enabled direct root respiration measurements without interfering heterotrophic respiration from soil microbes. Seedling death was assessed from leaf and bud observations and was assumed to have occurred after the complete loss of all leaves and the shrinking and darkening of buds following turgor loss. C-starvation was investigated by measurements of non-structural carbohydrate concentrations (NSCs, free sugars and starch) in wood. I expected a strong decrease in root respiration and a significant depletion of NSCs in seedlings at lethal C-starvation.

Materials and Methods

Experimental set-up

Two-year-old seedlings of the broad-leaved tree species *Betula pendula* ROTH, *Tilia platyphyllos* SCOP. and the conifer *Picea abies* (L.) H.KARST were grown in hydro-culture within walk-in phytotron chambers, which enabled complete exclusion of light with minor climatic changes. Hereafter, the species will be referred to by their genus name only. Hydro culture allowed sequential and non-destructive measurements of root CO_2 gas-exchange (see below), and largely avoided the influence of respiration from soil microbes. Initially, the conifer species *Pinus sylvestris* L. was included in the experiment, but showed complete mortality in both light treatments after a few weeks, possibly in response to hydro culture. Thus, further descriptions will only include *Betula*, *Tilia* and *Picea*. After delivery from a Swiss nursery (Forstgarten Lobsigen, Switzerland), the naked-rooted seedlings were stored in darkness at 4°C, to avoid bud break until May 27, 2015, when seedlings were "planted" into the hydro culture. 50 seedlings from each species were equally distributed to two phytotrons and each species was separately placed in 0.5 m deep opaque grey plastic containers filled with 72 L Knop's solution, consisting of tap water mixed with 0.65 kg m^{-3} $Ca(NO_3)_2$ * 4 H_20, 0.11 kg m^{-3} $MgSO_4$ * 7 H_2O, 0.11 kg m^{-3} KH_2PO_4, 0.11 kg m^{-3} KNO_3, and 0.01 kg m^{-3} $FeSO_4$. During the experiment, additional nutrient solution was added when necessary to maintain water electric conductivity at 1.5 mS cm^{-1}. Sufficient aeration of the water solution was enabled by permanently introducing air bubbles to each container with small aquarium pumps.

Soon after planting, seedlings produced typical white water roots with a different structure than the existing roots that have been produced in soil. At the start of the experiment, light was supplied to both chambers during 12 hours with a four-channel LED lamp system (blue, white, red and far-red, LED panels, DH Licht, Germany), providing a constant PPFD of *c.* 450 µmol m^{-2} s^{-1} at plant height with a blue : white : red mixing ratio of 50 : 100 : 50. Throughout the experiment, temperatures were kept at 15°C and 24°C during night and daytime hours, respectively, with a two hours ramp between the target temperatures after lights were switched on and off. The relative air humidity inside the phytotrons was set to constant 60%. Starting from July 1, 2015 (ca. 60 d after bud break in all species), one of the two phytotrons was kept

without light. Seedlings were monitored for dead individuals every second day within the darkened phytotron, until the experiment was stopped after 202 d in darkness on January 19, 2016.

Harvest and NSC analyses

Seedlings were considered dead when all leaves were shed and all buds turned from green to black and showed loss of turgor. *Picea* was considered dead when all needles turned brown and detached from the branch upon touching. Presumably dead seedlings were immediately harvested, and for each dead seedling in the darkening treatment one living individual of the same species was harvested from the illuminated phytotron. Seedlings were therefore separated in two groups for statistical analysis: "darkened and dead" versus "light and alive". However, 18 darkened *Tilia* individuals seemed still alive when the experiment was stopped, thus they were considered as a third treatment category: "darkened and alive". After harvest, stem wood at 5 cm above the root collar was separately sampled in paper bags, dried at 80 °C in a drying oven, ground to fine powder and analyzed for NSC concentrations. The NSC analyses followed a modified protocol based on Wong (1990) as given in detail in Weber et al. (2018).

Root respiration measurements

Root respiration was immediately measured after seedling harvest. Before measurements, roots were rinsed with water, and blotted dry with a cotton towel. No aboveground respiration was measured. The CO_2 efflux of entire roots stocks (with still intact seedlings) was quantified by enclosing the whole root system in a sealed plastic bucket with a volume of 5.3 L. The increase of CO_2 concentrations over time was recorded immediately after enclosing the roots. CO_2 concentrations were measured and recorded every five seconds with CO_2 gas analyzer (LI-820, LI-COR, Lincoln, NE, USA), and the slope of CO_2 increase was extracted from a linear model applied to the data points.

The root CO_2 efflux was calculated using the following formula:

$$E = \frac{\Delta C_{CO_2} V p}{t R T}$$

Where E is the root CO_2 efflux [nmol s^{-1}], ΔC_{CO2} is the change in air CO_2 concentration [ppm] over time t, V is the volume of the bucket, p is the air pressure, R is the gas constant and T is the air temperature. The root CO_2 efflux was related to the root dry matter of each seedling (nmol CO_2 g^{-1} dry root mass s^{-1}) to account for differences in the total root dry mass between individuals.

Calculations and statistics

Since root respiration and root biomass were log-normally distributed among treatments and species, both variables were log-transformed for statistics. The effect of species and treatment on root respiration was investigated with a full-factorial ANOVA with interaction ($p < 0.05$). Statistical analyses were performed using the software R (3.3.2).

Results

Visual appearance and survival in darkness

Around 20 days after the start of darkness, some individuals started shedding leaves, while in *Picea,* current-year shoots started to furl, combined with a pale appearance of needles. After around 60 days in darkness, a few *Betula* but most *Tilia* seedlings started with the production of long (up to 1 m) and white etiolated shoots (Fig. 4.1). Attached to these shoots were tiny, almost transparent leaves (below 5 mm length). Around 40 days after production, most etiolated shoots seemed to dry out, and their appearance changed to a light brown (Fig. 4.2), while some etiolated shoots did not visually change until the end of the experiment after 202 days in darkness. Most *Picea* died after 96-98 days, although one individual survived for 70 more days. *Betula* died after 99-196 days, and most *Tilia* were seemed still alive after 202 days in darkness at the end of the experiment.

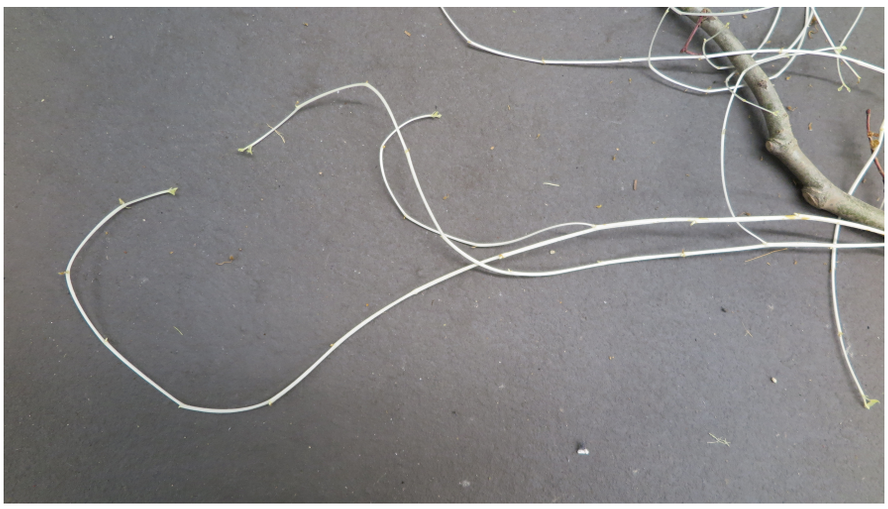

Fig. 4.1 Freshly produced etiolated shoots in *Tilia* after 90 d in darkness.

