You can't always get what you want
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Patterns and control mechanisms of carbon allocation in plants under stress and resource limitation

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1. From tree rings to ecophysiology

This thesis describes my research during the last seven years related to how trees cope with drought or carbon limitation. My PhD thesis research investigated tree mortality in response to anthropogenic (partial harvest) and natural disturbances (insect defoliation) in sugar maple (*Acer saccharum*) forests in western Quebec. Using both dendrochronological and stem wood isotope analysis as a means to retrospectively assess tree physiological condition my study suggested that mortality may have been related to carbon depletion from repeated insect defoliations. Heavily attacked trees had to use stored carbon to replace the predated foliage which reduced their reserve pools and predisposed them to the next epidemic herbivory attack until they succumbed to this series of disturbances. However, the retrospective dendrochronological approach I was using did not allow any direct proof of such a causal link because I could not analyze retrospectively the carbon reserve pool. I was somewhat disappointed and decided that I would use from that point on a more direct ecophysiological approach for my future research. I started a postdoc at the Max-Planck Institute for Biogeochemistry in Jena where I initially investigated tree mortality again – in response to drought. This research focused on carbon starvation during drought and incited me to also address more general questions regarding carbon relations in plants and was the starting point for my research group *Plant Allocation* that I founded in 2014.

Before laying out the scientific questions that have shaped my research during the last seven years, I dedicate a few pages to explaining why I have been and still am fascinated by research on carbon allocation in plants. I also give a general introduction on the basic concepts and methods I am using for my research. A brief section on my scientific framework precedes the actual research chapters which will be followed by a short conclusion that provides an outlook on my future research path that aims to improve our understanding of plant carbon allocation.

2. Carbon in the Earth system

Carbon is an amazing element as it creates conditions, with some few other elements, for life to develop on Earth, carries the energy required for terrestrial life and, overall, is the 'backbone' of life. Although it is the fourth most abundant element in the universe it is relatively sparse on Earth: Carbon makes up less than 1 % of the Earth crust's mass and is only the 15th most abundant element on Earth. Carbon in the Earth system exists in multiple forms that can be grouped into: (i) inorganic (including carbonate rocks, carbon dioxide in the atmosphere and inorganic carbon dissolved in oceans and terrestrial waters) and (ii) organic preserved in non-living material like rocks, in sediments and soils as well as in living organisms.

About 0.4 % of the atmosphere is carbon dioxide molecules, but due to land cover change (e.g., deforestation for agricultural production) and fossil fuel emissions this proportion is steadily increasing and can be expected to reach between 430 to 1000 ppm by 2100 (IPCC, 2013). Carbon dioxide (currently ~400 ppm) and methane (currently ~1850 ppb) are, besides water vapour occurring at a percentage range (~1 to 5%), the most important greenhouse gases of the atmosphere. The greenhouse effect is due to the fact that these gases absorb energy in infrared wavelengths that are radiated from the Earth's surface. The absorbed energy activates vibrational modes in the greenhouse gases that re-radiate energy to other molecules of the air, like O_2 and N_2 and to the Earth surface. Overall, the net effect is a warming of the atmosphere and without this greenhouse gas effect, the Earth's mean temperature would be around −19°C instead of the current 14°C (Le Treut *et al.*, 2007). Thus greenhouse gases allow temperatures that permit liquid water to persist at the surface, a condition that is required to support Earth's current life.

Carbon is also the main substrate for primary production and hence the vital source of energy to fuel terrestrial life, including millions of different species of bacteria, fungi, plants and animals. As noted above, atmospheric [CO_2] has been strongly increasing since industrialization (Etheridge *et al.*, 1996) with positive impacts on global primary production (Nemani *et al.*, 2003). However, as anthropogenic emissions continue to increase atmospheric [CO_2], the associated climatic changes can also have negative effects on current global vegetation. Climate-change induced temperature extremes combined with related, increasingly frequent drought episodes have been shown to offset the [CO_2] fertilization effect on primary production (Zhao & Running, 2010). Although changes in atmospheric [CO_2] and global temperatures have occurred frequently throughout the Earth's history, the current trend is disturbing because of the speed of change and due to increasingly frequent occurrences of extreme events, like heat waves and exceptional droughts (IPCC, 2012). Such extreme events, rather than changes in average temperature and in precipitation distribution, are responsible for decreasing regional ecosystems carbon stocks and that offset CO_2-mediated increases in the terrestrial carbon uptake (Reichstein *et al.*, 2013). Recent observations of elevated mortality rates in response to heat and drought in forest ecosystems worldwide (Allen *et al.*, 2010) raise concern about the vulnerability of global vegetation to these fast changes in climate (Allen *et al.*, 2015). The current global vegetation contains ~450-650 Pg of carbon and massive die-off events of the current global vegetation in response to rapid environmental change may liberate some of the carbon sequestered by vegetation – with likely severe consequences for biogeochemical cycles (Anderegg *et al.*, 2013) and feedback dynamics for global warming (Allen *et al.*, 2015).

3. Carbohydrates are both energy carriers and building blocks in plants

Much of the terrestrial carbon is constantly cycling among living components and the atmosphere. The global vegetation turns over large amounts of carbon every year, more than 120 Pg (IPCC, 2013), equivalent to that in a freight train filled with coal long enough to circle the equator more than 375 times. During photosynthesis, light energy is used to synthesize ATP (adenosine triphosphate), a cellular energy carrier, and reducing power in form of NAD[P]H (nicotinamide adenine dinucleotide [phosphate]). Energy and reducing power together allow the binding of CO_2 via RubisCo, the most abundant enzyme on Earth, to a 5-carbon molecule that immediately splits into half and releases two triose molecules. These are converted into a 6-carbon molecule, glucose, the building material for all other organic plant compounds and structures. Plants synthesize different forms of sugars called saccharides, all made of carbon, hydrogen and oxygen in constant proportions ($C_m(H_2O)_n$) but with varying numbers of atoms. These saccharides are classified depending on the number of individual units (e.g., monosaccharides like glucose, disaccharides like sucrose, oligosaccharides like raffinose, polysaccharides like cellulose) and are collectively called carbohydrates. Together with hydrogen-enriched sugar alcohols and other carbon-rich compounds like lipids, carbohydrates play a central role in plant functioning (Hartmann & Trumbore, 2016).

Carbohydrates are both energy carriers (non-structural carbohydrates) and the main building blocks for the synthesis of other compounds including structural plant biomass. Glucose molecules are bound together via β-glycosidic bonds to form long-chained, linear (amylose) or, via α-glycosidic bonds, branched (amylopectin) and non-branched spiral polymers, the latter called starch. Sugars and starch, once reconverted to glucose and oxidized to pyruvate during glycolysis, can be completely processed in the Krebs cycle to produce ATP and reducing equivalents for biosynthetic reactions while releasing CO_2. As such, carbohydrates (but also lipids and proteins) are the main energy source not only for plants but also for heterotrophic organisms and are thus the basis of the food chain. Without plants and their capacity to convert mineral carbon (CO_2) into organic forms like carbohydrates, life on Earth would not be possible. However, carbon also forms long chains of glucose units with glycosidic bonds that cannot be broken by plants due to a lack of the required enzymes. Such polymers, like cellulose, are the building material for plant structures (cell walls, woody tissues) and at the same time an important food source for other organisms. Symbiotic bacteria in ruminants and termites as well as some fungi and free-living bacteria produce enzymes allowing them to depolymerize organic lignocellulose (a composite of cellulose, hemi-cellulose, pectin and lignin) to glucose units that can then be metabolized during glycolysis (Cragg *et al.*, 2015) to make the sun energy, that has been originally stored in these polymers, available to metabolism.

Despite its central role for heterotrophic organisms much of the carbon captured during photosynthesis is directly released back into the atmosphere via plant respiration. Plant respire between 30 to 40% (Poorter *et al.*, 1990), sometimes even up to 80% (Amthor, 2000), on average roughly 50% (IPCC, 2013), of the carbon captured in the Calvin cycle and only the remaining carbon is spent for plant growth, development and reproduction (primary metabolism) and other functions like defence, communication and attraction (secondary metabolism). The distribution scheme of carbon across different functional pools is called carbon allocation, the central topic of this thesis.

4. Carbon allocation in terrestrial plants

Most plants are sessile organisms and cannot escape unfavorable environmental conditions or hostile attack by emigration. To cope with such situations, plants have to manage their available resources to optimize development and fecundity while securing survival and reproduction. For example, a young developing plant that is attacked by an herbivore must produce unpalatable or even toxic substances to deter attackers. However, these substances are carbon costly and divert resources away from primary metabolism, i.e. growth and reproduction (Massad *et al.*, 2012), as claimed by the "Growth-Differentiation-Balance Theory" (Herms & Mattson, 1992; Matyssek *et al.*, 2012a; Matyssek *et al.*, 2012b).

For more than 40 years, the control mechanisms of whole-plant carbon allocation have been of central interest to plant scientists (Mooney, 1972). Historically the regulation of plant carbon partitioning was thought to be driven by C supply (i.e. source activity), but recent evidence suggests that limitations of sink activity via physical constraints (e.g., cold) or stoichiometry (e.g., unbalanced nutrient supply) may be the main determinant for the amount of carbon allocated to different processes and functions (Fatichi *et al.*, 2014; Korner, 2015). Carbon allocation has three aspects: (1) the amount of material present at a given point in time, measured as biomass fractions; (2) the flow of carbon into the different functional processes (e.g., respiration, growth) per unit time, measured as a flux; and (3) the fraction of the total available carbon that is used by a functional process per unit of time (Fig. 1). From these three, only biomass is commonly assessed in many studies because fluxes are difficult to measure (e.g., root respiration) and/or the total amount of available carbon, i.e. the whole-plant carbon balance, is often unknown (Poorter *et al.*, 2012). However, without considering all three aspects of allocation, a thorough understanding of the control mechanisms, especially at the whole-plant level, cannot be achieved (Litton *et al.*, 2007). Predictions of plant responses to climate change cannot be realistic without a thorough understanding how plant manage their resources, especially non-structural carbohydrates (Dietze *et al.*, 2014). In order to

assess all three aspects research must apply a variety of tools, for example, isotopic labelling of plant carbon and measurements of the whole-plant carbon balance.

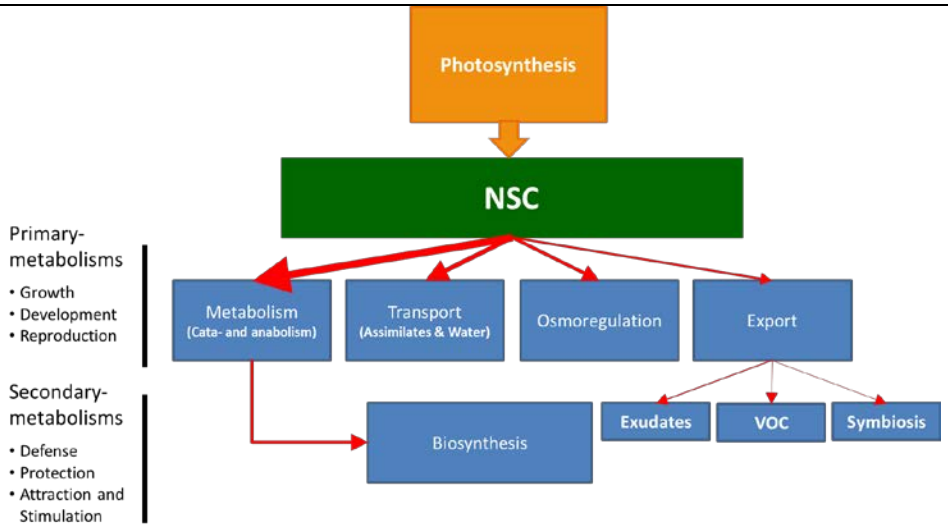

Figure 1 The central role of non-structural carbohydrates (NSC) in carbon allocation and plant functioning.
The boxes symbolize allocation pools (biomass) that can usually be easily measured but provide information on cumulative allocation at a given point in time only (e.g., seasonal growth). Arrows between pools represent fluxes, like respiration, that can be assessed either directly using, for example, gas exchange measurements or derived via tracers like isotopic markers (e.g., $^{13}CO_2$). The sizes of the arrows represent the fractions of available carbon (net carbon balance) that flows from one pool to another. Such information is extremely insightful for understanding plant allocation strategies but very hard to assess, especially in the field. To do so, one needs to know all components of the plant carbon balance.

5. Carbon isotopes – a unique tool for investigating allocation fluxes

Most carbon (98.93%) has a molar mass of 12 but two other naturally occurring isotopes exist: ^{13}C, a stable isotope (~1.07%) and radiocarbon (^{14}C, ~1.1 out of 10^{12} atoms) with a half-life of ~5730 years. There are many other known carbon isotopes (^{8}C to ^{22}C) but they do not occur naturally and are very unstable and decay within fractions of seconds, except ^{11}C with a half-life of ~ 20 minutes.

Isotopes are usually not reported in terms of absolute abundances because of difficulty in quantitative representation. Instead, the proportion of each isotope in a given sample is analysed and this ratio is often reported as a fraction of the source ratio (e.g., isotopic ratio of CO_2 in air, if known) or of an international standard with known isotope ratio (*VPDB*, Vienna Peedee Belemnite), defined as $\delta^{13}C$ (eq. 1).

$$\delta^{13}C_{Sample} = \left\{ \frac{\left(\frac{^{13}C}{^{12}C}\right)_{Sample}}{\left(\frac{^{13}C}{^{12}C}\right)_{Reference}} - 1 \right\} * 1000 \quad (1)$$

Plants take up carbon dioxide regardless of its isotopic composition but due to thermodynamic principles some of the processes involved in carbon assimilation by plants, like diffusion of CO_2 into leaves and carboxylation reactions in the chloroplast, discriminate against heavy carbon isotopes. The ratio of heavy to light carbon isotopes is hence smaller in plant biomass than in the source air. Discrimination against heavy carbon isotopes and the resulting isotope ratio are influenced by factors regulating carbon uptake, namely stomatal aperture and photosynthetic rates. Therefore changes in the isotope ratio of plant biomass can be used to infer on the underlying metabolic and environmental forcing of carbon uptake, like e.g. drought-induced stomatal closure.

Moreover, isotopes can also be used as tracers in plant carbon allocation. By 'feeding' plants with air having CO_2 with an isotopic ratio different than ambient air one can follow such a pulse of 'labelled' carbon from assimilation and into targeted processes (e.g., CO_2 emitted via respiration) or pools (e.g., non-structural carbohydrates). Depending on the scientific questions, the amount of carbon allocated to a specific process and the turnover time of the investigated carbon pool, the duration of exposure of the plant to the label and the strength of the label (i.e. the isotopic deviation from ambient air) can vary substantially (Epron *et al.*, 2012).

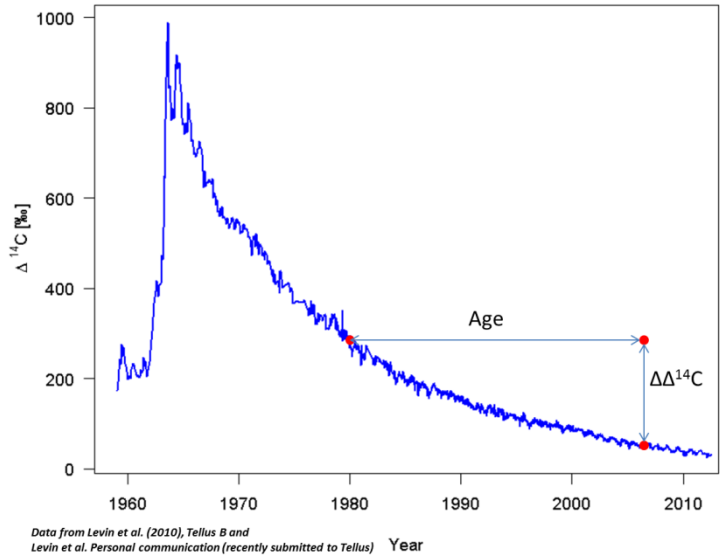

Figure 2 The atmospheric $\triangle^{14}C$ curve from 1960 to 2012.
When taking a plant sample the $\triangle\triangle$ (i.e. difference in $\triangle$ where $\triangle$ denotes the difference in $\delta^{14}C$ in atmosphere to pre-bomb $\delta^{14}C$) of that sample will likely be above the atmospheric curve. This signal can be traced back to deduce that this carbon has been accumulated when the atmospheric $\triangle^{14}C$ was still higher, in the shown example at ~1980.

Radiocarbon can also be a very useful tool in studies on carbon relations in plants. While the half-life of ^{14}C is commonly applied to estimate the age of material containing plant-derived carbon (e.g., wood beams, paint), an involuntary labelling of the atmosphere during nuclear bomb test in the 1960s is used to derive estimates of time elapsed since carbon assimilation with a high temporal resolution (Fig. 2). The derived 'carbon age' allows inferences on the use of older carbon for metabolism, for example, respiration (Muhr *et al.*, 2013) or estimations of turnover times of carbon pools (like non-structural carbohydrates in xylem sap, see chapter 9.8). Carbon isotope

analysis including both stable and radioactive forms will be a common and essential tool throughout most of the research chapters presented in this thesis.

6. Measuring the whole-plant carbon balance

Plants are somewhat like small enterprises. The have an income (carbon uptake), running costs (maintenance respiration), production costs (e.g., growth respiration, energy demands for nutrient uptake, synthesis of enzymes required for photosynthesis), other expenses like marketing (e.g., defence, attraction of pollinators) and they must strategically place investments (i.e. allocation) to ensure survival and reproduction. The plant carbon balance can therefore be viewed as the finance plan of an enterprise. However, some of the budget positions cannot be easily measured and must be deduced from other, more easily assessed but often concurrent functions or processes. For example, growth and maintenance respiration can usually not be differentiated directly and must be derived computationally by subtracting, as some kind of approximation, respiration during periods of zero growth from total respiration (Ryan, 1990). On the other hand, for estimates of the whole-plant carbon balance, differentiating between these two fluxes may not be important since both cost positions are summed in total respiration.