Fig. 4.2 Etiolated shoots in *Tilia* that have dried out after 131 d in darkness.

Root respiration and NSC concentrations

On average, root respiration was 2.8 nmol CO_2 g^{-1} s^{-1} across all species and treatments. *Tilia* showed a significantly lower CO_2 efflux per root biomass compared to other species (full-factorial ANOVA with interaction, $p < 0.001$, Fig. 4.3). Quite unexpectedly, seedlings that have been assumed to be dead within the darkened phytotrons, showed no significant decline of the measured root CO_2 efflux compared to the living controls (Fig. 4.3). When the CO_2 efflux was calculated per g root dry mass (without log-transformation of both variables), the average root respiration was lower in darkened *Picea* compared to controls, higher in darkened *Tilia* compared to

controls, and not significantly different between treatments in *Betula* (*t*-test, Table 4.1).

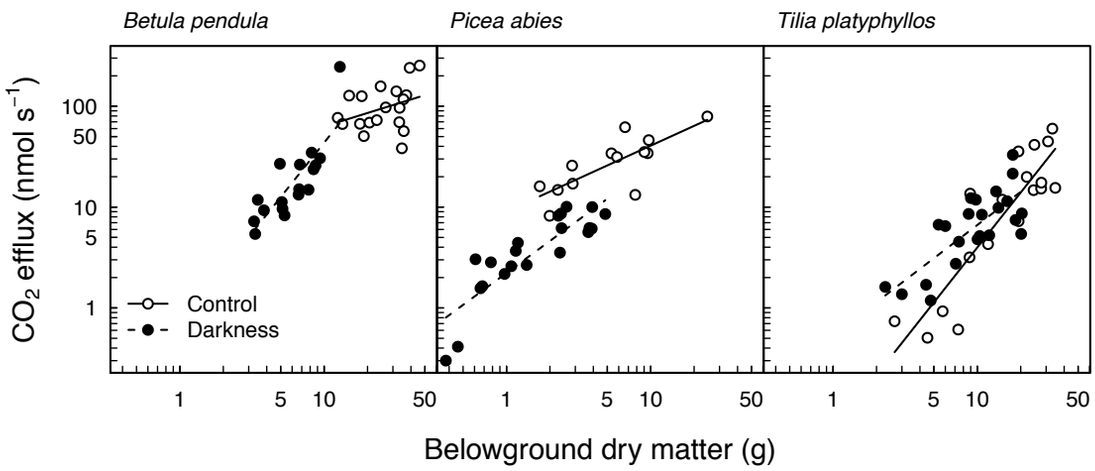

Fig. 4.3 Root dry matter (logarithmically displayed) of the three species versus the measured CO_2 efflux (logarithmically displayed) from roots. Slopes between living controls (open circles) and darkened individuals in all species did not differ significantly (full-factorial ANOVA with interaction, $p < 0.05$). Darkened *Tilia* seedlings (alive and dead ones) were pooled together. $n = 17$ per treatment.

Table 4.1 Mean root respiration per root dry mass (± SE, nmol CO_2 g^{-1} s^{-1}) in illuminated, alive (controls) and darkened, dead saplings of three species. Results from a *t*-test (*p* value and degrees of freedom) are given between controls and darkened seedlings of each species.

Species	Root respiration		*t*-test	
	Control	Darkness	*p*	df
Betula pendula	4.2 ± 0.5	4.0 ± 1.1	0.84	18.6
Picea abies	5.8 ± 0.7	2.5 ± 0.2	< 0.001	13.3
Tilia platyphyllos	0.8 ± 0.1	1.3 ± 0.1	0.01	18.2

Total stem wood NSC concentrations were significantly lower in *Picea* controls compared to *Betula* and *Tilia* (Table 4.2, Fig. 4.4). NSC concentrations in stem wood of all three species were substantially reduced at sapling death in darkness (Table 4.2, Fig. 4.4). This depletion of NSC was due to very strong reduction of the tissue concentrations of low molecular sugars and starch, respectively. Interestingly however, the tissue concentrations of sugars at sapling death (between 0.03 and 0.07 % DM) were even lower than that of starch (between 0.25 and 0.55 % DM, Table 4.2)

in all three species. At mortality in darkness, *Picea* stem wood had significantly lower starch, but similar sugar concentrations compared to the other species (ANOVA, $p < 0.001$, Table 4.2). *Tilia* that was harvested alive after 202 d in darkness had 55% lower stem wood sugar concentrations compared to controls, but still significantly higher concentrations than darkened, dead seedlings (Tukey's HSD, $p < 0.05$, data not shown). On the other hand, starch was not significantly different between darkened, living and darkened, dead *Tilia*, but decreased by 67% in darkened, alive seedlings compared to controls (Tukey's HSD, $p < 0.05$, data not shown). Dead saplings from the darkening treatment showed similarly low tissue concentrations independent of the time point of death (Fig. 4.4).

Table 4.2 Non-structural carbohydrate concentrations (NSC = sugars and starch, means ± SE) of the three different tree species in seedlings grown under full sunlight conditions and trees grown in darkness. The NSC samples in darkness treated trees were taken post mortem. *n* = 6-22.

Species	Sugars (% DM)		Starch (% DM)		Total NSC (% DM)	
	Control	Darkness	Control	Darkness	Control	Darkness
Betula pendula	1.75 ± 0.15	0.07 ± 0.02	10.0 ± 0.3	0.55 ± 0.09	11.8 ± 0.3	0.61 ± 0.10
Picea abies	1.11 ± 0.16	0.06 ± 0.02	1.30 ± 0.18	0.25 ± 0.03	2.42 ± 0.25	0.34 ± 0.02
Tilia platyphyllos	3.78 ± 0.26	0.03 ± 0.02	6.96 ± 1.58	0.48 ± 0.11	10.8 ± 1.7	0.55 ± 0.11

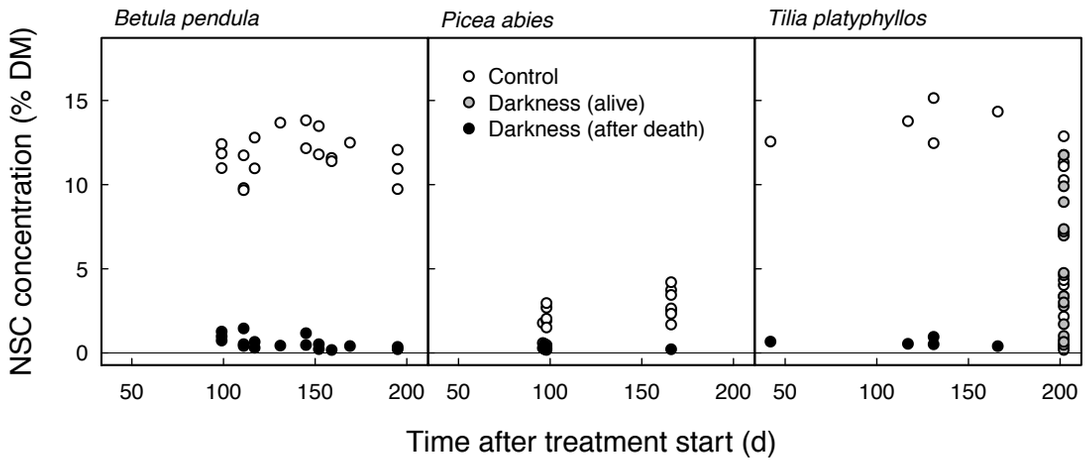

Fig. 4.4 Time course of non-structural carbohydrate (NSC) concentrations in stem wood of three different species (n = 6-22).

Discussion

Main findings

The observed NSC concentrations showed, that all darkened and dead seedlings had suffered lethal C-starvation. Since we still observed significant root CO_2 efflux in darkened seedlings with no visual life signs and NSC reserves below 1% DM, we could not directly account all CO_2 efflux to autotrophic root respiration. Thus, the applied method to quantify root respiration might show a significant bias from root-living heterotroph microbiota.

NSC concentrations

The strongly depleted NSC concentrations in stem wood of darkened seedlings compared to light-supplied seedlings, suggest that darkened seedlings experienced C-starvation. The measured sugar concentrations in stem wood of C-starved *Picea* (0.1% DM) correspond well to findings from in the first chapter of this thesis (Weber et al. 2018) where we measured 0.2% DM. Starch concentrations in *Picea* stem wood of this study (0.3% DM) were slightly lower than the 0.6% shown in chapter one.

Since I still observed significant amounts of NSC in darkened, but seemingly alive *Tilia*, NSC seem to well predict the C supply status in darkened tree seedlings. However, The first chapter of this thesis reports extremely low NSC concentrations already at the beginning of C-starvation, and thus considers NSC as early starvation markers. Therefore, NSCs do not seem to mark the point, after which death becomes

inevitable. However, Wiley et al. (2017) observed a linear decrease of NSCs with dieback of organs in *Populus tremuloides* seedlings following darkness, and they argued that there might be strong tissue-specific decreases in NSCs at the point when tissues die. Overall, regarding the very low NSC concentrations measured in this study, I conclude that all seedlings that were presumed dead, had likely suffered lethal C-starvation.

Root respiration

The lack of a significant difference in root respiration between dark/dead and light/alive seedlings, suggests that C availability is not a main driver of root respiration as quantified by the method used in this study. This hypothesis gains support from Wiley et al. (2017), who found a decrease of root respiration by only 40% in darkened, dead *Populus tremuloides* seedlings. However, it has to be questioned, if the quantification of root respiration is accurate enough, when the CO_2 efflux is calculated on the total root mass. Because roots, like stem sapwood, consist to a larger proportion of dead tissue, subtle changes of respiration in the living cells might be masked by the 'dilution' via the dead woody tissue in roots. To better determine if seedlings are able to down-regulate root respiration in response to C shortage, root CO_2 efflux should be related to the amount of living and respiring cells, which are rather found in the root cambium (in the outer part of the roots) than in the root wood. While dead root cells cannot be excluded, the amount of living cells might thus be quantified more accurately with root surface measurements than with measurements of root dry matter. In this study, *Tilia* roots appeared more sturdy (showing less surface per weight) than *Betula* and *Picea* roots, which might explain the significantly lower root respiration (related to dry matter) in *Tilia* compared to *Betula* and *Picea*. Wiley et al. (2017) measured 6-18 nmol CO_2 g^{-1} s^{-1} in fine roots, which is substantially higher than the 2.8 nmol CO_2 g^{-1} s^{-1} during this study. Thus, the inclusion of large and sturdy root wood pieces with low surface likely resulted in lower root respiration measurements.

Although the method used in this study was developed to be non-destructive, it can further not be excluded that the harvest of seedlings and blotting of roots before root respiration measurements significantly altered root respiration. Thus, further studies

seeking to quantify direct root respiration may use the comparison of soils with and without roots, which seem to be least disturbing (Hanson et al. 2000).

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4.2

Carbon allocation during drought

Introduction

It is still unclear if and under which conditions tree mortality under drought is caused by carbon (C)-limitation (McDowell et al. 2008, Hartmann and Trumbore 2016). This question is especially important since the current and future increase of atmospheric CO_2 concentrations might enhance the drought resistance of trees. For example, Paudel et al. (2018) showed a higher water use efficiency under drought in *Citrus limon* saplings when exposed to elevated CO_2 concentrations, but could not determine if survival increased with elevated CO_2. An important function during drought survival is generally accounted to the osmotic functions of free sugars, which can be quickly formed in plant cells by hydrolyzation of starch (Zwieniecki and Holbrook 2009, Dietze et al. 2014). A higher availability of stored non-structural carbohydrates (NSCs = free sugars and starch) therefore might increase tree drought tolerance. O'Brien et al. (2014) showed in tropical tree seedlings that shade exposition (2% of full sun-light) before drought decreases drought survival, and accounted these findings to the lower availability of NSCs in shaded seedlings. However, such responses are likely biased by unwanted shade effects, like decreased root growth under shade (Naidu and DeLucia 1997, Dias-Filho 2000). Hence, we still lack direct and unequivocal experimental evidence for the benefits of NSCs during drought, which hinders our understanding of the function and importance of C reserves for drought resilience of trees. While the abundance of NSCs within a plant might directly alter its drought resistance, reduced water availability might also cause higher phloem viscosity, leading to a slower downward transport of photo-assimilates (Sevanto et al. 2014). Ruehr et al. (2009) observed in a ^{13}C pulse-labeling experiment that recently fixed C was allocated significantly slower to roots during drought in *Fagus sylvatica* seedlings. If NSCs are required to maintain upward water transport, they likely also maintain phloem water content, and thus downward C transport. Hence, we should observe a feed-back effect of initial NSC reserves during drought, where higher NSC concentrations in sapwood of roots and stems before drought would also ameliorate the C allocation from leaves to roots during drought (Fig. 4.5).

Under well-watered conditions, lower NSC reserves should theoretically not lead to decelerated C allocation to the same extent as under drought, because less NSCs are required to maintain hydraulic integrity (Fig. 4.5).

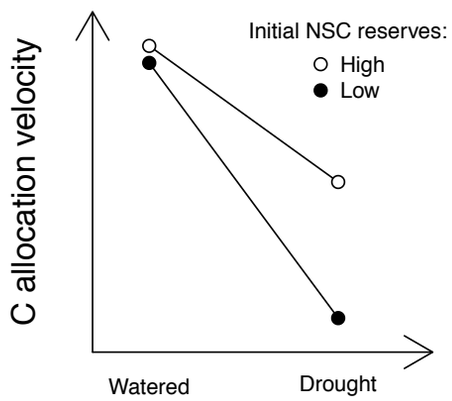

Fig. 4.5 Theoretical response of the C allocation in reaction to different initial NSC reserve sizes before drought.