During the last five years, my research group built up and employed an experimental design that allows us to assess the **whole-plant carbon balance** by measuring two major net **fluxes**: assimilation and respiration. We do this by growing plants in small chambers continuously flushed with air and by measuring [CO_2] of the air entering and the air leaving the chambers. The concentration difference multiplied by the flow rate of air through the chamber and summed over the whole day quantitatively yields the carbon gained (total daily assimilation > total daily respiration) or lost by the plant(s) (Fig. 3).

The resulting total available carbon can then be partitioned among functional **biomass** pools, yielding **fractions of available carbon** allocated to different functions or processes. Hence, our approach allows assessing all **three aspects of allocation**. To increase the number of flux measurements, we infer fluxes from repetitive measurements of biomass pools (e.g., non-structural carbohydrates), taking the difference in pool concentrations multiplied by the size of the pool (the biomass containing the pool, for example, leaf non-structural carbohydrate concentrations multiplied by leaf biomass) as the best estimate of the flux into or out of the pool. In addition, the use of isotopic markers, added to the air stream going into the chamber, allows inferences on the flux into different pools. The change in the isotopic composition of the pools indicates the amount of

carbon that went into that pool where the degree of change from the initial isotopic composition is proportional to the allocated amount of carbon (see chapter 9.4).

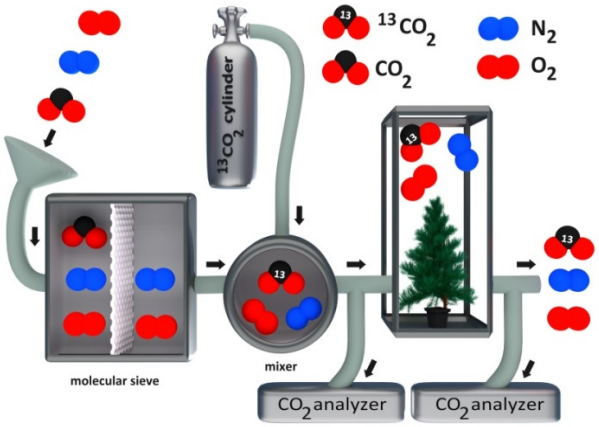

Figure 3 Schematic cartoon of the design for assessing the whole-plant carbon balance of plants during experiments.
The facility allows controlling [CO_2] and carbon isotopic composition of the air going into the chamber. Atmospheric CO_2 is first removed from ambient air, then CO_2 from a bottle is added to this air to achieve pre-set [CO_2]. Measurements of [CO_2] of the air entering and leaving the chambers and multiplied by the flow rate of the air yield the carbon balance of the plant. Modifications of the [CO_2] of the air entering the chamber also allow altering the plant carbon balance.

7. Using resource limitation to reveal allocation patterns and strategies

The facility described in the previous section allows not only assessing but also manipulating the whole-plant carbon balance. To do so, the [CO_2] of the air entering the chamber can be either enriched (with respect to current ambient concentration ~ 400 ppm) or depleted. Enrichment may be used to investigate plant responses to future atmospheric [CO_2], while depletion allows research on plant functioning under paleo-climatic conditions or on carbon allocation priorities. Reducing [CO_2] restrains plant carbon availability and also sets tighter boundaries for carbon allocation because the plant assimilates less carbon and can therefore also spend less. Monitoring of allocation fluxes (via direct measurements, isotopic markers or pool size changes over time) will indicate which of the fluxes are reduced the most and which the least. We assume that allocation sinks that are maintained (or less severely reduced than others) under carbon limitation have a high allocation priority. The resulting pattern of allocation can be taken as an indicator for allocation strategies (ensemble of responses to stress or environmental change) or used to derive cost-benefit scenarios during exchanges with symbiotic partners (see 9.10). This methodology is a central theme in several of the research chapters.

8. Carbon, carbon, carbon – mortality mechanisms and allocation strategies

When I started a postdoc at the Max-Planck Institute for Biogeochemistry in Jena, drought-induced tree mortality had become a hot topic in forest ecological research. In 2010 Allen et al. reported data suggesting an emerging climate change risks for forests and this publication

stimulated much discussion in many relevant international journals and at major ecological conferences and earth-science meetings. In their meta-analysis, Allen *et al.* (2010) documented that increasing tree mortality rates and more frequent forest die-off events were linked to heat spells and drought.

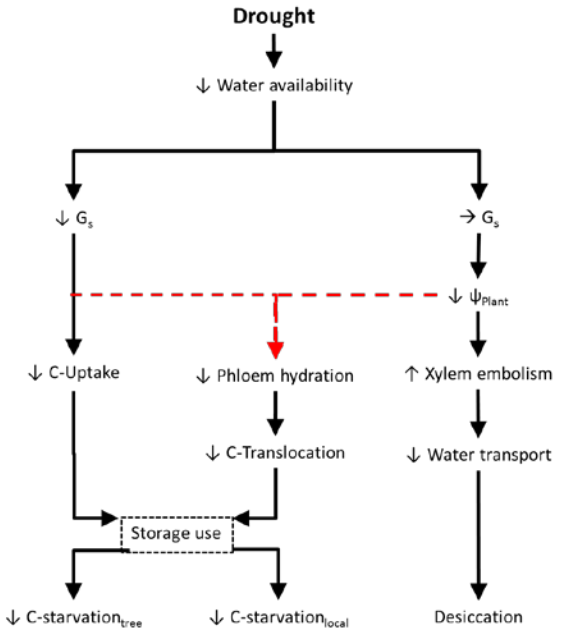

Figure 4 Potential drought-induced tree mortality mechanisms ('hydraulic framework', McDowell *et al.*, 2008).
Species that down-regulate stomatal conductance (G_S) early during drought (left) also hamper carbon assimilation and have to rely, especially during extended drought periods, on stored carbon to fuel metabolism. If these conditions persist, carbon reserves may be depleted, causing carbon starvation. By contrast species may not down-regulated stomatal conductance very early during drought (black arrows, right) and risk further water loss and decreasing water potential that can lead to xylem embolism, interruption of water transport and tissue desiccation, especially during severe droughts. However, given that drought will always cause reductions in plant water potential phloem functioning will also be negatively affected and thus a tree's ability to remobilize and transport stored carbon across organs, i.e. from storage tissues to metabolically active tissues. This may result in a depletion of carbon reserves locally, i.e. in sink tissues.

McDowell *et al.* (2008) proposed the hydraulic framework that links stomatal regulation of water loss during drought with reductions in carbon assimilation. For species that control water loss during drought with early stomatal closure this would lead, for extended drought periods, to carbon storage dependency and, ultimately, to carbon starvation. Species with less conservative stomatal control, however, would die of desiccation via hydraulic failure (irreversible interruption of water transport, Fig. 4).

Given my background in tree mortality I decided to contribute to this debate by laying out a strategy for ongoing research as a means to partition effects of potential mortality mechanisms. My research agenda expanded upon the hydraulic framework by including carbon translocation (i.e. storage mobilization and transport) as a potential additional mortality mechanism (Chapter 1: No water no transport no carbon). During drought plant water potential will decrease and independent of a species' stomatal regulation. This, in turn, negatively affects phloem functioning (Sevanto, 2014) and thus a tree's ability to remobilize and transport stored carbon across organs, i.e. from storage tissues to metabolically active tissues. This may result in a depletion of carbon reserves locally, i.e. in sink tissues (Fig. 4). I carried out a series of experiments to address the role of these mechanisms during lethal drought, with a particular focus on carbon relations (Table 1).

Table 1 Main research questions that will be addressed in the research chapters, categorized by major research topic, type of work (research or review/synthesis) and the corresponding reference.

Research question(s)	**Chapter Type**	**Reference**
Drought-induced tree mortality		
McDowell's hydraulic framework is incomplete	9.1 Review	Hartmann 2011 Global Change Biology
Does drought cause carbon starvation?	9.2 Research	Hartmann et al. 2013 Functional Ecology
Does reduced hydration prevent whole-tree carbon starvation?	9.3 Research	Hartmann et al. 2013 New Phytologist
Does drought prevent carbon allocation from source to sink organs?	9.4 Research	Hartmann et al. 2015 Tree Physiology
Is carbon starvation during drought impossible?	9.5 Review	Hartmann 2015 Journal of Plant Hydraulics
General carbon relations in forest trees during drought		
How does elevated temperature affect whole-tree carbon balance during drought?	9.6 Research	Zhao et al. 2013 New Phytologist
Can tree use alternative substrate for respiration during drought?	9.7 Research	Fischer et al. 2015 New Phytologist
How many growing seasons contribute to trees carbon reserves?	9.8 Research	Muhr et al. 2016 New Phytologist
What is the role of carbohydrates in tree functioning?	9.9 Review	Hartmann & Trumbore 2016 New Phytologist
Carbon allocation within plants and with symbiotic partners		
Are plants or mycorrhiza in charge during symbiotic resource exchanges?	9.10 Research	Zhang et al. 2015 Plant and Soil
Is carbon allocation to defence is constrained by carbon availability?	9.11 Research	Huang et al. 2017 Plant Cell Environment
How is whole-plant allocation patterns are orchestrated by phytohormones?	9.12 Research	Huang et al. 2017 Journal of Experimental Botany

The first experiment addressed whole-tree carbon dynamics in response to lethal drought based on tissue level measurements, thereby extending on the organism-centered hydraulic framework. The main question here was whether lethal drought would cause carbon starvation (Chapter 2: Only roots starve during drought). Using a unique and novel experimental approach I addressed in a follow-up manipulation the role of carbon translocation across organs as a drought mortality mechanism (Chapter 3: You can't starve while being thirsty) and also used the data set to investigate general allocation responses to drought, with a particular focus on allocation to carbon reserves

(Chapter 4: Saving your last penny – carbon storage during starvation). The accumulated information from these studies allowed me to make a unique contribution to resolve the debate in the literature about whether carbon starvation in trees occurs at all (Chapter 5: You can't starve while being thirsty – part II). Work carried out in my group also addressed interactive effects of drought and elevated temperature (often occurring concomitantly) on the tree carbon balance (Chapter 6: The dry and the wet drought) and drought effects on storage carbon metabolism (Chapter 7: Sweet drought and fatty shade). We further investigated the turnover time of carbon in reserves, i.e. the number of growing seasons contributing to the carbon in reserve pools, as a proxy for a tree's potential to endure extended disturbances that reduce carbon assimilation, like recurrent defoliations (Chapter 8: Old fresh sugar maple syrup). With these studies, I earned myself a robust reputation in this field of research and was able to lead the community in synthesizing existing knowledge and setting research priorities for future research in tree carbon relations in general (Chapter 9: New avenues for research on carbohydrates).

During these years, my research slowly evolved from addressing very specific questions regarding drought-induced tree mortality to the general topic of carbon allocation in plants (Table 1). After all, responses of forest trees to drought are, seen from a carbon perspective, just a specific case of strategic carbon investments to tolerate or overcome environmental change or stress. Hence, the more recent research in my group focuses not only on carbon allocation strategies in response to stress within the plant, i.e. allocation across tissues and functional pools, but also within a context of plant symbiotic interactions with mycorrhiza (Chapter 10: You just can't get enough but you still have to share). In addition, our work now focuses on allocation patterns including pools and fluxes that are difficult to assess, like production of secondary metabolites and emissions of volatile organic substances (Chapter 11: To defend or not defend, that is here the question) and also on the underlying control mechanisms of carbon allocation by including phytohormonal analysis at the whole-plant level (Chapter 12: Carbon and the works – orchestration of allocation).

9. Research chapters

9.1. Chapter 1: No water no transport no carbon

Hartmann H. (2011) Will a 385 million year-struggle for light become a struggle for water and for carbon? – How trees may cope with more frequent climate change-type drought events. *Global Change Biology*, **17**, 642-655.

Global Change Biology

Global Change Biology (2011) **17**, 642–655, doi: 10.1111/j.1365-2486.2010.02248.x

REVIEW

Will a 385 million year-struggle for light become a struggle for water and for carbon? – How trees may cope with more frequent climate change-type drought events

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Abstract

Trees are exceptional organisms that have evolved over some 385 million years and have overtaken other plants in order to harvest light first. However, this advantage comes with a cost: trees must transport water all the way up to their crowns and inherent physical limitations make them vulnerable to water deficits. Because climate change scenarios predict more frequent extreme drought events, trees will increasingly need to cope with water stress. Recent occurrences of climate change-type droughts have had severe impacts on several forest ecosystems. Initial experimental studies have been undertaken and show that stomatal control of water loss hinders carbon assimilation and could lead to starvation during droughts. Other mechanisms of drought-induced mortality are catastrophic xylem dysfunction, impeded long-distance transport of carbohydrates (translocation) and also symplastic failure (cellular breakdown). However, direct empirical support is absent for either hypothesis. More experimental studies are necessary to increase our understanding of these processes and to resolve the mystery of drought-related tree mortality. Instead of testing the validity of particular hypothesis as mechanisms of drought-induced tree mortality, future research should aim at revealing the temporal dynamics of these mechanisms in different species and over a gradient of environmental conditions. Only such studies will reveal whether the struggle for light will become a struggle for water and/or for carbon in drought-affected areas.

Keywords: carbon starvation, climate change, drought, impeded carbon translocation, tree mortality, xylem dysfunction

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Trees are exceptional organisms

The oldest tree-like plant fossil found in Gilboa, New York (USA) was dated to be 385 million years old. The plant had a long branchless trunk, a crown with frond-like branches and was about 8 m high (Stein *et al*., 2007). Subsequently, individual trees and evolving tree species had to compete for an increasingly limited and pre-emptable resource, light, and the development of the vertical structure of forest ecosystems can be considered the outcome of an evolutionary arms race (King, 1991; Falster & Westoby, 2003). Their large size (i.e. high canopy) creates environment conditions that allowed trees not only to persist but also to dominate terrestrial ecosystems for over 370 million years (Niklas, 1997). In their quest for light, tree size increased constantly, and contemporary tree species can grow up to impressive heights. For example, *Sequoia sempervirens* [(D. Don) Endl.] reaches up to more than 115 m into the sky (Van Pelt, 2002).

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Trees are also one of the most long-lived life forms on earth and Bristlecone pines (*Pinus longaeva* [D.K. Bailey]) are the oldest living trees. In Eastern California, a 4723-year-old individual (dated in 1957) was discovered by Schulman (1958) and Currey (1965) described an individual in excess of 4900 years (at the time) in Eastern Nevada. Moreover, trees may avoid mortality of individuals (as defined by their genetic assembly) altogether by means of vegetative reproduction. Quaking aspen (*Populus tremuloides* [Michx.]) can form clonal colonies of great spatial extent and old age. For example, the Pando colony in Utah (USA) is thought to be the world's most massive individual organism (47 000 stems weighing approximately 6615 tons and covering 77 ha) and is believed to be at least 80 000 years but potentially more than one million years of age (Mitton & Grant, 1996).

Trees have evolved over millions of years to dominate terrestrial ecosystems by shading out their competitors. They have sought refuge during unfavorable climatic conditions in past glacial cycles and survived harsh temperatures for thousands of years (Petit *et al*., 2008). However, recent environmental changes are much more

rapid than those experienced during normal glacial cycles. Industrial activities and land use changes have enriched the atmosphere with greenhouse gases and had significant impacts on the World's climate (Forster *et al*., 2007). Can trees cope with these changes? Will their competitive advantage gained from height growth remain a viable strategy in a future where water, not light, will be the limiting resource in many parts of a warmer world? Can trees adapt to the new conditions or will new environmental conditions put an end to their dominance?

In the following sections, I will cover general aspects of tree mortality, water relations in trees as well as physiological responses to alteration in water supply (one predicted outcome of climate change) particularly for tall plants like trees. I give examples of observed drought-induced forest mortality and will then discuss potential hypotheses of this mortality while highlighting the existing evidence but also knowledge gaps. Finally, I will discuss potential evolutionary consequences of being a tree in a drier world and indicate what we can do to gain a better knowledge of drought-induced tree mortality processes.

Tree mortality . . . and survival in a rapidly changing world

Trees, even though they are the most long-lived organisms on Earth, may not live forever and this despite the fact that the concept of senescence (i.e. cumulative changes to the molecular and cellular structure of an adult organism that will, when in a progressive state, increasingly disrupt metabolism, resulting in pathology and death) may not apply to trees (Mencuccini *et al*., 2005). For example, individuals of Eastern white cedar (*Thuja occidentalis* L.) grow oldest and with little evidence of senescence under the most environmentally adverse conditions (e.g., limited water supply, poor and very little exploitable soil volume, Larson, 2001) and a species like Bristlecone pine does not show signs of a slow-down or a disintegration of physiological processes with increasing age (Lanner & Connor, 2001). But, catastrophic events like wildfires, insect outbreaks or windthrow are responsible for widespread mortality and diseases that can kill individual trees (Kimmins, 2004). The probability of a tree to be involved in a stochastic, age-independent, accidental, and lethal event (e.g., disease, lightning, windthrow) accumulates with age and defines its intrinsic mortality risk (Keane *et al*., 2001). Depending on the frequency of these events in a given environment (i.e. the range of distribution of a species), this cumulative probability sets the expected maximum age for a tree species. Additionally, trees may succumb to resource depletion from neighboring vegetation through intra- and interspecific competition especially during the early phases of stand development, i.e. when many young individuals compete for increasingly sparse resources (Yoda *et al*., 1963; Luyssaert *et al*., 2008).

With respect to tree survival, the current climatic changes, exceptional in their dynamic and seemingly caused by human forcing mainly from anthropogenic greenhouse gases emissions (mostly CO_2 but also methane and nitrous oxide) since the industrial revolution (Forster *et al*., 2007), encompass a host of challenges for trees. Most alterations in environmental conditions from climate change (e.g., increased atmospheric water vapor and cloudiness, more diffuse radiation) may have indirect impacts on ecosystems through community dynamics. For example, increases in temperature are likely to integrate into different competitive interactions among ecotypes of a same species and among species (Bazzaz, 1990; Lau *et al*., 2007). Depending on the capacity to acclimate to the new conditions, this could force species to move along the environmental gradient towards their temperature optimum range (Norby *et al*., 2001). On the other hand, extreme events, such as droughts and heat spells or changes in precipitation patterns along with increased air temperatures, will have a direct impact on tree functioning and physiological processes (e.g., photosynthesis, water relations, carbon balance, growth rates) and ultimately on tree survival (Crawford, 2008).