Within this experiment, I investigated whether NSCs are beneficial for drought survival of tree seedlings, and additionally aimed to elucidate the involvement of C transport restrictions in drought mortality. To this, I first exposed *Quercus petraea* seedlings to either short-term darkness or ambient light before drought to alter the seedlings' NSC reserve pools without major effects on their morphology and anatomy. These seedlings were subsequently exposed to drought over a four week-period. During drought, all seedlings were ^{13}C pulse-labeled to quantify the speed of C allocation within seedlings. Especially, I addressed the following hypotheses:

1. One week of darkness decreases the NSC concentration in seedlings, and NSCs in darkened seedlings do not recover to control concentrations until the onset of drought.
2. During drought, previously darkened seedlings show higher mortality than constantly light-supplied seedlings.
3. Initial darkness has no significant effect on the phloem C transport velocity in well-watered seedlings.
4. C transport during drought is reduced in previously darkened seedlings with lower NSC reserves, compared to constantly light-supplied seedlings with higher NSC reserves.

Materials and Methods

Experimental set-up

The experiment was conducted during summer 2016 in a greenhouse at the University of Basel with 280 two-year-old seedlings of the deciduous broad-leaved tree species *Quercus petraea* (MATTUSCHKA) LIEBL. Before planting, seedlings were weighed and sorted by size, to later equally distribute them to the following four light x water treatment combinations: Consistently light-supplied (i.e. not darkened) and well-watered *(LW,* controls), initially darkened and well-watered *(DW)*, consistently light-supplied and drought stressed *(LD)*, and initially darkened with subsequent drought stress *(DD)*. On April 4, 2016, all seedlings were planted in clay pots with a volume of 3 L and grown in a greenhouse. The usage of clay pots ensured a faster drying during the drought treatment and thus a better control of soil water content than in plastic pots. Before the drought treatment, all pots were watered every 2-4 days, and this watering regime was continued for all watered seedlings after the start of the drought treatment. After all seedlings had flushed and established for several weeks at standard greenhouse and natural light conditions, DW and DD were placed into climatized dark chambers on July 25 for seven days. The chambers used for the darkening treatment were set to the same temperature condition as in the greenhouse. Complete darkening was achieved by covering the chambers with opaque plastic foil, as described in detail in chapter 1 of this thesis (Weber et al. 2018). The other 140 seedlings (LW and LD) were left in the greenhouse, with similar climatic conditions as described in chapter 1. Because I expected slower soil drying in darkness, watering was already reduced before darkness in DD to ensure a fast onset of drought after darkening, thereby not allowing recovery of NSC reserves after darkness. Thus, DD seedlings were only watered with 0.2 L per pot every two weeks starting from July 15. The drought treatment for LD and DD seedlings started on August 2*, i.e.* the date when DD had been released from darkness and reintroduced to light. To monitor drought conditions, soil water potential was logged at 10 cm depth in five pots per treatment (MPS-2 sensors, Decagon, Pullman WA, USA). Soil water content was measured on a weekly basis in four pots per treatment (ThetaProbe, Delta-T, Burwell, UK).

Assessment of drought reactions

To measure non-structural carbohydrates (NSCs), $\delta^{13}C$ and stem water potential (Ψ_{stem}), seedlings were harvested at the following dates: August 2 (only Ψ_{stem}), August 17 (1 h after ^{13}C pulse-labeling, see below), and August 18, 22 and 29. When seedlings were harvested, a small subsample (~1 g dry weight) of the following organs was harvested and processed as described in chapter 1 of this thesis: leaves, stem wood and root wood. Using these subsamples I measured NSC (as described in chapter 1) and $\delta^{13}C$ (see below). In drought treatments (LD and DD), first leaf damage was visible on August 15. Ψ_{stem} was measured during daytime in freshly harvested seedlings from the entire aboveground part of harvested seedlings, which entirely fitted into a Scholander pressure chamber (Model 1000, PMS, Albany OR, USA). On the same dates, C assimilation and stomatal conductance were measured on the leaf that appeared most vital on four seedlings per treatment at a PPFD of 500 µmol m^{-2} s^{-1}, 400 ppm CO_2 and ambient Temperature and humidity, using a LI-6400*xt* system (LI-COR, Lincoln, NE, USA). On August 17 before the ^{13}C pulse-labeling, C assimilation was measured in all seedlings.

^{13}C pulse-labeling

On August 17, seedlings were subjected to a 2 h long ^{13}C pulse-labeling treatment in the open air in front of the green house (Fig. 4.6). 200 seedlings were placed into a tent construction with a volume of 4.7 m^3, which was covered by a transparent plastic foil. To supply seedlings with ^{13}C-enriched CO_2, 1M-acetic acid (CH_3COOH) was dropped in four batches (see CO_2 concentration in Fig. 4.6) on 13 g of Calcium bicarbonate ($Ca(HCO_3)_2$) powder enriched with 50% ^{13}C. The developing 1:1 mixture of $^{12}CO_2$ / $^{13}CO_2$ was ventilated into the tent construction, while the following climatic parameters were monitored inside the tent (Fig. 4.6): CO_2 concentration (LI-820, LI-COR, Lincoln, NE, USA), photosynthetic photon flux density (PPFD; Li-190r Quantum sensor, LI-COR, Lincoln, NE, USA), and air temperature plus relative air humidity (HOBO pro V2 Sensors, Onset, Cape Cod MA, USA).

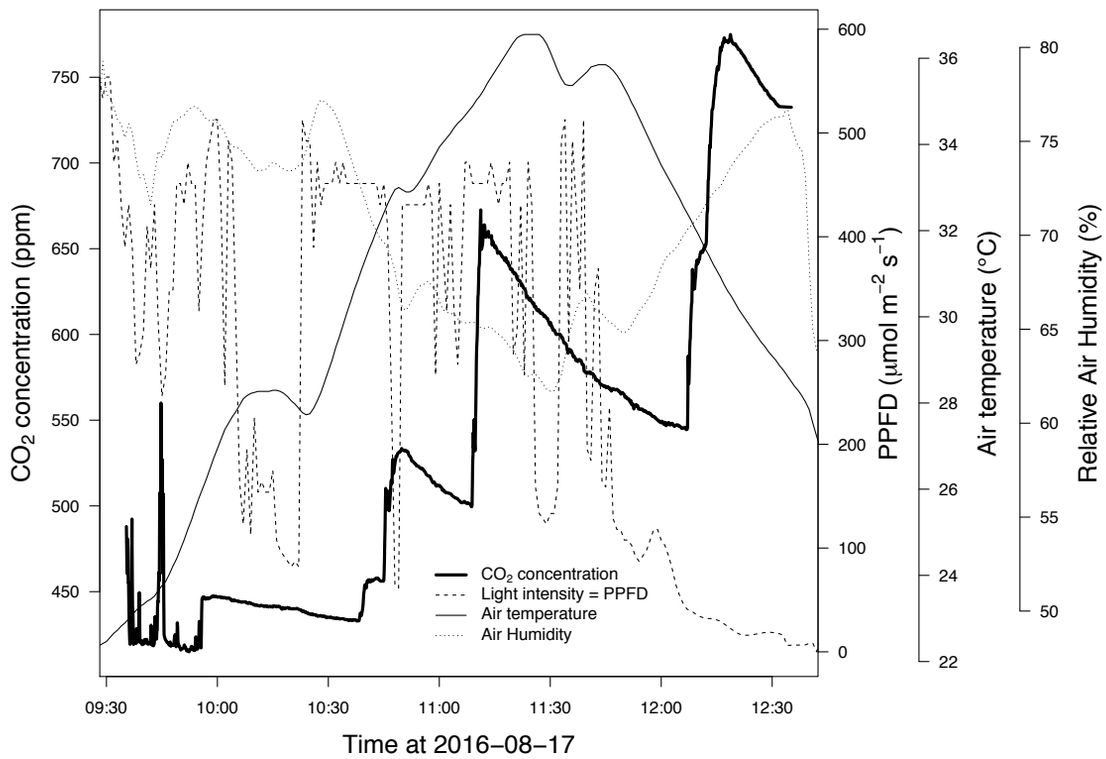

Fig. 4.6 Climatic conditions inside the tent construction during the ^{13}C pulse-labeling on August 17, 2016. The tent was closed at 09:55, and labeling started at 10:40.