Although total precipitation will not significantly change on a global scale, established patterns will change with increasing precipitation in high latitudes and decreasing precipitation in subtropical and mid-latitude regions (Trenberth *et al*., 2007). The tendency towards extreme weather events will have important impacts on agricultural production and ecosystem services. For example, Alcamo *et al*. (2006) showed that despite an expected increase in the average precipitation amount in Russia, increased runoff during extreme climatic events will reduce water availability in soils and for plants and will pose an increasing threat to the security of Russia's food system and water resources.

Moreover, drought events will be more frequent and the area affected by extreme drought will increase from a current (2006) 3% up to 30% by 2090 and for severe drought from a current 10% up to 40% by 2090 (Burke *et al*., 2006) with a potential for adverse impacts on numerous sectors, such as agriculture, water supply, energy production, and health (Trenberth *et al*., 2007). Drought can be defined in a strict sense, as a situation where a deficiency of precipitation over some period of time results in a water shortage for some activity, group, or environmental sector (Trenberth *et al*., 2007). For plants, drought represents a condition during which they suffer reduced functioning, growth or yield because of an insufficient water supply or a large humidity

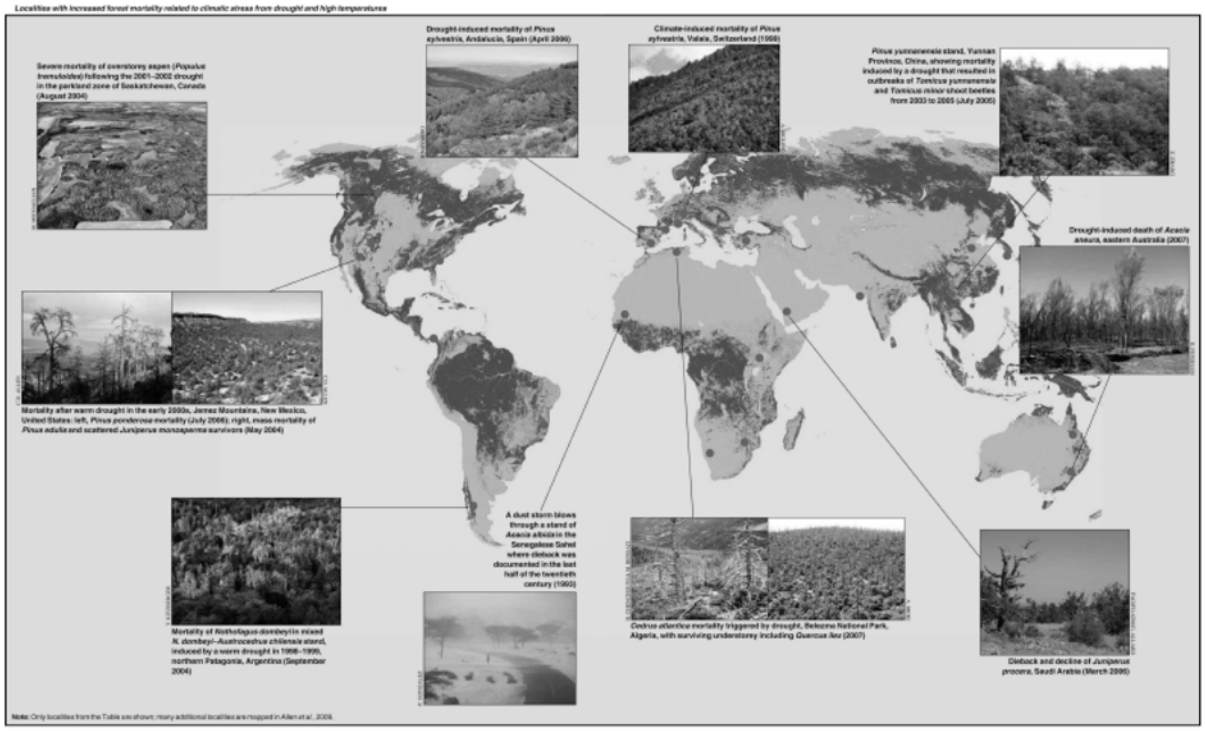

Fig. 1 World map showing some examples of increased tree mortality from drought and high temperatures during the last two decades. The figure shows localities (grey dots) and images of forests that show symptoms of increased mortality attributed to drought and high temperature. Note that increased mortality has been observed on all continents of the world and in different forest biomes. Courtesy of Allen (2009).

deficit (Passioura, 1996) and occurrences of intense drought and elevated temperature have been associated with increased mortality of trees in many regions of the world (Allen, 2009; Allen *et al.*, 2010, Fig. 1).

And why should we care about mechanisms of drought-induced mortality?

Predecessors of *Homo sapiens* dwelled in forests and human beings are still emotionally and economically dependent on the forest and its products (Kimmins, 2004). Forests offer a host of services to humans worth trillions of dollars each year (Constanza *et al.*, 1997). Tree mortality threatens the continuous flow of services from forests and may result in substantial loss of economic returns and of quality of life.

In recent years, enormous effort has been spent and continues to be spent on the modeling of vegetation dynamics. These models are usually well parameterized in terms of dispersal, establishment, and growth, but underdeveloped with respect to mortality algorithms (Hawkes, 2000) which, in part, is due to the difficulty of studying tree mortality, a process spanning years to decades. Mortality algorithms may be deterministic (beyond a specific threshold a tree dies) or stochastic (beyond a specific threshold a tree has an increased probability of dying) but are usually empirically derived (i.e. driven from data) and not mechanistic. Empirical mortality algorithms are data intensive, spatially and temporally specific, and the resulting simulations are prone to error and are inflexible to environmental change (Hawkes, 2000). A better understanding of the physiological responses of trees to environmental stresses can yield mechanistic relationships between biologically meaningful predictors (Hawkes, 2000) and would be beneficial for linking mortality to new environmental conditions associated with climatic change (Keane *et al.*, 2001).

Furthermore, trees and forests are a major component of the Earth's carbon cycle. They comprise nearly 90% of the terrestrial biomass which is equivalent of about two-thirds of the atmospheric carbon (Körner, 2003). Forests circulate about 8% of atmospheric CO_2 back and forth into the biosphere every year (Malhi *et al.*, 2002). The net carbon assimilation of a forest ecosystem, the difference between CO_2 uptake by photosynthesis and its release by autotrophic and heterotrophic respiration,

is allocated to several pools of above- and belowground biomass, such as leaves, branches, and roots (Litton *et al.*, 2007). Dead biomass (e.g., dead leaves in autumn, defunct branches) is returned to the forest floor, decomposes and accumulates, especially in temperate and boreal forests, to form enormous carbon stores in forest soils and associated peat deposits that can make up two-thirds of the carbon in forest ecosystems (Dixon *et al.*, 1994).

The net contribution of any forest to the global carbon cycle is determined by major releases of CO_2 from disturbances, such as fire, insect outbreaks, and droughts but also from deforestation and harvest. Changes in disturbance regimes from climate change can make a significant difference in whether forests are carbon sinks or sources (Kurz *et al.*, 2008). Failing to account for fluxes from this pool, for example those caused by tree mortality, will necessarily yield uncertain predictions of feedback loop dynamics (Malhi *et al.*, 2002).

Drought and forest ecosystems

Droughts have always been a normal recurrent disturbance in many forest ecosystems but predictions of climate change foresee more frequent catastrophic climate events with important impacts on these ecosystems (Jentsch *et al.*, 2007). Forests will be affected by several means, such as reduced net primary production (NPP), and increased susceptibility to fire, insects, or disease (Dale *et al.*, 2001). Also, because different sensitivities to drought can determine species distribution along the axis of environmental variation, changes in soil moisture availability from climate change may alter species distribution, community composition, and species diversity (e.g., Mueller *et al.*, 2005; Engelbrecht *et al.*, 2007).

These changes may be mediated by community dynamics such as species-specific trade-offs to drought sensitivity and shade tolerance (Huston, 1994). Extreme events may cause shifts in species distributions, community assemblages, and ecosystem structure and function through the creation of selective pressures for the evolution of locally adapted physiologies (Parmesan *et al.*, 2000). These changes may push the system beyond the threshold of dynamic equilibrium and towards a novel system trajectory (Jentsch *et al.*, 2007). However, severe drought events can also directly affect tree health and increase regional mortality rates in many forest ecosystem types, such as in temperate hardwoods of North Carolina (Elliott & Swank, 1994), neotropical forests of Panama (Condit *et al.*, 1995), tropical Amazonian rainforest of Brazil (Williamson *et al.*, 2000), subalpine forests of the Rocky Mountains (Bigler *et al.*, 2007), montane Mediterranean-type conifer forests of the Pacific US (van Mantgem *et al.*, 2009), and east-Mediterranean conifer forests on the islands of Samos (Körner *et al.*, 2005). Furthermore, a regional forest die-off has been linked to a severe 'global change-type drought' in montane conifer forests of the southwestern US (Breshears *et al.*, 2005) and in many other regions across the world (Allen, 2009; Allen *et al.*, 2010, Fig. 1).

Because there is still only indicative support on the relationship between drought-mediated changes in tree functioning and mortality (Table 1), I will focus the following sections on the impact of extreme drought events on the physiology of individual trees. Further on, I will return to processes mediated by population and community dynamics and to evolutionary implications of extreme drought events.

Water stress in trees

Water transport in vascular plants

Water plays an essential role in several vital processes of plants. It supplies electrons to the photosynthetic reaction, transports minerals and nutrients from the soil to the growing plant tissue, gives mechanical stability to nonlignified tissues, prevents overheating in sun-exposed leaves and is by itself the basis of the cellular solution (cytoplasm). For every gram of plant organic matter produced, a plant needs to take up approximately 500 g of water (Taiz & Zeiger, 2006).

Carbon assimilation in leaves requires that atmospheric CO_2 diffuses into intercellular airspaces of leaves and early plants developed pores within the epidermis to facilitate CO_2 diffusion into leaves (Raven, 2002). Unfortunately, this diffusive pathway also allows water to diffuse out of the leaf, so the resulting water loss must be continuously replaced with water extracted from the soil (Brodribb, 2009). To optimize carbon fixation per unit water loss higher vascular plants have evolved stomata, i.e. pores equipped with guard cells that allow closure of the pore aperture in response to water deficit (Raven, 2002).

Water transport in plants is a physical process that requires no energy input from the plant and has been described as a soil–plant–atmosphere continuum where a continuous water column links soil water through the plant vascular system (xylem) with atmospheric humidity (Sperry *et al.*, 2002). Water evaporates from leaves and diffuses out into the atmosphere. The evaporative demand is transmitted to mesophyll and bundle sheath cells and to the vascular system where adhesive and capillary forces of water molecules maintain the flow (Pickard & Melcher, 2005).

Table 1 Examples of some tree mortality causes

Mortality cause	Covariate or vector	Relationship	Scale	Support	References
Senescence	Age	Theoretical	Individual	No	Larson (2001), Lanner & Connor (2001), Mencuccini *et al.* (2005)
Accidental events	Age occurrence of extreme events	Probabilistic	Individual	Yes	Xi *et al.* (2008), Busing *et al.* (2009)
Catastrophic events	Age occurrence of extreme events	Probabilistic	Population community	Yes	Peterson & Pickett (1995), Turner *et al.* (1997), Dalziel & Perera (2009)
Resource depletion	Stand development	Mechanistic	Population community	Yes	Yoda *et al.* (1963), Luyssaert *et al.* (2008)
Resource depletion	Global change	Mechanistic	Population community	Yes	Lau *et al.* (2007)
Xylem failure	Occurrence of extreme events	Mechanistic	Individual	Indicative	Sparks & Black (1999)
Carbon starvation	Occurrence of extreme events	Mechanistic	Individual	Indicative	McDowell *et al.* (2008), Adams *et al.* (2009), McDowell & Sevanto (2010)

The table indicates covariates or vectors of mortality, the nature of the relationship between cause and mortality, the scale of action of the cause, and whether this relationship is supported in the literature as well as selected references of support.

Regulation of water relations during drought

Water uptake by roots reduces soil moisture content surrounding them and if soil water conductance is lower than the rate of water uptake by the plant, the soil dries out. As it does, air replaces water in the soil pores and increasing adhesive forces between water and soil particles reduce the availability of soil water for root uptake (Sperry *et al.*, 2002). A constant evaporative water demand must pull harder on the vascular water column in order to overcome the adhesive forces between water and soil particles. This creates a negative pressure within the xylem and when this pressure exceeds a given threshold, embolisms within the vascular cells occur from air seeding through pit members of neighboring air-filled wood cells. The water column ruptures and this leads to a (partial) loss of xylem conductance (Tyree & Zimmermann, 2002). If the water deficit persists over an extended period of time, a total loss of conductance may occur and cause alterations of physiological processes (e.g., Galmés *et al.*, 2007), reduced photosynthetic rates (e.g., Flexas *et al.*, 2006), growth reductions (e.g. Lecoeur *et al.*, 1995), decreased cell turgor pressure, wilting of nonlignified tissues (leaves) and eventual cell death (Taiz & Zeiger, 2006).

Like all vascular plants, trees possess regulatory mechanisms to reduce water loss and thereby maintain functional plant–water relations and xylem functioning when water supplies become limited. Short-term adjustments of transpiration rates are achieved mainly by stomatal closure, but will also cause decreases in CO_2 diffusion rates from the atmosphere to the site of carboxylation (Farquhar & Sharkey, 1982). The xylem pressure difference between stomatal closure and partial xylem dysfunction (50% loss of conductance) defines a plant's safety margin. Isohydric species (large safety margin) close their stomata in advance of any danger to xylem dysfunction thereby maintaining a nearly constant leaf water potential. On the other hand, anisohydric species (small safety margin) close their stomata only when there is an immediate risk of hydraulic failure but have leaf water potentials fluctuating with evaporative demand (Tardieu & Simonneau, 1998). Although the isohydric strategy may appear advantageous, it will be shown later that this strategy may have an enormous cost (McDowell *et al.*, 2008).

Medium-term and long-term adjustments to water deficits include either morphological acclimations or evolutionary adaptations (Chaves *et al.*, 2003). To the former belong adjustments of the root : shoot ratio. Under extended drought trees can increase the ratio of roots vs. aboveground biomass by either allocating more carbon to root growth (Leuschner *et al.*, 2001) to increase whole-plant water uptake or by decreasing the

leaf area (i.e. leaf shedding, Bréda *et al.*, 2006). Both of these mechanisms come with the cost of reduced net carbon assimilation rates due to lower plant-level photosynthetic carbon gain (stomatal closure, leaf shedding) or increased respiration rates (increased root biomass) by constant photosynthesis. Long-term adaptations include cavitation resistant xylem anatomies (e.g. small vessel size), thick leaf morphology (e.g. sclerophylls), or deep root systems which may play an important rule in a species' drought escape or resistance strategy (Chaves *et al.*, 2003).

Impacts of tree height on drought–water relations

Because of their great height, trees have a competitive advantage over other terrestrial plants in intercepting light (Falster & Westoby, 2003). This great height requires a costly investment of carbon into growth and maintenance of structural tissues and the height itself comes with an additional cost. Leaves high up in the crown require water to maintain the moist environment within leaves that is necessary for photosynthesis, a process evolved in an aquatic environment (Brodribb, 2009). Because water movement through the vascular system encounters resistance to flow, water transport within trees has been hypothesized as a physically limiting factor to height growth in big trees. In fact, increasing leaf water stress due to gravity and path-length resistance may ultimately limit leaf expansion, leaf hydraulic conductance, and stomatal conductance and will reduce photosynthesis for further height growth in the tallest trees (Ryan & Yoder, 1997; Koch *et al.*, 2004).

Hence, large trees may experience hydraulic limitations even with an ample water supply within the soil (McDowell *et al.*, 2005), they operate close to the edge of hydraulic dysfunction and more cavitation occurs at increasing tree height (McDowell *et al.*, 2002). Xylem structure of large trees is tailored to satisfy the conflicting requirements for water transport and water column safety but the avoidance of runaway embolism imposes increasing constraints on water transport efficiency (Domec *et al.*, 2008). Big trees are therefore more vulnerable to drought-induced cavitation than small trees and must fine-tune transpiration rates more tightly to more closely match stomatal control of water loss with water supply. Hence, an isohydric stomatal control strategy seems necessary for trees to grow tall but this may have important consequences on the carbon balance of tall trees (see ''Drought and carbon starvation'').

On the other hand, large trees have more root volume (Magnani *et al.*, 2000) which may allow them to exploit more profound water pools during drought. Because of their greater bole volume, tall trees have a higher capacitance, i.e. water storage, which may allow them to draw on these resources during periods of reduced soil water uptake. Stored water could make up between 30% and 50% of transpired water over short periods (Waring *et al.*, 1979) and the proportion of stored water used for transpiration increases with tree size (Phillips *et al.*, 2003). However, because tall trees may respire hundreds of liters of water a day (Phillips *et al.*, 2003), the role of stored water as a means of survival during (prolonged) drought may be questionable.

Since their first appearance on Earth, trees have overtaken other plants in order to harvest light first. I will show in the following sections that recent rapid changes in the environmental conditions, especially those changes involving extreme drought events, may turn this evolutionary advantage against them. Trees, in general and tall trees in particular may be affected by drought either through reduced carbon assimilation and depletion of their carbon storage during prolonged drought (carbon starvation), through catastrophic xylem dysfunction during severe drought, by means of impeded long-distance transport of photoassimilates in the phloem or because of symplastic failure, i.e. low tissue water potentials during drought constraining cell metabolism.