Measurements of stable carbon isotopes

0.5 mg dry powder obtained from leaves, stem wood and root wood was weighed into tin capsules. The total C content and the ^{12}C to ^{13}C ratio were analyzed in a Flash 2000 elemental analyzer coupled to a Delta V Plus continuous-flow isotope ratio mass spectrometer (IRMS) via a Conflo IV interface (Thermo Fisher Scientific, Bremen, Germany). Samples went through flash combustion at ~ 1800 °C in the presence of oxygen, before the emerging CO_2 was introduced into the IRMS. Stable isotope data are expressed in the delta notation ($\delta^{13}C$), relative to the ^{12}C / ^{13}C ratio of Vienna Pee Dee Belemnite standard (R_VPDB = 0.0111797).

Results

Drought intensity and mortality

Soil water content and soil water potential were significantly decreased in drought (LD and DD, Fig. 4.7). As a consequence, DD and LD seedlings exhibited increasingly lower Ψ_{stem} along the experimental drought period. Throughout the drought treatment, LD seedlings had significantly lower Ψ_{stem} than DD seedlings, except on August 18 (Fig. 4.7). However, also 25% of well-watered controls (LW and DW), showed a Ψ_{stem} below -2.2 MPa. C assimilation and stomatal conductance in drought were only reduced after August 22 (two-way ANOVA, $p < 0.01$). Darkness and drought treatments did not have significant effects on seedling mortality, since several seedlings died also within the well-watered treatments for unidentified reasons. Until the first harvest on August 2, only 40-52 of initially 70 seedlings per treatment had survived.

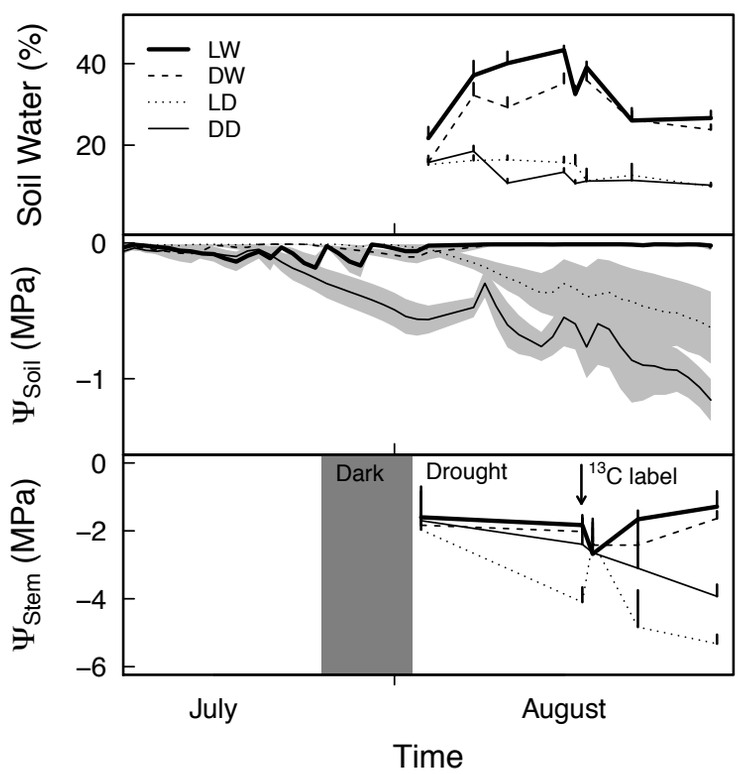

Fig. 4.7 Time course of hydraulic parameters (soil water content + SE, above, n = 4; soil water potential ± SE, middle, n = 5; stem water potential + SE, below, n = 4) from four different treatments: *LW*, controls; *DW*, darkness and watered; *LD*, light and drought; *DD*, darkness and drought. The 7 d long darkening period in DW and DD is indicated by a dark rectangle in the lowest diagram.

Non-structural carbohydrates

The darkness treatment between July 25 and August 2 failed to induce significantly lower total NSC concentrations in DW and DD until the first sampling date for NSCs on August 15, when the first drought damage became visible on leaves (Tukey's HSD, $p > 0.05$). Also in the subsequent harvests, NSC concentrations were not significantly lower in darkness treatments (data not shown). However, drought significantly increased the concentrations of free sugars in woody tissues (Fig. 4.8). Sugar concentrations in root and stem wood were significantly higher in LD compared to well-watered seedlings (LW and DW), and this difference became larger and more significant with proceeding drought (Tukey's HSD, $p < 0.05$, Fig. 4.8). In contrast to the investigated woody tissues, no difference in sugar concentrations were observed between treatments in leaves. Stem and root wood sugar concentrations in DD were slightly (not significantly) decreased compared to LD 5 d after the ^{13}C-labeling (Tukey's HSD, $p = 0.08$, Fig. 4.8). However, root starch concentrations in DD and LD at this point were 16% and 18% DM, respectively, and stem starch concentrations were 9% and 8% DM (data not shown). Thus, the decreased sugar concentrations in DD compared to LD were unlikely caused by the darkness treatment, but rather by the earlier onset of drought in DD compared to LD (Fig. 4.7). Root wood sugar concentrations in DD increased towards the end of the experiment, and were significantly higher than concentrations in well-watered treatments (LW and DW; Tukey's HSD, $p < 0.001$, Fig. 4.8).

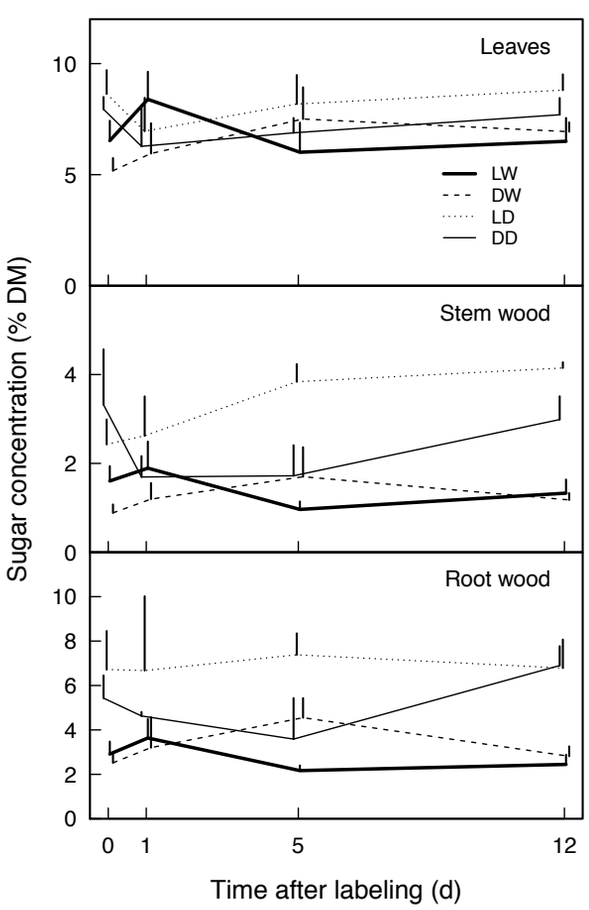

Fig. 4.8 Time course of sugar concentrations per dry matter (% DM, means + SE) in three different tissues of *Quercus* seedlings in four different light and water treatments: *LW*, controls; *DW*, darkness and watered; *LD*, light and drought; *DD*, darkness and drought. $n = 5$.

The free sugar concentrations in stem wood showed a strong negative correlation with Ψ_{stem}, when data were pooled from all treatments (Fig. 4.9): A 1% increase in sugar concentration per dry matter corresponded to a 1.1 MPa decrease in Ψ_{stem} (Fig. 4.9). On the other hand, starch concentrations were very weakly positively correlated with Ψ_{stem} (Fig. 4.9).

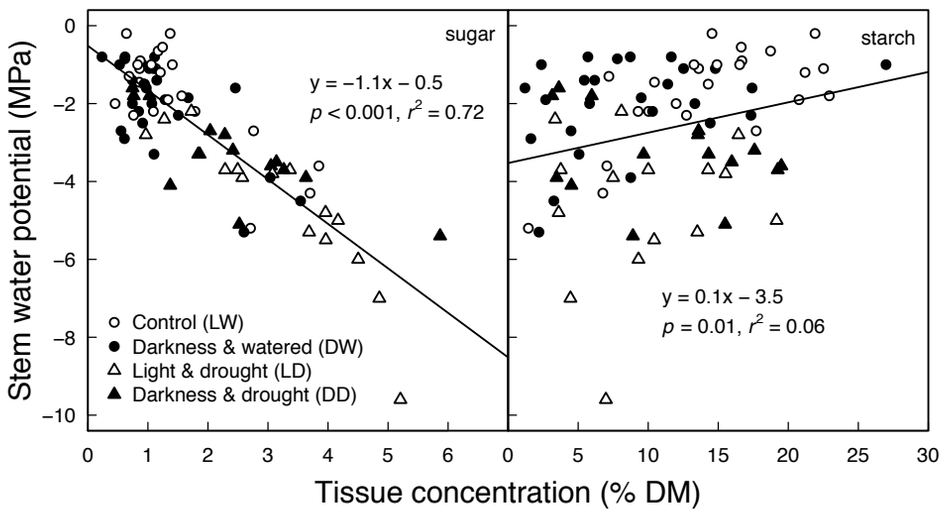

Fig. 4.9 Correlation of sugars (left) and starch (right) concentrations per dry matter (% DM) in stem wood or harvested seedlings with the Ψ_{stem} of entire seedlings measured at harvest.

^{13}C pulse labelling

After ^{13}C pulse-labeling, leaf $\delta^{13}C$ in all seedlings was above the natural level (around 28‰ as reported in Hoch et al. 2013; Fig. 4.10). However, ^{13}C enrichment in leaves showed a log-normal distribution with only a few strongly and many weakly to moderately labeled individuals (Fig. 4.10). C assimilation before labeling was positively correlated with log (leaf $\delta^{13}C$) after labeling (one-way ANOVA, Fig. 4.11), but not with log (root $\delta^{13}C$) 5 d or 12 d after labeling (one-way ANOVA).

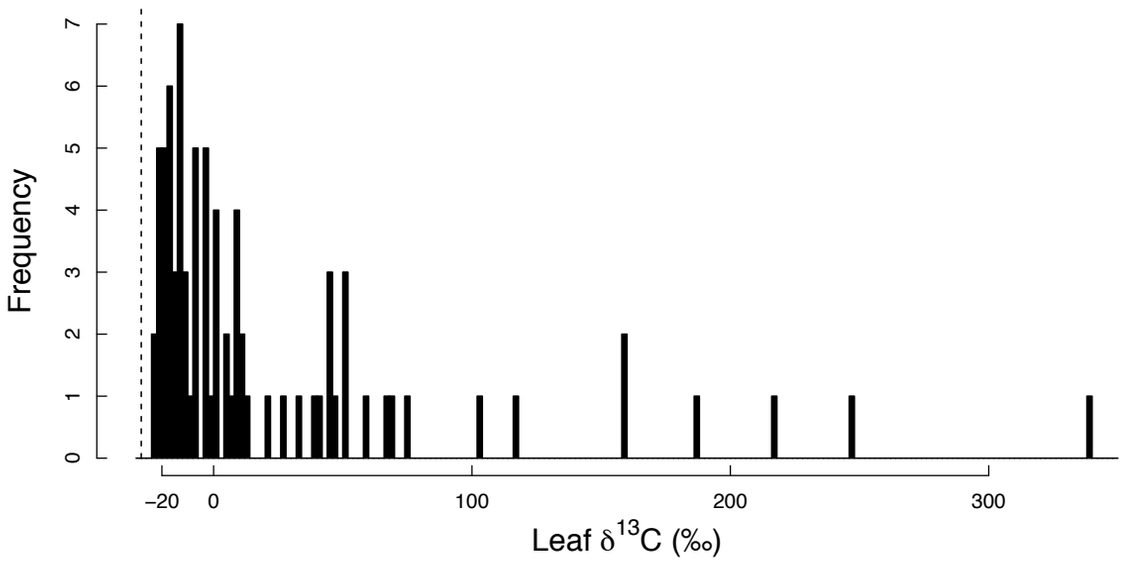

Fig. 4.10 Histogram of leaf $\delta^{13}C$ from all treatments and measurement dates pooled. The dashed line indicates an ambient $\delta^{13}C$ of -28‰ that is reported for *Quercus petraea* (Hoch et al. 2013).

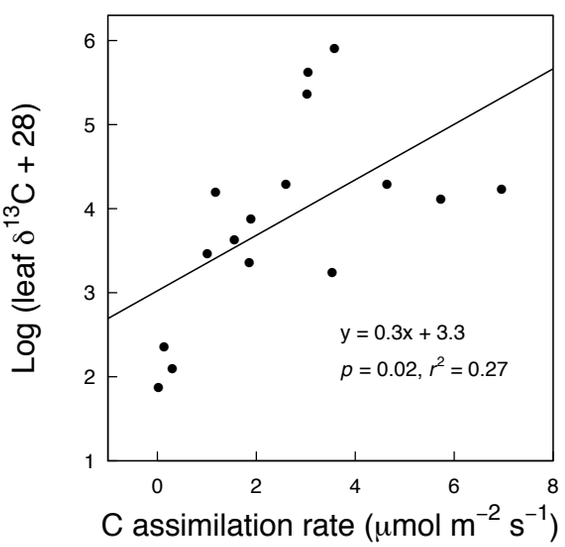

Fig. 4.11 Leaf C assimilation rate (at a PPFD of 500 µmol m^{-2} s^{-1}, from all treatments pooled) 4 h before the ^{13}C pulse-labeling, compared to the logarithmized leaf $\delta^{13}C$ 1 h after labeling. $n = 16$.