Drought and carbon starvation

Drought is not always immediately lethal and tree mortality may occur years to decades after the actual drought event (e.g., Bigler *et al.*, 2007). This observation is consistent with a conceptual tree disease and decline model (Manion, 1981) that links initial tree condition (i.e. vigor) to stress vulnerability. According to this model, long-lasting predisposing factors such as soil compaction, genetic potential, or air pollutants reduce initial tree vigor and predispose affected trees, now less resistant against further disturbance, to more severe inciting stresses such as defoliating or bole boring insects (e.g., bark beetles, spruce budworm or pinyon ips, Shaw *et al.*, 2005; Kurz *et al.*, 2008; Raffa *et al.*, 2008), or drought. Inciting stresses cause sharp and irreversible vigor declines, while contributing stresses such as secondary pathogens, may accelerate the decline and act as the 'coup de grâce'.

Until recently, it was commonly assumed that tree vigor is related to the amount of carbohydrates stored as reserves in a tree's storage compartments (e.g., starch accumulation in chloroplasts of leaves; sugar loading of stem ray cells, and root parenchyma cells) and that trees die as a consequence of exhausted carbon reserves (Waring, 1987). However, Körner (2003) showed that carbon assimilation, structural growth, and carbon storage loading are actually reversely linked where

concentrations of nonstructural carbon (i.e. water-soluble sugars, lipids, and starch) are indicative of environmental constraints (e.g., water deficit) on tree growth (Körner *et al*., 2005) and, therefore, not necessarily of tree vigor. Nevertheless, storage compounds, such as sugars or starch, are important for several physiological processes related to tree vigor such as the synthesis of defense mechanisms against pathogens or leaf resprouting after insect defoliations (e.g., Gregory & Wargo, 1986), adjustments of osmotic potentials for cold hardiness (e.g., Wong *et al*., 2003), respiration during heterotrophic seasons (Pallardy, 2008), or protection against desiccation (Crowe *et al*., 1992; Rorat, 2006).

Carbon depletion has been proposed as one of the underlying mechanism of drought-induced mortality either directly from a negative carbon balance where respiration > carbon assimilation (e.g., Bréda *et al*., 2006; Güneralp & Gertner, 2007), or in combination with a carbon-depletion-mediated predisposition to other agents of mortality such as pathogens (McDowell *et al*., 2008). During nonconstrained normal functioning with ample water, light and nutrient supply and under normal temperature, plants can spend more than 50% (and maybe even up to 90%) of their assimilated carbon for maintenance and growth respiration (Ryan *et al*., 1997; Nogués *et al*., 2006). In autotrophic tissues, water stress reduces carbon assimilation through stomatal closure but has little effect on respiration and this leads to negative net carbon assimilation rates (Flexas *et al*., 2006). The sustained maintenance respiration in heterotrophic tissues during drought (Meir *et al*., 2008) requires that carbon is supplied from sources (storage) to theses sinks and this will further and negatively influence the whole-plant carbon balance. Because isohydric species maintain a large hydraulic safety margin and close their stomata at a relatively well-hydrated state, they develop a negative carbon balance relatively early during drought and may therefore be forced earlier to rely on carbon reserves and thus may be more vulnerable to carbon starvation than anisohydric species (Fig. 2). In support of this idea, McDowell *et al*. (2008) showed that, theoretically, isohydric species are prone to carbon starvation during moderate but long-lasting droughts (Fig. 2), whereas anisohydric species may only be affected by intense droughts and by means of catastrophic xylem dysfunction.

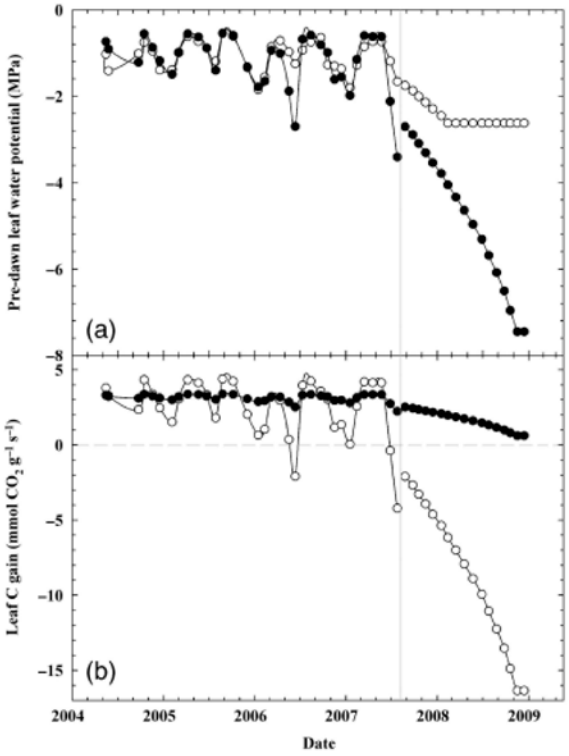

Fig. 2 (a) Monthly predawn water potentials as indicator of tree water status measured from 2004 to 2007 (left of grey vertical line) and simulated from 2007 to 2009 (right of grey vertical line) for isohydric *Pinus edulis* (open circles) and anisohydric *Juniperus monosperma* (filled circles). (b) Estimated leaf carbon gain for both species using leaf water potential in (a) and stomatal conductances (data not shown). The horizontal line in (b) indicates the zero assimilation point. The figure shows how isohydric species run short of carbon because of their greater safety margin whereas anisohydric species maintain a positive carbon gain even during sustained drought periods. Courtesy of McDowell *et al*. (2008). Permission granted by Wiley & Sons.

Could tree height facilitate carbon starvation?

Because an isohydric strategy is necessary for trees to grow tall (see 'Impacts of tree height on drought–water relations'), tall trees also show a narrow range of gas exchange rates. This narrow range of gas exchange may prevent tall trees from 'filling the pantry' (carbon loading) under sufficient water supply. When water supply is limited, the reduced carbon loading could play a role as a predisposing mechanism in carbon starvation by reducing the length of time a tree can survive with a negative net photosynthesis and until carbon starvation occurs (McDowell *et al*., 2010). Tall trees have also proportionally more heterotrophic tissue than small trees and therefore a higher cost of maintenance respiration (Ryan *et al*., 1995). Although higher maintenance respiration cannot explain declines in NPP in aging forest stands (Ryan & Waring, 1992; Gower *et al*., 1996), it may have a significant effect during times of reduced carbon assimilation (as occurring during drought) by making tall trees more prone to carbon

starvation than small trees with proportionally less heterotrophic biomass. According to this reasoning, trees may also be more vulnerable to drought than cooccurring, smaller woody vegetation.

Tall trees had higher mortality rates than small trees following drought (Floyd *et al.*, 2009) and although these were attributed to higher carbon expenditures associated with reproduction (Mueller *et al.*, 2005) the underlying mechanism would still be carbon starvation. In other cases, higher mortality rates in taller trees following drought were attributed to a lower return in carbon gains from their investment in stem growth (Zhang *et al.*, 2009), indirectly also supporting the hypothesis that tree height facilitates carbon starvation. Several other studies have found an inverse relationship between tree size and drought-induced mortality, i.e. higher mortality in small- and medium-sized trees (Condit *et al.*, 1995; Nakagawa *et al.*, 2000; Suarez *et al.*, 2004; Guarín & Taylor, 2005). However, tree size was most often measured as tree stem diameter and not tree height (Condit *et al.*, 1995; Nakagawa *et al.*, 2000; Guarín & Taylor, 2005) and trees were lumped into only two broad classes (1–10 and >10 cm diameter, Condit *et al.*, 1995). On the other hand, when Condit *et al.* (1995) analyzed mortality rates as a function of growth form (large trees, mid-size trees, understory trees, and shrubs) they found higher mortality rates in large and mid-size trees during drought than during the non-drought period. Furthermore, there were no significant differences in mortality rates between drought and nondrought periods in understory trees and shrubs (Condit *et al.*, 1995). Suarez *et al.* (2004) measured tree height directly but mortality rates were analyzed at the stand level. Hence, while higher mortality rates were found in stands with greater average canopy height (Suarez *et al.*, 2004) other stand characteristics, such as an almost threefold prior mortality rate (mortality occurring before the analyzed drought event), seemed to indicate that stand health or prior disturbances may have confounded causal factors of tree mortality. These findings are therefore not necessarily counter-indicative of a potential link between tree height and carbon starvation. Furthermore, because none of these studies assessed nonstructural carbohydrates as a function of tree height the potential link between tree height and drought-induced carbon starvation remains speculative. Further experimental studies should help close this knowledge gap.

Experimental evidence for the carbon starvation hypothesis

Given that respiration rates are temperature dependent (Atkin *et al.*, 2005), Adams *et al.* (2009) investigated responses of trees to drought and increased temperature under controlled environmental conditions. If carbon starvation was the mechanisms of tree mortality during drought, trees growing under elevated temperature should die earlier from carbon starvation than trees growing under ambient temperature. As expected, trees grown in higher temperatures had higher cumulative respiration rates and died sooner than trees grown under ambient temperatures and, therefore, Adams *et al.* (2009) interpreted the results as confirmatory evidence for the carbon starvation hypothesis (see Fig. 3). Although the methodology (Leuzinger *et al.*, 2009) as well as the strong inferences drawn from this study (i.e. that expected increases in extreme drought events coupled with predicted higher temperatures would lead to fivefold increases in mortality rates) were criticized (Leuzinger *et al.*, 2009), the study represents a first empirical test of the respiratory component of the carbon starvation hypothesis. However, Sala (2009) argued correctly that alternative hypotheses were not addressed in the study and that the decreased survival of trees grown under elevated temperature conditions does not imply that carbon reserves had been depleted (Sala *et al.*, 2010). The variable of interest – nonstructural carbohydrates – had not been measured directly by Adams *et al.* (2009) and hence carbon starvation (carbohydrate depletion) could not be inferred as mortality mechanism from the experiment (Leuzinger *et al.*, 2009). In summary, the generalizations drawn from this study are still speculative and more experimental studies are needed to elucidate the role of nonstructural carbohydrates in drought-induced mortality mechanisms.

Is catastrophic xylem dysfunction a plausible alternative hypothesis for drought-induced tree mortality?

Catastrophic xylem dysfunction can decrease or even disrupt water supply to aboveground plant parts and can cause cell structural or plant metabolic breakdown (Chaves *et al.*, 2003). However, for mature trees, there is very little evidence linking xylem dysfunction to mortality (Cinnirella *et al.*, 2002, but see Sparks & Black, 1999 on small trees) other than mechanistic relationships between drought condition and theoretical 'point of death'-xylem hydraulics (Brodribb & Cochard, 2009). Furthermore, xylem dysfunction can be repaired by refilling embolized vessels (Hacke & Sauter, 1996) and even if cavitation does persist, trees sacrifice more often small expandable distal parts (i.e. branches) rather than proximal parts (i.e. stem) because cavitation occurs more readily in the former than in the latter (e.g., Rood *et al.*, 2000). By doing so, mortality is restricted to branches and twigs and does not affect the whole

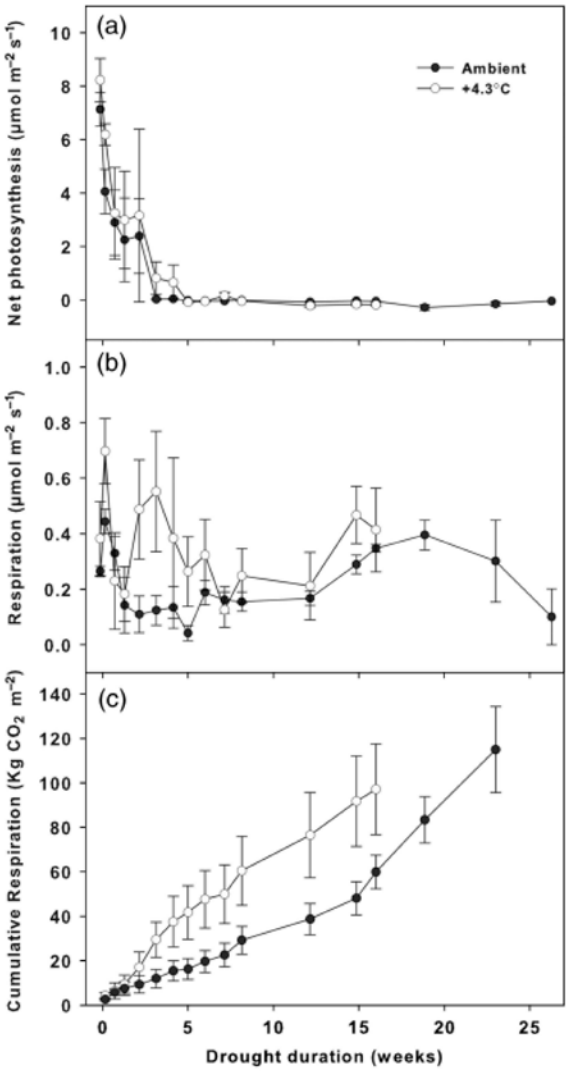

Fig. 3 Net photosynthesis (a), respiration rates (b) and cumulative respiration rates (c) of small *Pinus edulis* trees grown under ambient (black circles, $n = 5$) and elevated (+ 4.3 °C, white circles, $n = 4$) temperature. Although there is not a great difference in net photosynthetic rates between the temperature treatments respiration rates and cumulative respiration rates are higher under elevated temperature. Trees grown in elevated temperature died sooner than trees grown in ambient temperature (data not shown). Courtesy of Adams *et al.* (2009).

organism. However, intense drought could be a potential driver of mortality in anisohydric tree species and whose hydraulic conductances are further constrained by soil properties or tree size (i.e. trees at their maximum height with a long hydraulic path length, trees growing on shallow soils, or tree seedlings with little root volume, McDowell *et al.*, 2008). Under these conditions, recurrent occurrences of drought could cause regular drought-induced stand thinnings from above, i.e. killing the biggest trees with the most intense strain on their hydraulic system, or from below, i.e. killing the shallow-rooted regeneration.

What else could explain drought-induced tree mortality?

There are two more potential explanations for drought-induced tree mortality which are intrinsically linked to hydraulic failure (catastrophic xylem dysfunction), impeded long-distance transport (translocation) of photoassimilates in the phloem (Sala, 2009; Sala *et al.*, 2010) and symplastic failure, i.e. low tissue water potentials during drought constraining cell metabolism (Woodruff *et al.*, 2004; Ryan *et al.*, 2006). The latter could cause reductions in carbon assimilation and may thereby render trees more vulnerable to pathogen attack from a decreased production of defensive compounds (McDowell *et al.*, 2008, 2010). However, it has been shown that nonstructural carbohydrates increased with increasing tree height under limited water supply which would indicate the absence of constraints on carbon acquisition (Sala & Hoch, 2009). Instead these results could favor the impeded translocation hypothesis. Higher xylem tensions in the upper xylem and apoplast of tall trees could limit water movement from the xylem to the phloem and thereby impede photoassimilate distribution throughout the tree which would then accumulate close the sites of synthesis, i.e. in the crown (Sala & Hoch, 2009). Unfortunately, there is no empirical support for either hypothesis with respect to tree mortality and, once again, further investigations are necessary to elucidate the role of these mechanisms in drought-induced tree mortality.

Will a 385-million year struggle for light become a struggle for water, for carbon or for both water and carbon?

Trees have evolved over the last 385 million years and have become a dominating terrestrial life form. Trees gained this dominance because of their height which makes it possible for them to intercept light before competing plants can do so. However, rapidly changing environmental conditions may turn this competitive advantage against them.

In the future, the number of extreme and severe drought events is projected to double and there will be a significant increase in the mean event duration for all forms of drought (Burke *et al.*, 2006). Extreme events such as droughts may lead to marked changes not only in individual tree survival but also in community assembly (e.g., Mueller *et al.*, 2005; Engelbrecht *et al.*,

2007). Because trees grow (tall) only where stresses are minor and resources are abundant and competition for light places a premium on height growth (Koch *et al.*, 2004) one could expect a change in community composition of current terrestrial ecosystem. Regional vegetation die-offs following severe drought have had differential impacts on vegetation (Breshears *et al.*, 2005, 2009) and could, if reoccurring periodically, lead to a regional extinction of the less drought-resistant species and thereby to a shift in species composition of the forest ecosystem (Mueller *et al.*, 2005; West *et al.*, 2008).

In more extreme cases, there could be a shift from forests to a different vegetation type. Dryer climate is expected for Amazonia, the United States, northern Africa, southern Europe, and western Eurasia (Burke *et al.*, 2006), hence for regions (at least partly) covered with forests. As has been demonstrated above, height growth is limited by soil water supply and by water transport in tall vegetation such as trees. Regions that will receive less precipitation or that will suffer from frequent and prolonged droughts may therefore not be able to sustain tall vegetation formations such as forests in the future.

Furthermore, the dramatic increases in drought conditions predicted for the 21st century present significant adaptation challenges (Burke *et al.*, 2006). Strong responses of organisms, populations and communities to extreme events could lead to a strong selection driven by changes in fitness (Gutschick & BassiriRad, 2003). Trees are unique in that they maintain high levels of genetic diversity and have a large juvenile population sizes that allows strong and variable selection with great potentials for local adaptations (Petit & Hampe, 2006). For example, a tree's ability to recycle carbon by means of corticular (stem) photosynthesis reduces the carbon cost of these structures and may be an effective carbon conserving strategy and a substantial component of carbon gain during periods of severe water stress (Teskey *et al.*, 2008). Similarly, intraspecific genetic variation in water-use efficiency (unit of carbon assimilated per unit of water transpired) may influence ecosystem functioning through physiological pathways (Fischer *et al.*, 2004) and could create environmental bottlenecks that could impact a species' future capacity to adaptation (Petit & Hampe, 2006). On the other hand, elevated atmospheric CO_2 concentrations may increase water-use efficiency by lowering stomatal conductance (Ceulemans & Mousseau, 1994) and may lead to higher growth rates during drought stress (Townend, 1993). At least for agricultural crops this could alleviate the impact of drought (Robredo *et al.*, 2007). However, stomatal responses to elevated CO_2 concentrations vary across species and do not necessarily reduce drought sensitivity in trees (Beerling *et al.*, 1996). Furthermore, if reductions in stomatal conductance do not compensate for increases in total leaf area in response to elevated atmospheric CO_2 concentrations whole plant water consumption will increase and trees may be even more severely exposed to drought stress (Saxe *et al.*, 1998).

However, whether and how such species-specific differences in stomatal control, water-use efficiency and whole-plant leaf area can make a difference with respect to survival (and hence for selection) during an extreme drought event remains uncertain. Trees are not a phylogenic distinct group of plants but a life form relatively loosely defined based on an ensemble of common (but nonexclusive) features. Trees usually have a modular structure, indefinite growth, no clear separation between germline and soma (there are no early predefined reproductive cells), reversible cellular differentiation, pronounced phenotypic plasticity and physiological tolerance and, very important, all still-existing trees are vascular plants (Petit & Hampe, 2006). During their evolutionary history, trees have developed vascular systems with increasingly efficient water transport capacities, i.e. maximizing cavitation resistance and hydraulic conductance while minimizing carbon investment, although water-for-carbon exchange rate is still poor (Sperry, 2003). Because maximizing gas exchange rates while avoiding hydraulic failure means operating on the edge of dysfunction, some-mostly ancestral-plant forms (like lycopods and pteridophytes but also many gymnosperms) use conservative water conservation strategies (Sperry, 2004).

Most still-existing tree species fall within one of two divisions, gymnosperms and angiosperms, with distinct xylem structures. The xylem of gymnosperms, the evolutionary older division, is composed uniquely of tracheids whereas the xylem of angiosperms also features vessels with higher conductivity than tracheids (Sperry, 2003). The impact of drought on tree physiology depends on a species' particular set of above-mentioned features with xylem structure being a prominent one. While vessels mark the pinnacle in water transport efficiency and provide a competitive advantage when an ample water supply must be quickly pumped to sustain fast growth, this advantage may become a burden when water supply is sparse. Vessels are more expensive in terms of carbon investment and may compromise refilling capacity more than tracheids (Sperry, 2003) thereby setting undesirable conditions under the carbon starvation or catastrophic xylem dysfunction hypothesis of drought-induced mortality mechanisms.

Extreme events are inevitable for long-lived organisms like trees and future extinctions of tree species in response to climate change are probable, especially for species with already limited geographic distribution or

climatic range (Petit *et al.*, 2008). Hence, evolutionary progress may be reversed by rapidly changing climatic conditions, at least where these changes are extreme. Rapid climatic changes during the last deglaciation period have led to the extinction of at least one North American tree species (*P. critchfieldii*, Jackson & Weng, 1999). Local extinctions of several tree species in the Mediterranean Basin were driven by rapid climatic changes during the Quaternary, however, it still needs to be shown whether these extinctions took place during glacial (chilling) or interglacial (warming) periods (Petit *et al.*, 2005). The climatic transition between the Late Tertiary to the Quaternary has triggered extinctions of many tree genera (Latham & Ricklefs, 1993) and anticipated rapid climatic changes pose a particular challenge for many taxa. Species must adapt quickly enough to new climate regimes or they may be threatened by extinction (Davis & Shaw, 2001).

Under these assumptions and given the predictions of more frequent, more severe and longer-lasting occurrences of drought events, it can be suggested that the trees' struggle for light will become a struggle for water and/or carbon in affected areas. Consequences of this struggle could span from shifts in community assemblages to local extinctions and all the way to the loss of already rare tree species. However, the mechanisms for drought-induced tree mortality are still elusive and further research is needed (McDowell & Sevanto, 2010; Sala *et al.*, 2010). Ultimately, reduced carbon assimilation, impeded long-distance translocation, carbon depletion, symplastic failure, and carbon starvation represent a cascade of mechanisms that can be conceptually linked to a tree's water status and hence to hydraulic failure (Sala *et al.*, 2010). Which of these mechanisms is the final step leading to drought-induced mortality may be determined by a species' hydraulic makeup and strategy (i.e. xylem structure, isohydry vs. anisohydry), the underlying environmental and climatic conditions (e.g., soil type and depth, duration and severity of drought) and the attributes of individual trees (e.g., tree height, rooting depth, predrought storage loading). Instead of testing the validity of particular hypotheses as mechanisms of drought-induced tree mortality, future research should aim at revealing the temporal dynamics of these mechanisms in different species and over a gradient of environmental conditions.

Where to go from here?

Although a better understanding of tree mortality mechanisms may not save trees from their fate of climate change-induced mortality, it could assist in accounting for the future implications of current actions. By doing so, management actions and policy decisions can be guided as to minimize undesirable future conditions whether they are ecological or economic in nature.

In light of the reviewed literature, I advocate the need for more experimental studies on the mechanisms of drought-induced tree mortality. Specifically, studies should address alternative hypotheses of drought-induced mortality such as xylem failure but also insufficient carbon translocation during stress. In order to do so, carbon assimilation and compartmental respiration rates (leaves, stem, roots), xylem and carbon translocation functioning, and storage depletion should be monitored. Also, internal reassimilation of respired CO_2 should be evaluated as a potential mechanism in survival during drought.

Drought experiments using species with contrasting drought tolerance (e.g. *Populus* vs. *Pinus*) should monitor xylem fluxes with the heat dissipation method (e.g., Granier, 1987; Do & Rocheteau, 2002) and could detect the onset and evolution of xylem cavitation using acoustic signals (e.g., Perks *et al.*, 2004; Rosner *et al.*, 2006). Limitations on carbon translocation in phloem can be estimated by modeling phloem fluxes based on xylem water potential (Hölttä *et al.*, 2009) but also by measures based on phloem-related shrinking and swelling of tree stems (Zweifel *et al.*, 2005). Stable (^{13}C) or radio carbon (^{14}C) isotope labeling could be used to track the fate of (predrought) assimilated carbon in tree metabolism using analyses of respired CO_2 (see Mencuccini & Hölttä, 2010 for the potential but also for the limitations of these methods). If carbon starvation results from depletions of mobile carbon storage pools, drought-exposed individuals should have, at the end of the experiment (death), very low concentrations of nonstructural carbon (sugars, starch, lipids) in their tissues (leaves, stem and root sapwood), at least lower than those of control trees. If impeded translocation causes carbon starvation, i.e. if trees cannot utilize their reserves due to reduced transport functioning, drought-exposed individuals and control trees should have similar concentrations of nonstructural carbon in their tissues.

Furthermore, carbon starvation under well-watered conditions (hence no impeded translocation) could be forced by growing trees in a (nearly) CO_2-free atmosphere. Isotopic signatures of respired CO_2 could then indicate whether and which carbon pools are used to for maintenance (Badeck *et al.*, 2005). Such an experiment would ultimately show if carbon storage pools are actually used as a buffer to ride out a period of reduced carbon assimilation or if this carbon loading results merely from an accumulation of unused carbon due to an environmentally limited sink activity (Körner, 2003) and also if there is an inherent lower limit to the depletion of these pools (Li *et al.*, 2002).

The role of carbon reassimilation in tree stems may be tested by comparing survival times in species with photosynthetically active stems, such as *Populus*. Using stem covering as a treatment, time-to-death in drought-exposed individuals would indicate whether corticular photosynthesis may become a substantial component of a tree's carbon gain during periods of severe water stress or after drought-induced leaf shedding (Teskey *et al.*, 2008).

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9.2. Chapter 2: Only roots starve during drought

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Lethal drought leads to reduction in nonstructural carbohydrates in Norway spruce tree roots but not in the canopy

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Summary

1. Heat waves and droughts are expected to increase in frequency and severity in many regions with future climate change, threatening the survival of a number of forest ecosystems. However, our understanding of the physiological processes and mechanisms underlying drought-induced tree mortality is incomplete. Here, we present results on the physiological response of young Norway spruce trees exposed to lethal drought stress.

2. We applied three levels of drought treatment (control, drying–rewetting, complete drought) and monitored relevant physiological functions and processes of carbon and water relations at high temporal resolution until tree death occurred.

3. Only trees subjected to continuous drought died in our experiment. Trees subjected to drying–rewetting cycles consistently recovered in their ability to transport water, indicating that these trees do not suffer permanent damage to the hydraulic system. In all cases, drought reduced carbon assimilation, caused changes in carbon allocation and appeared to have severely reduced phloem functioning and carbon translocation. Structural growth was sacrificed for carbon investment in maintenance respiration and osmoprotection. Severe drought caused trees to rely on stored carbon reserves but, in contrast to above-ground tissues, only root carbon pools were strongly reduced when trees died.

4. Our results indicate that drought-induced changes in carbon allocation, use and transport differ between above- and below-ground tissues in trees. While root death may have been caused by carbon depletion, this was definitely not the case in above-ground tissues. Our findings indicate that mortality mechanisms are not defined at the organism level but rather within tree compartments.

Key-words: carbon assimilation, carbon balance, carbon reserves, drought-induced tree mortality, respiration, sapflow, stable carbon isotopes

Introduction

Drought-induced tree mortality has received much attention in the recent past (Zeppel, Adams & Anderegg 2011) and occurrences of increased tree and forest mortality from drought and heat have been observed within all major types of forest ecosystems (e.g. Elliot & Swank 1994; Condit, Hubbell & Foster 1995; Dutilleul, Nef & Frigon 2000; Williamson *et al.* 2000; Breshears *et al.* 2005; Körner, Sarris & Christodoulakis 2005; Bigler *et al.* 2007; van Mantgem *et al.* 2009; Brodersen *et al.* 2010; Peng *et al.* 2011), indicating emerging climate change risks for forests (Allen *et al.* 2010). Large-scale forest mortality can cause significant releases of CO_2 into the atmosphere (Kurz *et al.* 2008) and alter the local energy balance (Bala *et al.* 2007) and thereby influences feedbacks between climate, forests and the global carbon cycle (Malhi, Meir & Brown 2002). Hence, there is a need for a thorough understanding of the processes and mechanisms underlying drought-induced tree mortality.

During drought (defined here as water demand >> water supply for extended periods), plants downregulate transpiration rates by reducing stomatal conductance (Bréda *et al.* 2006) but this also reduces the rate of CO_2 diffusion to the sites of carbon fixation in leaves and thus carbon assimilation rates (Farquhar & Sharkey 1982). Because maintenance respiration cannot be sustainably reduced under prolonged drought (Meir *et al.* 2008), reduced CO_2 diffusion into leaves can lead to negative carbon assimilation rates (Flexas *et al.* 2006; Güneralp & Gertner 2007).

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In response, trees may remobilize stored carbon reserves to fuel maintenance respiration during drought (McDowell *et al.* 2008; McDowell & Sevanto 2010).

Hypothetical frameworks of drought-induced tree mortality suggest that drought duration and intensity determine the cause of tree death depending on a tree's hydraulic strategy. Isohydric species close their stomata and maintain nearly constant leaf water potential while reducing CO_2 diffusion into leaves early during drought (Tardieu & Simonneau 1998; Lambers, Chapin & Pons 2008). On the other hand, anisohydric species keep stomata open longer during drought and allow leaf water potential to fluctuate with evaporative demand, permitting higher CO_2 diffusion rates into leaves. If drought and the resulting negative carbon balance are sufficiently lengthy, isohydric tree species relying on stored carbon reserves may be more susceptible to carbon starvation (McDowell *et al.* 2008).

An alternative explanation for tree mortality is hydraulic failure that occurs if drought intensity promotes irreversible xylem cavitation, especially in anisohydric species. The lack of water supply causes plant desiccation before carbon starvation can occur (McDowell *et al.* 2008). However, the validity of this conceptual framework has been challenged because other potential mechanisms, such as impeded carbon translocation with declining plant water potential, are not considered (Sala, Piper & Hoch 2010). Translocation of stored carbon from sources (storage organs) to sinks (respiring tissue) requires phloem loading and phloem transport – two processes that are negatively affected by decreasing plant water potential (Hölttä, Mencuccini & Nikinmaa 2009). Hence, declining plant water potential could impede the redistribution of stored carbon and cause local depletions of carbon reserves instead of whole-tree carbon starvation. Moreover, the hydraulic framework defines mortality mechanisms at the scale of the organism and does not consider potential differential responses across tree compartments (e.g. leaves, twigs, branches, stem, roots) although it has been shown that hydraulic failure during drought may be constrained in some species to distal parts of the crown (i.e. leaves, twigs, branches) allowing the rest of the tree (i.e. stem, roots) to maintain functional water relations (Rood *et al.* 2000). We are not aware of any studies reporting similar differential responses during drought-induced mortality with respect to the tree carbon budget.

Despite the increasing body of literature on observed impacts of drought on forest ecosystems, few experimental studies have evaluated changes in tree physiology during drought-induced mortality for individuals. Recent investigation of drought and temperature effects on tree mortality in *Pinus edulis* (Englem.) showed that elevated temperature during drought increased respiration rates and accelerated tree death (Adams *et al.* 2009), but direct evidence for carbon depletion was not found (Sala 2009). More recently, Anderegg *et al.* (2011) linked results from experimental manipulations of aspen saplings with assessments of widespread aspen mortality and discovered that hydraulic failure of roots was linked to landscape patterns of canopy and root mortality. Yet, because saplings did not die from drought during their study, strict experimental support for either mortality mechanism (carbon starvation, hydraulic failure, impeded carbon translocation) is still missing. In a recent study by Plaut *et al.* (2012), hydraulic constraints on gas exchange, rather than hydraulic failure *per se*, have been found to promote drought-induced mortality in *P. edulis*. Although taken together these findings seem to point towards carbon limitation (starvation) as mortality mechanism, no assessment of storage pool loading had been carried out (Plaut *et al.* 2012). Despite the recent increase in research on drought-induced tree mortality (Zeppel, Adams & Anderegg 2011), further studies specifically designed to investigate mortality processes by killing plants are needed (McDowell 2011). Moreover, because plant hydraulics and carbohydrate metabolism are interdependent (McDowell *et al.* 2011), experiments explicitly designed to distinguish between drought effects on tree water status and carbon dynamics are needed (McDowell & Sevanto 2010).

We established an experimental facility that can deliver high-resolution tree physiology data during drought-induced Norway spruce (*Picea abies* L.) sapling mortality and separately evaluate the carbon and water dynamics of different tree compartments (leaves, branches, roots). The general climatic trend in Europe is expected to cause important shifts in the distributional range of Norway spruce and a strong reduction in forest biomass of this economically very important tree species (Hlásny *et al.* 2011a). Also, anticipated increasing drought may enhance its susceptibility to secondary agents (Dutilleul, Nef & Frigon 2000) and, combined with climate change-driven changes in pest population dynamics (Hlásny *et al.* 2011b), poses another threat to the survival of Norway spruce in Central Europe. Cavitation occurs in this species at relatively high xylem water potentials while stomatal control against cavitation is considerably tight (Bréda *et al.* 2006) making Norway spruce a rather isohydric species.

Our experiments were designed to maintain water deficit until tree death occurred while continuously monitoring relevant physiological functions and processes, including (i) xylem transport, (ii) leaf carbon assimilation, (iii) root respiration and its isotopic signature, (iv) structural growth, (v) whole-tree carbon exchange and (vi) carbon storage (starch, sugars) loading and isotopic signature of needles, branches and roots. Our aim was to test whether carbon pools are reduced during severe drought stress and whether such a reduction was strong enough to explain drought-induced tree mortality. Furthermore, by assessing carbon dynamics across all tree compartments, we wanted to evaluate whether storage pools were affected similarly in all compartments. We discuss our findings with respect to proposed mechanisms and highlight the importance of impeded carbon translocation during drought-induced tree mortality.

Materials and methods

STUDY LOCATION

The study was carried out at the Max-Planck Institute for Biogeochemistry in Jena, Germany (50°54′36″N, 11°33′59″E). Mean annual temperature was 9·3 °C (1961–1990), but it has increased in the last decade to *c.* 10·5 °C (1999–2010). The institute has an outdoor experimental site that was divided into two sections, one for a rain exclusion experiment and the other for whole-tree chambers (WTC). Considering the many problems in upscaling of ecological processes (Jarvis 1995), the latter served as an alternative for upscaling point measures of carbon metabolism (e.g. leaf gas exchange, root respiration; see below) to the tree level.

RAIN ROOF

We constructed a 10 × 10 m wooden structure covered with transparent acrylic roof sheeting (light transmittance factor *c.* 0·90 of photosynthetically active radiation, PAR, 400–700 nm). In early spring, we planted 18 half-sibling Norway spruce of compatible provenances from a nursery (*c.* 1·5 m tall from root collar, 7 years old, *c.* 65 mm diameter at stem base) in pots (*c.* 25 L volume, insulated with aluminium-coated styrofoam sheeting) in a 2:1 vermiculate–sand mixture. Nutrients were supplied by an instant fertilizer (Manna® Wuxal Super 8-8-6 with microelements, Wilhelm Haug GmbH & Co. KG, Düsseldorf, Germany), combined with a slow-release conifer fertilizer (Substral® Osmocote 11-8-17, Scotts Celaflor GmbH, Mainz, Germany). We placed 12 trees under the roof in a completely randomized design and surrounded these with a row of unmonitored border trees to create similar neighbourhood conditions for all sample trees (Fig. S1, Supporting Information).

WHOLE-TREE CHAMBERS

Six trees were placed in WTC. These chambers were approximately 2·5 m high and 1·3 m in diameter and were covered with 180-μm greenhouse film (Folitec® UV 4, Scotts Celaflor GmbH) attached to a bottom and top plate (Fig. S2, Supporting Information). They were continuously flushed with ambient air at a rate of 130 m^3 h^{-1}, which exchanged the air approximately every 1·5 min, preventing excessive heating. Even with this flushing, however, maximum daily temperatures within the WTC were *c.* 2 °C higher than ambient air temperatures during the course of the experiment.

TREATMENTS

Watering system

Soil water potential measurements from tensiometers were automatically read once an hour and compared to a preset 'field capacity' threshold (−0·005 MPa). When the actual value decreased below this threshold for individual trees, they were watered with a magnetic valve–activated watering system. The amount of water added per period was proportional to the deviance from the threshold. This watering regime was applied to all trees in the pretreatment period, which lasted from 1 month (WTC) to 2 months (rain roof trees), and to trees of the control treatment throughout the experiment.