Leaf $\delta^{13}C$ decreased exponentially after labeling but did not differ significantly between treatments (Tukey's HSD, $p > 0.05$, Fig. 4.12). However, leaf $\delta^{13}C$ was always significantly higher than stem wood or root wood $\delta^{13}C$ (one-way ANOVA, $p < 0.001$, Fig. 4.12). Root wood $\delta^{13}C$ increased over time (one-way ANOVA, $p < 0.01$, Fig. 4.12), but was never significantly different between treatments (one-way ANOVA, $p > 0.05$, Fig. 4.12). Thus, C allocation to roots was not strongly impaired by drought or darkness. Yet, C allocation to roots seemed impaired across all treatments including the control, given that root wood was never substantially ^{13}C-enriched, being still at a $\delta^{13}C$ of -21.8 ± 0.7‰ 12 days after the pulse labeling when pooled among treatments (Fig. 4.12). Nevertheless, control seedlings seemed to exhibit a slightly faster C transport to roots during the first 5 days after labeling, indicating a weak additional reduction to C phloem transport by the darkening and/or the drought treatment (Fig. 4.12).

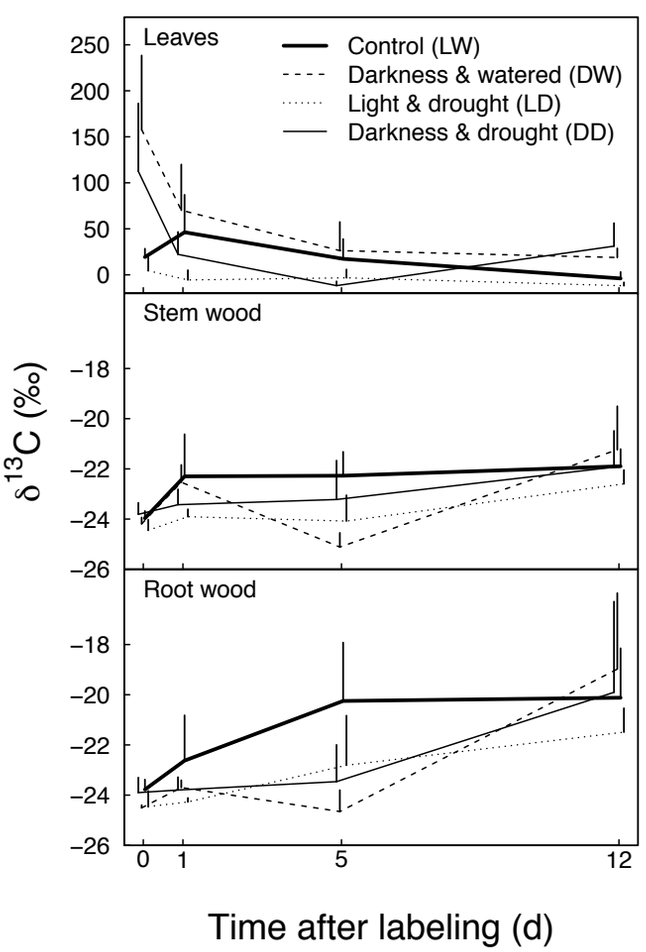

Fig. 4.12 Time course of $\delta^{13}C$ (means + SE) in four different treatments after ^{13}C pulse labeling. Note the different scale for the y-axis for leaves (above). $n = 5$.

Discussion

Main findings

Fast recovery of NSCs after darkness likely prevented a further investigation of the usefulness of NSCs during drought. Against my initial hypothesis, NSC concentrations were not significantly different from light controls in darkened seedlings by the beginning of the drought treatment. The study was therefore not able to address the direct effects of low vs. high NSC pools on the drought resilience of seedlings. I conclude that fast replenishment of NSC reserves after C-limitation is a challenge when aiming to artificially deplete NSC reserves before drought. Moreover, the observed high mortality (presumably caused by insufficient production of new roots after potting in many individuals, pers. observation) even in well-watered controls limited the ability of this study to draw unequivocal conclusions about the real drought stress status of seedlings under the different treatments. However, a negative correlation of free sugars with Ψ_{stem} and a positive correlation of starch with Ψ_{stem} confirmed the necessity of the presence of higher sugar concentrations for drought resistence, and indicates the active conversion of starch to free sugars during drought.

Were all seedlings drought stressed?

The measurements of mid-day Ψ_{stem} revealed surprisingly low values also in the well-watered seedlings, which further indicates that many seedlings had limited water uptake after transplantation. The observed Ψ_{stem} below -2.2 MPa in well-watered controls were in fact close to the -2.5 MPa mid-day Ψ_{stem} reported for branches of adult *Quercus petraea* during a dry period (Dietrich et al. 2018). In addition, Ruehr et al. (2009) showed a significant increase of root $\delta^{13}C$ after pulse labeling in drought-stressed *Fagus sylvatica* already 8 d after a 4 h-pulse labeling with approximately 70 atom% ^{13}C. In the current study, root $\delta^{13}C$ was hardly increased and still below -20‰ even 12 d after labeling in drought stressed and watered seedlings. In combination, the high mortality, low water potentials and limited phloem transport to roots, indicate that water stress likely occurred in all seedlings including water controls.

Sugars during drought

Despite the high mortality throughout the experiment and inconsistent responses of stomatal conductance and Ψ_{stem} to drought treatments, I observed a strong correlation between sugar concentrations and Ψ_{stem}. The linkage between these two parameters and the accumulation of free sugars as osmotically active compounds to prevent dehydration and turgor loss in drought stressed plants is a well documented stress reaction in plants (Christy and Ferrier 1973, Cernusak et al. 2003, Dietze et al. 2014). These previous data suggest a high importance of NSC to overcome drought. However, up to date there are still patchy and very limited indications for a direct beneficial effect of high NSC pools for drought resistance in trees. The best evidence so far comes from a factorial shading x drought experiment on tropical tree seedlings (O'Brien et al. 2014). However, also in the study by O'Brien et al. (2014) NSC concentrations in originally NSC deprived seedlings increased during the drought treatment, leaving some open questions about the real effect size of NSC on the seedlings' survival time during the drought period. Unfortunately, the ambiguous results from the current study did largely prevent more detailed conclusions on the interplay between the C reserve supply status of a tree and its resilience under drought. The fast refilling of NSC pools after temporal depletion in tree seedlings, that was also one of the most important results in chapter 1 of this thesis (Weber et al. 2018), probably limits the suitability of darkening for experiments on NSC x drought effects. A promising alternative avenue might be the additional usage of low CO_2 chambers like used by Hartmann et al. (2013) and Schmid et al. (2017) in parallel with drought treatment to prevent NSC refilling during drought. Such modified experiments would help to better understand the functional interrelation between the C and water household of trees under drought.

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General summary and conclusions

Observed decreases of NSC tissue concentrations in times of low C-uptake have led to a wide, although probably not sufficiently founded usage of NSCs as an indicator for C-limitation in stressed trees. In this thesis, I studied the indicative value of NSCs to predict the C supply status of temperate tree species. I aimed at quantifying the species- and tissue-specific dynamics of NSC concentrations and growth i) at severe and eventually lethal C-limitation and ii) during long-term and less severe (non-lethal) C-limitation. Further, I aimed at (iii) assessing the long-term acclimation of photosynthesis and C allocation to shade in tree saplings, thereby improving the predictive value of short-term shading experiments for understory trees.

In general, the results confirm the importance of C storage during C-limitation, because NSCs increased in the long term under less severe (non-lethal) C-limitation, while NSCs were almost completely depleted during severe (lethal) C-limitation. The potential of C-limited trees to up-regulate NSC pools in trade-off with other C sinks, like growth, is most likely underestimated using short-term shading experiments.

Dynamics of NSCs before, during and after release from C-starvation

During complete darkness and before lethal C-starvation, NSC concentrations (the sum of starch, sucrose, glucose and fructose) decreased quickly in tree seedlings, and reached extremely low concentrations in all organs (*c.* 1-3% DM, a decrease by at least 80% of control concentrations; chapter 1, chapter 4.1), which are scarcely observed under natural conditions (chapter 2). The low NSC concentrations were maintained, while seedlings presumably also used alternative C sources like lipids, hemicelluloses or proteins (chapter 1). During that time, broad-leaved species were still capable to produce etiolated shoots, which underpins the availability and remobilization of substantial C storage compounds (chapter 1, chapter 4.1). After weeks with such low concentrations and without sufficient photosynthetic C uptake, seedlings died after a final, non-significant decrease in NSC concentrations (chapter 1). During scenarios of C limitation where no mortality emerged (*e.g.* in *Abies, Carpinus* and *Picea* at 6% of full sunlight, or in defoliated *Carpinus*; chapter 2), I also observed minimum NSC concentrations of *c.* 1% DM, but these concentrations were

highly species-specific, and were never decreased by more than 70% compared to those in saplings with high C supply growing in unshaded conditions (chapter 2, Fig. 2.9). Therefore, NSC concentrations can be used to predict lethal C-starvation under sustained C-limiting conditions already weeks before death, but such measurements always require comparative measurements of NSC concentrations in potentially C-limited and fully C-supplied control individuals at the same time (chapter 1, chapter 2). On the other hand, NSC concentrations cannot be used as indicators for the exact point of death of an individual plant (chapter 1).

When darkened seedlings were re-illuminated before lethal C-starvation emerged, NSC concentrations recovered quickly to pre-darkness concentrations. The refilling of NSC pools occurred even at a faster rate than observed for its depletion under darkness (chapter 1). Such recovery was accompanied by re-flushing of leaves, finally emphasizing that lethal C-starvation cannot be assumed, when NSC concentrations are still higher than the minimum concentrations found in individuals that indeed died in darkness (chapter 1). Apparently, re-growth after re-illumination was restricted by a preferential up-regulation of NSCs, because growth only occurred when an end-season "target" NSC concentration was met (chapter 1). Combined with the observed absence of growth after darkness, I conclude that NSC storage has a strong priority over growth after release from severe C-limitation until NSCs are largely re-filled (chapter 1). However, the observed production of etiolated shoots despite depletion of NSCs during darkness suggests that NSC storage is not prioritized over growth before and during lethal C-starvation.

Preferential NSC formation during non-lethal C-limitation

Besides a strong increase of NSCs after release from severe C-limitation (lethal if sustained; chapter 1), NSC pools were also up-regulated during less severe (non-lethal) long-term C-limitation, like at 6% light (chapter 2). Despite an initial two-fold decrease of NSC concentrations after the first growing season in temperate tree saplings under shade and a reduction of the relative growth rate (g g^{-1} a^{-1}) by at least 70% in shaded individuals, the average winter NSC concentrations in shaded saplings of all investigated species recovered to those of un-shaded controls after three years (chapter 2). Because I observed such strong up-regulation against growth in all investigated species including those with no mortality during three years at 6% light

(*Abies*, *Carpinus* and *Picea*, chapter 2), I suggest that NSC storage has a priority over structural growth even when the experienced C-limitation is not lethal.

Besides the observed maintenance of NSCs under shade, saplings at 6% light exhibited significantly decreased NSC concentrations (compared to controls) during the first half of each growing season (when most of the tree growth occurs), or after additional C limitation by defoliation. Hence, in shaded saplings, strong declines of NSC concentrations were observed in all tree organs a few weeks after leaf flush in deciduous species, as well as five months after a defoliation treatment, and during a natural aphid infestation (chapter 2). Thus, it can be assumed that even under significant C limitation, NSC storage loses its priority over growth at situations were growth is essential for survival (e.g. formation of leaves in spring, regrowth after defoliation).

Based on my findings, I conclude that measurements of similar NSC concentrations during winter in potentially C-limited tree saplings compared to well C-supplied controls do not exclude the presence of C-limitation in trees, while measurements during the growing season predict C-limitation more reliably. However, NSC measurements during the growing season should also be compared between potentially C-limited and well C-supplied saplings (as suggested in the previous chapter, but for a different reason).

Reliability of short-term shading experiments

The observed long-term acclimation of NSC storage to shade highlights that NSC measurements from short-term (one year) shading experiments cannot provide data to make predictions for the C relations of long-term acclimated tree saplings in a forest understory (chapter 2). However, the photosynthetic apparatus in tree saplings was acclimated to 6% light already within one growing season, thus, photosynthesis measurements from short-term shading experiments provide a realistic estimation of the photo-assimilation in tree saplings naturally occurring in the forest understory (chapter 3).

Conclusions

This thesis showed unequivocally, that measurements of substantial NSC concentrations in potentially C-limited tree seedlings or saplings do not exclude the presence of non-lethal C-limitation, while they clearly exclude lethal C-starvation. Thus, lethal C-starvation can be predicted more easily from NSC measurements, than non-lethal C-limitation. These findings improve the predictive value of NSC concentrations, and hopefully help to prevent erroneous assessments about the C-relations of trees. Because non-lethally C-limited trees showed reduced NSC pools only during mid-season or after disturbance, NSC concentrations can only be used as a proxy for a tree's C-supply, if their seasonal dynamics are captured, or if measurements from a single time point are compared to those from well C-supplied controls. Finally, in addition to NSC measurements, predictions about a plant's C-balance should be complemented by other physiological measurements, or strengthened using CO_2 enrichment studies. For example, the higher NSC pool of trees at the alpine tree line compared to trees of the same species occurring at lower elevations, had led to the interpretation that the cold limit of tree growth is mostly not caused by C-limitation (Hoch and Körner 2012). This hypothesis has been supported by independent investigations on photosynthesis and growth of tree line trees, which showed that C-uptake is significantly less sensitive to cold temperatures than tissue formation (Körner 2015). To give another example, growth in defoliated *Pinus uncinata* growing at cold limits could be shown to be C-limited despite significant NSC reserves, because growth was enhanced under elevated CO_2 (Handa et al. 2005). Constantly high NSC pools in mature temperate forest trees, led to the assumption that the productivity of these trees is unlikely limited by C-assimilation (Hoch et al. 2003). This assumption could be finally confirmed by a long-term CO_2 enrichment experiment (Körner et al. 2005).

In conclusion, the comparative analysis of tissue concentrations of NSC has been proven a useful tool to infer an integrative assessment of the C source-sink relations in trees. However, because the formation of C reserves can also occur against prevailing C sink demands in trees, caution has to be taken if NSC concentrations are used as indicators for C-limitation.

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