Watering regimes

Groups of four trees were randomly assigned to the following treatment levels: (i) sufficient watering ('control'), (ii) drying–rewetting cycles ('cycle') and (iii) complete drought ('drought'). The different watering regimes were initiated in July, once longitudinal growth had ceased for the year, at a time when natural droughts can occur in this region. Trees in the 'control' treatment were watered regularly to maintain soil moisture at field capacity. Trees in the 'cycle' treatment were left unwatered until the relative extractable soil water content (REW, see below) decreased to below 3%. Rewetting aimed at saturating the substrate and was carried out in two watering events. A fixed amount of water was added to the pots (3·5 L), and they were left for several hours before adding more water (1·5 L). After the second watering event, water temporarily accumulated in the saucer below the pot and was slowly absorbed by the substrate. Trees exposed to complete drought did not receive any water from the beginning of treatment until tree death occurred. Because there is no unequivocal definition for tree death, we assumed that trees were dead when vital signs were no longer detectable, that is, foliage had wilted and fallen completely and leaf gas exchange and root respiration rates remained at near-zero levels. These symptoms occurred in drought trees from 13 September onwards. However, continuous data collection and tissue sampling for all treatments continued until 23 September.

ENVIRONMENTAL CONDITIONS

Soil water availability was assessed with TDR sensors (CS645, 7·5 cm three-rod probes with a TDR 100 connected to a SDMX50-series multiplexer; Campbell Scientific Inc., Logan, UT, USA) on a subset of trees within each treatment group (two control, three cycle and three drought trees). Volumetric soil water content measured by the TDR (SW_i) was converted to relative extractible soil water:

$$REW_i = \frac{SW_i - SW_{min}}{SW_{max} - SW_{min}} \quad \text{eqn 1}$$

where SW_{max} and SW_{min} are the volumetric soil water content at field capacity and the minimum volumetric soil water content after several weeks of drought.

Photosynthetically active radiation photon flux density (μmol s^{-1} m^{-2}) was measured with high-stability silicon photovoltaic detectors equipped with acrylic diffusers. Twelve of these sensors were distributed evenly under the roof, and three sensors were place at mid-height in each of the WTC. One more PAR sensor was installed above the roof to measure total incident radiation. All sensor readings were transmitted to a Campbell® AM16/32 relay multiplexer and then recorded with a Campbell® CR23X micrologger.

TREE MEASUREMENTS

All trees

On each tree, we installed a steel wire dendrometer to measure stem diameter variation. The steel wires of the dendrometers wrapped around a portion of the tree stem at *c.* 1/3 tree height and were attached to a push-rod potentiometer (Fig. S3, Supporting Information). We used variation in stem diameter as an indicator for phloem hydration and carbon allocation (stem growth). Although changes in plant hydration affect both phloem and xylem tissues, most of the initial shrinking and swelling occur in the nonlignified cells of the phloem making stem diameter variation a reliable indicator for phloem hydration (Zweifel, Zimmermann & Newbery 2005). Variations in bark thickness included in stem diameter variation were neglected because bark tissue is very thin in these young trees.

We also installed a Granier-type heat dissipation sapflow sensor (3 cm long) on each tree. The two probes of the sapflow sensors were installed *c.* 10 cm apart vertically on the lower portion of the stems with a slight horizontal offset. We computed a sapflow index from the temperature differences between the two probes (eqn 2):

$$K_i = \frac{\Delta T_{max} - \Delta T_i}{\Delta T_i} \quad \text{eqn 2}$$

where K_i is the sapflow index at time i, ΔT_{max} is the maximum temperature difference between the two sensors measured on a daily basis to account for drought-induced drifts of T_{max} (Lu, Urban & Zhao 2004) during an assumed zero-flow (0h - 5h) period, and ΔT_i is the temperature difference at time i. Hence, K_i is dimensionless and indicates relative sapflow velocity (Wang & Stutte 1992). While the assumption of night-time zero-flow may not hold when equilibrium between plant and soil moisture takes a long time to establish (e.g. large trees, dependency on deep soil water pools) (Regalado & Ritter 2007), our experimental design (i.e. small trees in pots) resulted in much shorter required time periods for night-time water recharge.

To avoid physically damaging the experimental trees, we measured leaf water potential with a pressure bomb (Skye®, model SKPM 1400/80, Skye Instruments Ltd, Powys, UK) on separate trees undergoing soil drying to below 3% REW. We did not measure branch conductivity to assess hydraulic failure because to do so would require harvesting of large branch portions that would have substantially affected tree hydraulic functioning and carbon balance in the experimental trees. Instead, we assumed that the absence of functional hydraulic failure (irreversible and complete xylem cavitation) at the whole-tree level could be inferred from the recovery of sapflow in the cycle treatment.

Rain roof trees

Each rain roof tree was equipped with a branch and a root chamber. The transparent cylindrical branch chambers (*c.* 0·8 L volume) were made of methyl methacrylate resin and suspended from the roof. We inserted a terminal branch section of each tree into these chambers through a round opening at the chamber base. To maintain air tightness, the branches were sealed using closed-cell foam plugs slit open to accommodate the branch and inserted tightly into the chamber openings. The chambers were flushed with ambient air at a rate of 3 L min^{-1} to prevent depletion of CO_2 during times of high photosynthetic activity. However, at this flow rate, both $\delta^{13}C$ and night-time respiration of branches/needles could not be measured with adequate precision and are not reported here.

Root chambers were made by sealing off the head space of the pots with a stainless steel cover. This head space was also continuously flushed with ambient air at 3 L min^{-1} to prevent accumulation of CO_2 respired by roots. Flow rates were kept constant with electronically controlled mass flow controllers (M+W Instruments®, model D-6211, M + W Instruments GmbH, Leonhardsbuch, Germany). Each branch and root chamber was also equipped with a thermocouple for measuring air and soil temperature, respectively.

Leaf gas exchange, root respiration rates and $\delta^{13}C$

Each tree was measured during a 5-min period per hour, so the whole cycle of all 12 trees was completed after 1 h, with timing controlled by a Campbell® CR10X micrologger and a custom-made valve switching unit. Within each 5-min interval, a second valve switching unit rotated in 100-s intervals among inlet streams of ambient air and outlet streams of branch and root chambers and directed these to a Picarro® G2101-*i* isotopic CO_2 cavity ring-down spectrometer gas analyser (Picarro, Santa Clara, CA, USA). Within each 100-s measuring interval, a core period of 20 s was used to compute an average $[CO_2]$ for ambient air and for each sample air type. We then computed leaf gas exchange and root respiration rates as the difference in $[CO_2]$ of branch ($[CO2]_{leaf}$) or root ($[CO2]_{root}$) chambers minus ambient air within each 5-min cycle.

The Picarro® G2101-*i* measures $[^{12}CO_2]$ and $[^{13}CO_2]$ individually at a very high temporal resolution (*c.* 1 data point/2 s) and computes $\delta^{13}C$ (relative to the Vienna Pee Dee Belemnite standard) from these. However, because of short-term variations in source $\delta^{13}C$ (i.e. of ambient air) due to fossil fuel emissions in this suburban area, we report $\delta^{13}C_{Sample}$ (root respiration) as deviations ($\Delta\delta^{13}C$) from the source (ambient air), hence:

$$\Delta\delta^{13}C = \delta^{13}C_{Sample} - \delta^{13}C_{Source} \quad \text{eqn 3}$$

We computed $\delta^{13}C_{root}$ directly as:

$$\delta^{13}C_{root} = \left(\frac{[^{13}CO_2]_{root}/^{12}CO_2]_{root}}{^{13}C/^{12}C_{PDB}} - 1\right) * 1000 \quad \text{eqn 4}$$

where $[CO_2]_{root}$ was computed for both isotopic species individually.

The nominal volumetric flow rate of ambient air through the chambers (VFR, 3 L min^{-1}) was corrected to account for variations in ambient temperature, air pressure and partial water vapour pressure:

$$VFR_{corr}(L\ min^{-1}) = VFR_{nom}(L\ min^{-1}) * \frac{D_{Air}(g\ L^{-1})}{D_{Air\_max}(g\ L^{-1})} \quad \text{eqn 5}$$

where D_{Air} is the actual air density (g L^{-1}) computed from data collected at the institutional meteorological station and D_{Air_max} is the maximum density of dry air at normal temperature (0 °C) and pressure at sea level (1·293 g L^{-1}). This flow rate was then used to compute the net carbon exchange (C_{net}) by the branch section or the roots within the chamber:

$$C_{net}(gC\ min^{-1}) = \Delta[CO_2] * 10^{-6} * \frac{VFR_{corr}(L\ min^{-1})}{22.4\ L\ mol^{-1}} * 120.1\ gC\ mol^{-1} \quad \text{eqn 6}$$

where $\Delta[CO_2]$ was measured in p.p.m. and represents the changes in the $[CO_2]$ in the air flow due to photosynthesis or respiration. The molar fraction of carbon in this flow difference multiplied by the molecular weight of carbon then yielded the net carbon exchange by the branch section or the roots in the chamber per minute. These estimates were considered constant for each 5-min interval and integrated over the whole 1-h cycle.

Leaf area contained in the branch chambers was estimated with digital images at the end of the experiment. Fresh and oven-dried needles from each chamber were placed on a light table and photographed. We estimated the projected leaf area as the count of pixels beyond a specified RGB threshold (i.e. dark area against bright background). The shrinking factor from fresh to dry state in green needles was applied as a 'growing' factor for needles from dead trees, and this allowed estimating their projected leaf area before the onset of drought stress. The area was calibrated with an object of known area (1 Euro coin) and then used to estimate specific leaf gas-exchange measurements for each chamber. Because longitudinal growth had ceased before the start of the experiment, we assumed needle biomass (and leaf surface) to be constant over the time course of the experiment.

WHOLE-TREE NET CARBON EXCHANGE

A valve switching unit controlled by a Campbell® CR10X micrologger was also used for directing the air stream of each of the six individual WTC to a LI-COR® 6262 IRGA (LI-COR Environmental, Lincoln, NE, USA). The reference cell was constantly flushed with N_2, yielding absolute [CO_2] measurements. As for the branch and root chambers on rain roof trees, nominal flow rates were corrected for temperature, air pressure and water vapour and converted to net carbon exchange rates. We report whole-tree net carbon exchange of the two drought levels (cycle, drought) as relative deviations from control trees. Trees were removed from WTC after the experiment, separated into structural/functional units (leaves, branches, stems, roots), completely dried at 70 °C and weighed.

NONSTRUCTURAL CARBOHYDRATE CONCENTRATIONS AND MOBILE NSC $\Delta^{13}C$

We considered glucose, fructose, sucrose and starch to be the main physiologically important carbon storage compounds and determined their concentrations in leaf, branch and root tissue. Root tissues were sampled before and at the end of the experiment; branch and needle samples were collected twice a week before and during the experiment. Samples were cut from trees with a sharp branch cutter, immediately deep-frozen in liquid nitrogen and kept on dry ice until they were placed in −80 °C freezer for longer storage. For nonstructural carbohydrate (NSC) extraction, frozen samples were vacuum freeze-dried for 72 h and milled with a ball mill (Retsch® MM200; Haan, Germany) to fine powder (Raessler *et al.* 2010).

Water-soluble sugars

We added 50 mg of the ground samples to 1 mL distilled water and vortexed the mixture until it was homogenized. This mixture was incubated for 10 min at 65 °C in a thermomixer and then centrifuged for 15 min at 2300 ***g***. The supernatant was removed with a pipette and stored on ice. The procedure was repeated twice, and supernatants were pooled. Water extracts were stored frozen at −20 °C for later measurement (Raessler *et al.* 2010).

Starch

We added 50 mg of the ground sample to 0·35 mL distilled water, vortexed this mixture for 1 min and treated it for another 10 min in the thermomixer at 65 °C (1050 rpm). We then added 0·5 mL of 33% perchloric acid and let it incubate in an orbital shaker for 20 min. The mixture was then centrifuged at 14 300 ***g*** for 6 min and the supernatant removed with a pipette. This procedure was then repeated on the remaining pellet, and the supernatant from the two extractions was pooled.

NSC concentration measurements

An aliquot of the sugar/starch extract was diluted (1:20 for soluble sugar and 1:55 for starch extracts) before measurement of sugars by high-pressure liquid chromatography–pulsed amperometric detection (HPLC – PAD), using a Dionex® ICS 3000 ion chromatography system equipped with an autosampler (Thermo Fisher GmbH, Idstein, Germany; Raessler *et al.* 2010).

Mobile NSC $\delta^{13}C$

An aliquot of the sugar extracts was pipetted into tin cups and assayed with a Finnigan MAT DeltaPlus XL EA-IRMS (ThermoFinnigan GmbH, Bremen, Germany), coupled to an autosampler (Koppenaal, Tschaplinski & Colombo 1991). Measurement error of $\delta^{13}C$ analyses was <0·1‰ based on measurements of an internal laboratory standard (NBS 22: –30·03‰ on VPDB scale) (Coplen *et al.* 2006). However, residuals of perchloric acid in the hydrolysed starch extracts prohibited their analysis by mass spectrometry, so we have no data for stable isotopic signatures of this fraction.

STATISTICAL ANALYSIS

Where appropriate, we compared treatment means for each time step with an ANOVA (response *c.* treatment) followed by Tukey's honest significance test ($\alpha < 0.05$) to detect significant differences between the treatment levels. We used the Levene test to check for heteroscedasticity across groups (Kozlowski & Pallardy 2002) and report significant test results of pairwise comparisons only when variances were homoscedastic. The temporal trend in NSC concentrations from beginning vs. end of the experiment was statistically assessed with repeated measures ANOVA, after checking for sphericity with Mauchly's test. A significant ($P < 0.05$) treatment*date interaction indicated that observed differences between groups developed over the duration of the experiment. All analyses were carried out with R (v. 2.13.0, R Foundation for Statistical Computing 2011).

Results

TREATMENT AND ENVIRONMENTAL CONDITIONS

For the duration of the experiment, light availability, ambient temperature and water vapour pressure deficit were quite variable. The cumulative daily sum of PAR photon flux density was, on average, 29·4 mol m^{-2} day^{-1} during the experimental period and varied between 4·6 and 52·7 mol m^{-2} day^{-1}. Average daily maximum temperature was 21·7 °C and varied between 13·1 and 33·1 °C, while average daily vapour pressure deficit was 5·8 hPa and varied between 1·0 and 14·0 hPa (Fig. 1a).

The drought treatment started on 1 July (last day of watering for cycle and drought trees) and caused rapid declines in REW. REW of the control treatment remained at *c.* 80% (=field capacity), while REW in the drought treatment declined to <3% after *c.* 3·5 weeks. Cycle trees were given water four times during the experiment. However, between rewetting events, REW declined to values similar to those of complete drought (Fig. 1b).

TREATMENT EFFECTS ON SAPFLOW AND STEM DIAMETER VARIATION

Sapflow was strongly affected by both drought conditions. Shortly after the beginning of the experiment, sapflow declined in both cycle and drought trees and was near-zero after *c.* 2 weeks. Sapflow rates never went down to true zero, which may be due to overestimations of low sapflow rates with thermal dissipation probes (Burgess *et al.* 2001). Rewetting events in cycle trees caused a rapid recovery of sapflow but showed an increasing delay with each rewetting cycle (Fig. 2a). Stem

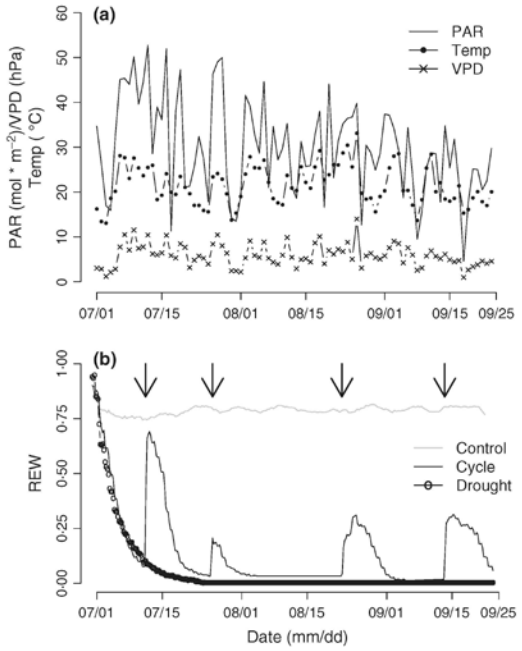

Fig. 1. Climate forcing (a) and relative extractable soil water (b) during the experimental period. Shown in (a) are the cumulative daily sum of PAR photon flux density (PAR, mol m^{-2} day^{-1}, line only), the daily maximum temperature (TEMP, °C, line with dots) and water vapour pressure deficit (VPD, hPa, line with crosses). Relative extractable soil water (b, REW, see text) for the three treatments. Arrows indicate dates when cycle trees were given water. Note that REW never regained field capacity following rewetting.

diameter increased in control trees by *c.* 50%, while cycle trees maintained their initial stem diameter during the experiment. Complete drought caused stem shrinkage of *c.* 33%, indicating a severe decline in phloem and xylem hydration (Fig. 2b). Daily stem diameter variation was highest in cycle trees, peaking during rewetting events but also during periods of declining REW and sapflow (Fig. 2c). Both stem diameter and stem diameter variation showed significant temporal trends (repeated measures ANOVA, $P < 0{\cdot}05$), and stem diameter in both drought treatments was significantly smaller than in control trees (ANOVA, followed by Tukey's HSD, $P < 0{\cdot}05$) from *c.* 1 week after the start of the experiment onwards. Midday leaf water potential under nondrought conditions ranged between −0·8 and −2·2 MPa but decreased to less than −3·5 MPa when soil moisture was at a minimum and even decreased to less than −4·5 MPa on hot and sunny days (data not shown).

LEAF GAS EXCHANGE

Carbon assimilation ceased rapidly after the onset of drought in both drought and cycle trees. Average daily carbon assimilation rates approached zero after <2 weeks in these trees (Fig. 3a). Rewetting events caused instantaneous increases in carbon assimilation of cycle trees lasting *c.* 2 weeks, while carbon assimilation in drought trees ceased completely after *c.* 6 weeks. Early morning assimilation rates were similar between all treatment levels and remained positive until *c.* August 10 (Fig. 3b). Furthermore, during mild, cloudy and humid days, midday (12–14 h) assimilation rates remained remarkably high in drought trees even after *c.* 4 weeks of drought stress (e.g. July 30, Fig. 3c).

ROOT RESPIRATION AND $\Delta^{13}C$ OF ROOT-RESPIRED CO_2 ($\Delta^{13}C_{ROOT}$)

Root respiration in control trees was driven largely by temperature (correlation coefficient 0·46, Fig. 4a). In cycle and drought trees, respiration rates were less controlled by temperature (correlation coefficient 0·15 and 0·16, respectively). Respiration rates in cycle trees increased after each rewetting event and also on mild, cloudy and humid days in drought trees. These were also the dates when carbon assimilation rates increased (Fig. 3). $\delta^{13}C_{root}$ increased in both cycle and drought treatments beginning just after the start of the experiment and continuing until tree death (drought trees) or the end of the experiment (cycle trees, Fig. 4b). In cycle trees, $\delta^{13}C_{root}$ decreased temporarily by *c.* 5‰ after the second and third watering event indicating downward transport of fresh assimilates. In contrast, $\delta^{13}C_{root}$ in drought trees remained enriched but became more variable from August 25 onwards (Fig. 4b).

WHOLE-TREE NET CARBON EXCHANGE

To make comparison easier, we report the differences in whole-tree net carbon exchange for cycle and drought conditions relative to control trees. Positive deviations (smaller whole-tree C loss compared to control trees) occurred mainly during night-time in drought trees, indicating that respiration was reduced by drought (Fig. 5a). However, cycle trees showed increases in night-time respiration after watering and overall lost more carbon at night than control trees (Fig. 5a).

During the daytime, however, carbon exchange rates were strongly and negatively affected by complete drought and to a lesser degree in cycle trees (Fig. 5b). Overall, the negative daytime deviation in net carbon exchange rates more than compensated for smaller losses during the night in drought trees, and this led to a substantial overall carbon loss. Rewetting events in cycle trees dampened the negative effect but only for *c.* 1 week (Fig. 5b). Root development was also strongly affected by drought. Control trees had well-developed root systems at the end of the experiment, while root systems of both cycle and drought trees were smaller and shallower (Table S1, Fig. S4, Supporting Information).

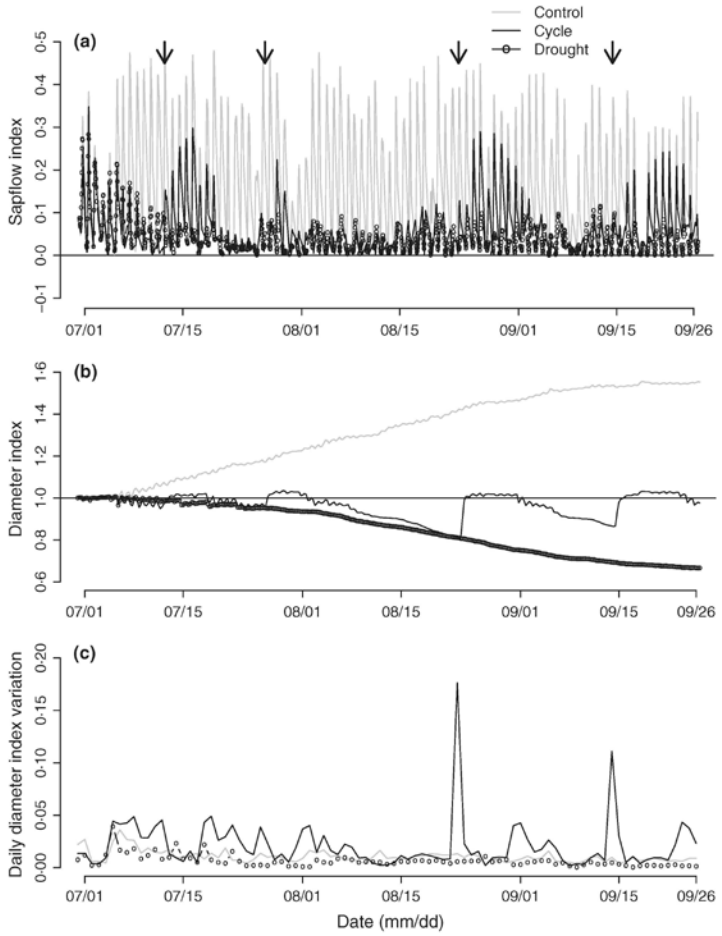

Fig. 2. Smoothed daytime sapflow index (a), diameter index (b) and diurnal diameter index variation (c) of the three treatment groups (control – grey line, cycle – black line, drought – black line and round symbols). The sapflow index is proportional to xylem flux velocity, and the diameter index represents the cumulative diameter variation from initial diameter at the beginning of the experiment. Diurnal diameter index variation is the daily variation in stem diameter and is therefore indicative for stem capacitance. Arrows indicate dates when cycle trees were given water. Note the apparent negative sapflow indices during periods of drought stress for both drought and cycle tree.

NONSTRUCTURAL CARBOHYDRATE POOLS AND MOBILE NSC $\Delta^{13}C$

Carbon pools in the above-ground tissues were not affected by drought. NSC concentrations in droughted needles and branches were similar to those of control trees (Tables 1 and 2). In cycle trees, this was also the case for sucrose. However, glucose and fructose concentrations in branches and needles were higher in cycle trees than in control trees at the end of the experiment (Tables 1 and 2). Root NSC concentrations, especially sucrose, were reduced in the drought treatment (Table 3). However, starch was almost completely depleted in roots of droughted trees while showing large increases in cycle and control trees over the course of the experiment (Table 3). Total NSC concentrations in needles were not affected by drought treatments (repeated measures ANOVA, $P > 0{\cdot}05$, Fig. 6a) but significantly increased in branches of cycle trees (ANOVA, followed by Tukey's HSD, $P < 0{\cdot}05$, Fig. 6b) and decreased in roots of trees in the complete drought treatment (Fig. 6c).

Mobile NSC $\delta^{13}C$ of needles and branches showed a clear treatment effect (repeated measures ANOVA, $P > 0{\cdot}001$). Over the growing season, the initial $\delta^{13}C$ values in needles and branches (*c.* $-25{\cdot}5‰$ and $-26{\cdot}5‰$, respectively) decreased in control trees to *c.* $-29‰$ and $-27{\cdot}5‰$, respectively, while they increased slightly in needles of both drought treatments (*c.* $-26{\cdot}5‰$) and somewhat more in branches (*c.* $-25{\cdot}5‰$ in cycle trees, *c.* $-26‰$ in drought trees, Table 4). Mobile NSC $\delta^{13}C$ remained

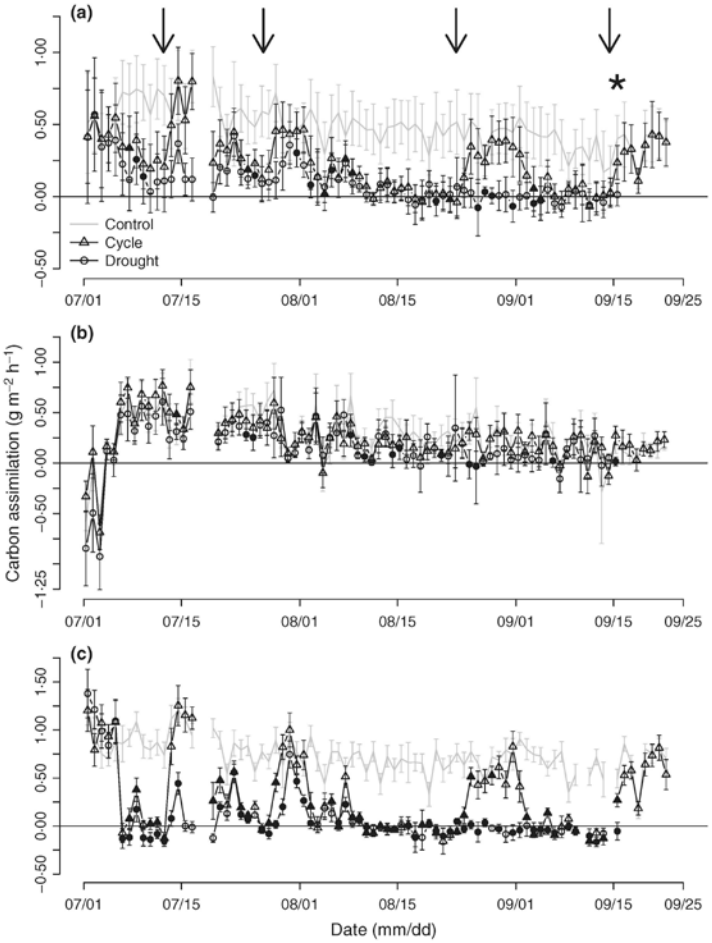

Fig. 3. Daily (a), early morning (6–8 h, b) and midday (12–14 h, c) averages (±1SEM) of specific carbon assimilation rates (g m^{-2} h^{-1}) of the three treatment groups (control – grey line, cycle – black line, drought – black line). Filled symbols in cycle and drought trees (triangles and circles, respectively) indicate dates where differences are significant from control trees (ANOVA and Tukey's HSD, $\alpha < 0{\cdot}05$). Arrows indicate dates when cycle trees were given water, and the asterisks (*) indicate when trees in the complete drought treatment apparently died.

constant in roots of cycle trees and increased by *c.* 0·7‰ in drought trees, while it decreased in control trees by almost 2‰ (Table 4). However, there was no overall significant treatment effect over the run of the experiment (repeated measures ANOVA, $P > 0{\cdot}05$).

Discussion

DROUGHT EFFECTS ON TREE HYDRAULIC FUNCTIONING

We found no indication for irreversible xylem cavitation in cycle trees although their sapflow declined before rewetting events as much as in drought trees (Fig. 2a). Recovery of sapflow after rewetting indicated that trees seemingly refilled cavitated vessels (Sperry *et al.* 1994; Hacke & Sauter 1996) although we observed an increasing delay it took to recover. Xylem refilling under tension, as occurred in our rewetted cycle trees, is an energy-driven process (Zwieniecki & Holbrook 2009) and imposes an additional burden on the carbon budget, potentially triggering a negative feedback on xylem functioning (McDowell *et al.* 2011). However, cycle trees showed high soluble sugar concentrations in branch tissue at the time of the last rewetting event (Table 2), which makes it very unlikely that the increasing delay in sapflow recovery was driven by carbon limitation, but instead could result from a water deficit impeding the signalling for xylem refilling (Brodersen *et al.* 2010).

We observed strong diameter variation especially in cycle trees during periods of severe water deficit (i.e. before rewetting events) and explain this observation with decreased phloem hydration and the use of stored stem water (capacitance) (Offenthaler, Hietz & Richter 2001; Meinzer *et al.* 2009). In our experiment, this phenomenon

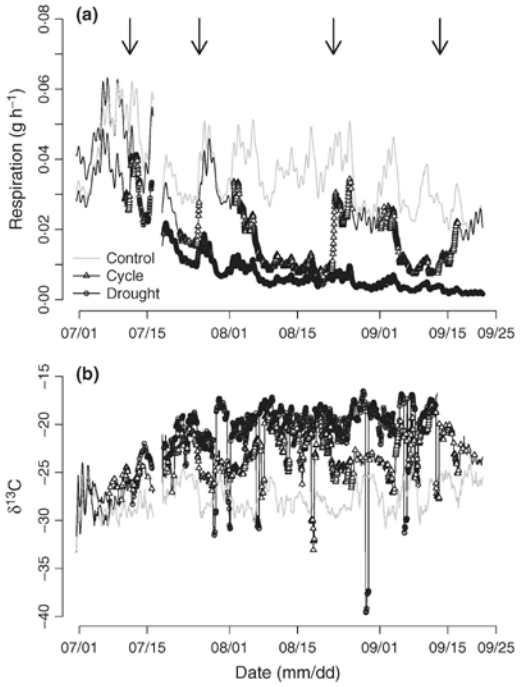

Fig. 4. Smoothed hourly averages in root respiration rates (a, g h^{-1}) and root δ^{13}C (b, adjusted for mixing ratio in air from root chamber, in ‰, see eqn 5) of the three treatments (control – grey line, cycle – black line, drought – black line). Symbols on lines in cycle and drought trees (triangles and circles, respectively) indicate where differences are significant from control trees (ANOVA and Tukey's HSD on raw unsmoothed data, $\alpha < 0{\cdot}05$). Arrows indicate dates when cycle trees were given water, and the asterisk (*) indicates when trees in the complete drought treatment apparently died.

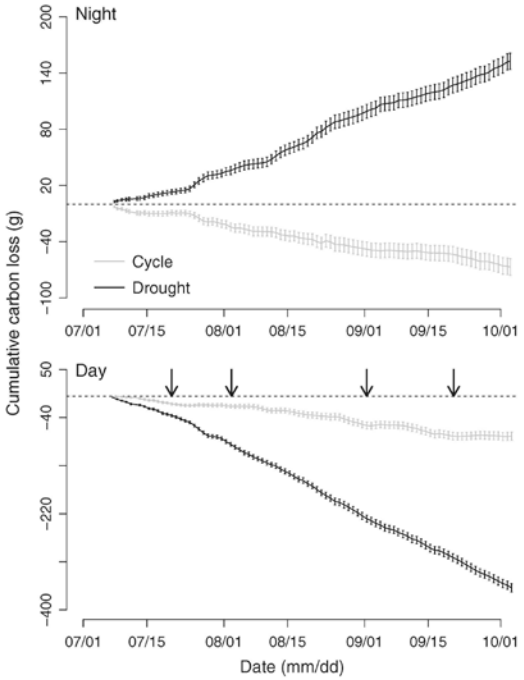

Fig. 5. Average cumulative carbon loss (g, ±1 propagated SEM) for cycle (grey line) and drought (black line) relative to control trees during night-time (upper panel, 18 h00–6 h00) and daytime (lower panel, 6 h00–18 h00). Arrows indicate dates when cycle trees were given water, and the dotted line indicates zero carbon loss. Note that each group represents only two individual WTC. WTC, whole-tree chambers.

may have contributed to the avoidance of runaway embolism and catastrophic (complete and irreversible) xylem cavitation in cycle trees by buffering fluctuations in xylem pressure. However, because a sustained use of capacitance requires the xylem to be recharged (Meinzer *et al.* 2008), its functionality as a mechanism of drought tolerance or even as a survival strategy was dysfunctional for complete drought trees where no soil water was available for xylem refilling.

Conclusions about the absence of a functional hydraulic failure (i.e. complete and irreversible xylem cavitation) in drought trees cannot be drawn from sapflow and diameter variation dynamics in cycle trees. Moreover, even the absence of a functional hydraulic failure would not exclude the possibility that droughted trees suffered from an effective hydraulic failure, that is, permanently discontinued water transport to the canopy. Both the lack of soil water (Fig. 1b) and near-zero sapflow rates (Fig. 2c) indicate a complete loss of water transport to the canopy and hence the possibility of an effective hydraulic failure in aboveground tissues.

DROUGHT EFFECTS ON TREE CARBON METABOLISM

Carbon starvation may occur when availability of free (i.e. not bound to metabolic needs like respiration) mobile NSC fails to meet the amount needed to maintain osmotic potential and cell turgor (McDowell 2011). The exact threshold where NSC availability falls below demand and causes death is not defined, but is expected to result from a simultaneous decrease in carbon acquisition and maintained carbon expenditure (McDowell 2011). As expected, carbon assimilation decreased in the droughted trees quite rapidly after the onset of drought. While drought trees reduced respiration and took advantage of favourable environmental conditions (early mornings, mild cloudy days, Fig. 3b,c) for optimizing carbon assimilation in relation to water supply (Chaves *et al.* 2002), there was a net carbon loss compared to control trees overall (Fig. 5). The fact that cycle trees did not show any increase in diameter during the growing season may be interpreted as an indication for either strong carbon limitation (no carbon left for stem growth) or a shift in carbon allocation from structural growth to maintenance respiration (Maunoury-Danger *et al.* 2010).

The increase in the isotopic signature of root respiration could reflect two different processes: a decrease in the

Table 1. Needle nonstructural carbohydrate (NSC) concentrations (in mg g^{-1} of dry biomass) for glucose (glc), sucrose (suc), fructose (fruc) and starch during the beginning (early July) and towards the end (mid-September) of the experiment

		Treatment mean (SE)			Comparison (*P*)		
Date (dd/mm)	NSC	Control	Cycle	Drought	CT-CYC	CT-DR	CYC-DR
01/07	Gluc	11·49 (1·82)	14·69 (0·36)	15·49 (3·72)	.	.	.
	Fruc	8·71 (1·76)	11·82 (0·65)	14·01 (5·03)	.	.	.
	Sucr	22·86 (7·64)	15·82 (7·57)	11·15 (3·77)	.	.	.
	Starch	10·32 (3·44)	7·15 (3·41)	5·04 (1·7)	.	.	.
05/07	Gluc	19·73 (2·47)	16·76 (3·15)	21·76 (1·93)	.	.	.
	Fruc	12·84 (2·1)	13·12 (3·1)	16·76 (2·8)	.	.	.
	Sucr	25·91 (4·34)	21·99 (5·05)	14·89 (5·85)	.	.	.
	Starch	11·7 (1·96)	9·93 (2·28)	6·73 (2·64)	.	.	.
08/07	Gluc	18·97 (0·73)	13·71 (2·61)	23·03 (1·29)	.	.	0·011
	Fruc	15·61 (1·93)	13·68 (2·63)	18·56 (1·97)	.	.	.
	Sucr	14·24 (3·58)	9·16 (2·46)	7·75 (5·28)	.	.	.
	Starch	6·44 (1·62)	4·15 (1·11)	3·52 (2·38)	.	.	.
09/09	Gluc	18·37 (1·37)	28·04 (2·15)	25·3 (1·87)	0·012	.	.
	Fruc	11·66 (1·54)	23·57 (4·27)	19·56 (2·42)	.	.	.
	Sucr	19·19 (5·63)	11·98 (6·02)	18·13 (4·83)	.	.	.
	Starch	8·68 (2·54)	5·44 (2·71)	8·2 (2·17)	.	.	.
13/09	Gluc	21·11 (1·12)	29·16 (2·26)	23·33 (0·86)	0·012	.	.
	Fruc	13·87 (1·79)	24·63 (2·78)	17·35 (0·95)	0·01	.	.
	Sucr	15·09 (4·32)	5·19 (0·85)	18·02 (5·76)	.	.	.
	Starch	6·83 (1·95)	2·37 (0·38)	8·16 (2·59)	.	.	.
16/09	Gluc	21·47 (1·86)	30·88 (3·44)	22·17 (1·73)	.	.	.
	Fruc	15·01 (3·19)	24·7 (2·82)	15·53 (2·06)	.	.	.
	Sucr	18·02 (8·00)	3·13 (0·77)	19·86 (6·53)	.	.	.
	Starch	8·16 (3·60)	1·45 (0·35)	8·98 (2·94)	.	.	.

Pairwise comparison between treatments (Tukey's HSD following ANOVA) were considered significant when $P < 0{\cdot}05$. For easy reading, *P*-values > 0·05 were replaced with a period ('.').

discrimination during CO_2 fixation in drought and cycle trees and/or a shift to the increased use of NSC that are more enriched in ^{13}C as substrates for respiration. We expect decreased fractionation during photosynthesis because of reduced stomatal conductance and ensuing declines in intercellular [CO_2] (Farquhar & Sharkey 1982). During periods of active phloem transport, the increase in $\delta^{13}C_{root}$ (isotopic ratio of root-respired CO_2) would then indicate the use of fresh ^{13}C-enriched assimilates for respiration (Duranceau *et al.* 1999). However, sapflow and stem diameter strongly decreased in drought trees from mid-August onward and in cycle trees before the third rewetting event, which suggests that stem water potential was very low during these periods (Offenthaler, Hietz & Richter 2001). Given the link between xylem water potential and phloem functioning (Hölttä, Mencuccini & Nikinmaa 2009) and considering that *c.* 90% of the diurnal variation in stem diameter (minus radial growth) can be attributed to changes in phloem water deficit (Zweifel, Zimmermann & Newbery 2005), it is likely that phloem functioning was severely impeded in drought trees from mid-August onward and maybe this similarly applied to cycle trees when stem shrinkage was greater than *c.* 10% (Fig. 2b). A lack of phloem transport would impede carbon translocation, and thus, the source of ^{13}C-enriched respiration from roots would not reflect changes in the signature of C fixed in leaves. This idea is further supported by an observed decoupling of photosynthetic fractionation from the isotopic signal of respiration and mobile NSC in roots. Increases observed in the $\delta^{13}C$ of mobile NSC in needles and branches (indicating less discrimination) were not mirrored in root mobile NSC of drought trees (Table 4), and when cycle trees were watered, $\delta^{13}C_{root}$ decreased suddenly and substantially (Fig. 4b) indicating the use of fresh assimilates and hence a relief of drought-induced limitation of carbon transport from above- to below-ground.

Starch compounds are more enriched in ^{13}C than free sugars (Badeck *et al.* 2005), so the increase in $\delta^{13}C_{root}$ during drought, combined with the probable lack of transport of new photosynthetic products through the phloem to the roots, likely indicates starch remobilization and use (Gessler *et al.* 2007; Maunoury-Danger *et al.* 2010). Very low starch and total NSC concentrations in droughted tree roots at the end of the experiment (Table 3, Fig. 6) also suggest that roots were metabolizing ^{13}C-enriched starch compounds. Because carbon assimilation (and apparently carbon transport) decreased during drought, roots had to rely progressively more on *in situ* carbon reserves for respiration, and during the following weeks of increasingly severe drought stress, trees seemed to have switched to alternative respiration substrates. We observed sudden negative shifts of up to *c.* 10‰ in $\delta^{13}C_{root}$ in droughted and cycle trees (during drought periods) compared to controls (Fig. 4b), which may indicate the use of lipids during severe stress

Table 2. Branch nonstructural carbohydrate (NSC) concentrations (in mg g^{-1} of dry biomass) for glucose (gluc), sucrose (sucr), fructose (fruc) and starch during the beginning (early July) and towards the end (mid-September) of the experiment

		Treatment mean (SE)			Comparison (*P*)		
Date (dd/mm)	NSC	Control	Cycle	Drought	CT-CYC	CT-DR	CYC-DR
01/07	Gluc	11·283 (2·12)	16·094 (4·87)	10·41 (2·79)	.	.	.
	Fruc	10·149 (2·65)	16·686 (6·04)	10·86 (2·65)	.	.	.
	Sucr	28·329 (3·99)	23·006 (6·78)	24·67 (5·24)	.	.	.
	Starch	12·775 (1·80)	12·606 (4·31)	10·55 (3·87)	.	.	.
05/07	Gluc	14·673 (1·46)	10·535 (1·69)	11·21 (1·00)	.	.	.
	Fruc	16·134 (1·34)	9·26 (1·37)	11·76 (2·04)	0·036	.	.
	Sucr	30·219 (3·65)	26·902 (2·55)	22·55 (3·80)	.	.	.
	Starch	13·617 (1·65)	12·132 (1·15)	9·84 (2·35)	.	.	.
08/07	Gluc	16·257 (1·50)	12·963 (1·52)	16·29 (0·71)	.	.	.
	Fruc	15·741 (1·13)	13·64 (2·45)	17·39 (2·99)	.	.	.
	Sucr	28·122 (1·47)	27·016 (0·75)	34·75 (3·32)	.	.	.
	Starch	12·668 (0·94)	11·888 (0·20)	14·5 (0·12)	.	.	.
09/09	Gluc	10·442 (0·87)	18·23 (1·43)	9·73 (0·51)	0·001	.	0·001
	Fruc	6·096 (1·40)	17·745 (2·35)	2·29 (1·27)	0·003	.	<0·001
	Sucr	28·517 (2·31)	31·21 (1·96)	25·26 (3·05)	.	.	.
	Starch	12·864 (1·04)	14·086 (0·88)	11·4 (1·38)	.	.	.
13/09	Gluc	11·925 (0·56)	21·144 (0·73)	11·19 (1·05)	<0·001	.	<0·001
	Fruc	8·481 (0·72)	21·794 (0·96)	5·77 (1·21)	<0·001	.	<0·001
	Sucr	26·046 (2·97)	27·443 (1·20)	18·92 (3·28)	.	.	.
	Starch	11·76 (1·34)	11·96 (0·46)	8·54 (1·48)	.	.	.
16/09	Gluc	11·65 (0·90)	20·10 (2·33)	11·69 (1·67)	0·018	.	0·019
	Fruc	7·28 (1·29)	17·20 (2·29)	4·83 (1·18)	0·006	.	0·001
	Sucr	25·65 (4·11)	23·76 (1·39)	23·81 (1·34)	.	.	.
	Starch	11·58 (1·85)	10·73 (0·63)	10·75 (0·61)	.	.	.

Pairwise comparison between treatments (Tukey's HSD following ANOVA) were considered significant when $P < 0·05$. For easy reading, *P*-values > 0·05 were replaced with a period ('.').

Table 3. Root nonstructural carbohydrate (NSC) concentrations (in mg g^{-1} of dry biomass) for glucose (gluc), sucrose (sucr), fructose (fruc) and starch before (17/06/2011) and at the end (28/09/2011) of the experiment

		Treatment mean (SE)			Comparison (*P*)		
Date (dd/mm)	NSC	Control	Cycle	Drought	CT-CYC	CT-DR	CYC-DR
17/06	Glc	10·98 (2·01)	15·79 (1·62)	9·75 (4·90)	.	.	.
	Suc	29·81 (3·20)	22·99 (3·32)	26·06 (3·20)	.	.	.
	Fruc	9·02 (0·93)	12·97 (1·82)	8·49 (3·00)	.	.	.
	Starch	13·47 (1·44)	9·42 (0·93)	11·77 (1·45)	.	.	.
28/09	Glc	8·58 (1·63)	10·72 (1·76)	5·25 (1·40)	.	.	.
	Suc	38·57 (3·97)	31·58 (2·23)	5·41 (2·30)	.	<0·001	<0·001
	Fruc	10·22 (2·07)	9·82 (2·23)	4·15 (1·90)	.	.	.
	Starch	17·40 (1·79)	14·95 (0·73)	2·44 (1·06)	.	<0·001	<0·001

Pairwise comparison between treatments (Tukey's HSD following ANOVA) was considered significant when $P < 0·05$. For easy reading, *P*-values > 0·05 were replaced with a period ('.').

(Tcherkez *et al.* 2003). Further research on the identity and availability of putative substrates and their isotopic signature is necessary to assess the dynamics of storage use under drought stress.

We did not find any indication for soluble sugar depletion in above-ground tissues but rather a general increase in branch glucose and fructose concentrations (Tables 1 and 2). Increases in fructose concentrations have been explained in olive plants as a mechanism of osmotic adjustment to increase drought tolerance (Dichio *et al.* 2009), and laboratory experiments confirm that reducing sugars (e.g. glucose and fructose) play an important role in cell osmotic adjustment during water stress (Handa *et al.* 1983). While the mechanisms as well as the compounds involved in osmotic adjustment seem to be highly species specific (Kozlowski & Pallardy 2002), a concurrent decrease in starch and sucrose and increases in glucose and fructose concentrations indicated that monosaccharides were the most important osmotica for adjustment in leaves of *Malus domestica* (Borkh.) (Wang & Stutte 1992) as well

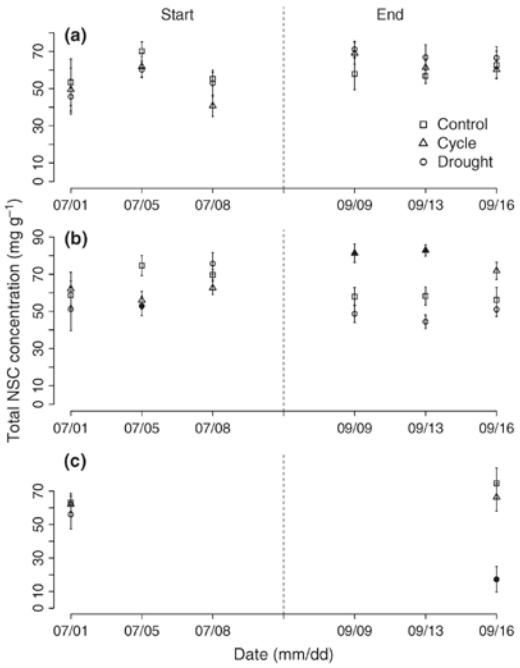

Fig. 6. Total (glucose + fructose + sucrose + starch) concentrations (mg g^{-1} dry biomass) in needles (a), branches (b) and roots (c) of the three treatments (control – squares, cycle – triangles, drought – circles). Repeated measures ANOVA showed a significant ($P < 0{\cdot}01$) temporal trend in total NSC concentrations of branches and roots (not shown). Filled symbols indicate dates where differences are significant from control trees (ANOVA and Tukey's HSD, $\alpha < 0{\cdot}05$). Note that actual sampling dates of root tissues (06/17, 09/28) are reported here for simplicity as 07/01 and 09/16, respectively. NSC, nonstructural carbohydrate.

as in shoots and roots of *Pinus banksiana* (Lamb.) and *Picea glauca* (Moench) Voss) (Koppenaal, Tschaplinski & Colombo 1991). The fact that soluble sugar concentrations were generally higher in cycle than in control trees seems to corroborate their importance as osmotica; concentrations of both glucose and fructose increased in needles and branches; in needles, this seemed to have occurred at the expense of sucrose (Table 1). We speculate that the cycle trees may have acclimated to drought stress in earlier drying cycles and optimized carbon partitioning for osmotic adjustment. Given an average increase of *c.* 20 mg of glucose and fructose per gram of dry needle and branch biomass (Tables 1 and 2), a 1:3 ratio of dry/fresh weight (data not shown) and assuming a symplastic water content of *c.* 75% (Gross & Koch 1991), the osmotic pressure would have been increased by 0·18 MPa at full turgor in cycle trees. We are currently investigating the temporal dynamics of this water-driven carbon partitioning in more detail in a separate study.

Overall, our NSC and starch data suggest that above-ground tissues and roots respond differently to drought stress in terms of carbon allocation and balance. While above-ground tissues were not carbon depleted even when the drought trees died, roots in droughted trees showed severe declines in carbon pools (Fig. 6c), especially starch (Table 3), and likely lost biomass (or at least gained very little compared to control trees). This finding is interesting because other studies have found that severe drought increased root NSC concentrations in poplar seedlings (Galvez, Landhäusser & Tyree 2011) and in many other tissues of droughted plants (Muller *et al.* 2011).

Sink limitation to growth can explain high NSC concentrations in trees even in stressful environments (Körner 2003) because growth declines faster than photosynthesis

Table 4. Mobile nonstructural carbohydrate (NSC) $\delta^{13}C$ (in ‰) in different functional units (FU) at the beginning (June–July) and at the end (September) of the experiment

		Treatment mean (SE)			Contrasts		
FU	Date (dd/mm)	Control	Cycle	Drought	CT-CYC	CT-DR	CYC-DR
Needle	01/07	−27·39 (0·17)	−27·08 (0·19)	−27·1 (0·25)	.	.	.
	05/07	−27·5 (0·15)	−27·2 (0·71)	−27·5 (0·4)	.	.	.
	08/07	−27·39 (0·34)	−26·77 (0·45)	−27·12 (0·45)	.	.	.
	09/09	−28·62 (0·15)	−26·53 (0·68)	−26·59 (0·29)	0·002	0·001	.
	13/09	−28·78 (0·37)	−26·23 (0·3)	−26·52 (0·22)	0·001	0·001	.
	16/09	−28·94 (0·2)	−26·24 (0·55)	−26·46 (0·17)	<0·001	<0·001	.
Branch	01/07	−26·41 (0·47)	−26·52 (0·36)	−25·86 (0·21)	.	.	.
	05/07	−26·32 (0·39)	−25·57 (0·56)	−26·31 (0·58)	.	.	.
	08/07	−26·65 (0·55)	−26·03 (0·86)	−26·47 (0·19)	.	.	.
	09/09	−27·55 (0·14)	−25·47 (0·59)	−25·99 (0·29)	0·001	0·003	.
	13/09	−27·42 (0·26)	−25·41 (0·23)	−25·99 (0·21)	<0·001	0·003	.
	16/09	−27·76 (0·34)	−25·2 (0·56)	−25·99 (0·21)	0·001	0·006	.
Root	17/06	−25·94 (0·57)	−24·81 (0·39)	−25·6 (0·4)	.	.	.
	28/09	−27·88 (0·33)	−24·87 (0·54)	−26·34 (1·04)	0·012	.	.

Pairwise comparison between treatments (Tukey's HSD following ANOVA) were considered significant when $P < 0{\cdot}05$. For easy reading, *P*-values > 0·05 were replaced with a period ('.').

and causes substrate accumulation (Muller *et al.* 2011). Increased glucose, fructose and total NSC concentrations occurred in above-ground tissues of cycle trees during periods of substantial source activity (Fig. 3) and concurrent near-zero growth sink activity in stems (Fig. 2b) and roots (Fig. S4, Supporting Information). Although other sink activities (e.g. maintenance respiration) are less affected by drought (Flexas *et al.* 2006) and heterotrophic tissues in distal parts from sources, like stems and roots, were still in need of metabolic energy, periods of reduced phloem functioning may have prevented carbon import from tissues close to source activity (i.e. needles, branches) and hence to local carbon accumulation.

Moreover, the particular conditions in our experiment (e.g. limited rooting space without access to deeper soil water pools, extreme drought) not only decreased carbon assimilation but seemingly also carbon translocation from above- to below-ground in the complete drought treatment (Ruehr *et al.* 2009). The drought may have been too severe for carbon storage remobilization, and this may have prevented sustained storage use in above-ground components. The trees' metabolism collapsed in these tissues seemingly before storage pools could be substantially decreased, and studies with less severe but prolonged drought should be undertaken to allow storage remobilization. On the other hand, stress periods may have been too short in cycle trees to cause strong carbon storage dependency, which may also explain, besides sink limitation, why these trees did not show symptoms of above-ground carbon depletion but instead increases in carbon pools. In our experiment, reduced phloem functioning clearly caused roots to be isolated from carbon sources and made them dependent on locally stored carbon for metabolic needs. Reduced root growth (Fig. S4, Supporting Information) seemingly did not offset the reductions in carbon supply and reductions in carbon pools ensued.

SUMMARY AND OUTLOOK

In above-ground tissues of cycle trees, hydraulic constraints were not irreversible as indicated by the recovery of xylem transport in tree stems and of carbon assimilation in needles when they were watered. Nevertheless, hydraulic failure from a lack of available water cannot be refuted as mortality mechanism in above-ground tissues of drought trees. In the roots of these trees, the continued use and depletion of stored carbon suggest carbon starvation as a potential cause of death. Stored carbon fuelled maintenance respiration from locally available carbon pools but ultimately root respiration rates dropped to near-zero and death ensued. Decoupled isotopic signatures of respired and mobile NSC in leaves and branches vs. roots indicate that carbon translocation was also impeded but further studies will be necessary to validate its role during drought stress.

Care should be used in the extension of our results to trees growing in a natural setting. Our trees could not avoid drought by means of accessing deeper soil water (Dawson 1996) or by taking advantage of hydraulic lift from deep-rooting neighbours (Dawson 1993). Because the effects of drought on tree physiology are influenced by the specific set of environmental condition and tree characteristics (Hartmann 2011), the results obtained here may have been impractical in a study with naturally growing trees. There are many potential physiological causes for tree decline (Franklin, Shugart & Harmon 1987), and the interplay of predisposing, inciting and contributing factors makes tree mortality a complex process (Manion 1991). In the light of this, we underscore that neither of the hypothesized mortality mechanism (carbon starvation, hydraulic failure or carbon translocation failure) acts over the entire organism, whether individually or interactively. Our results suggest that physiological responses to drought stress (and hence maybe also mortality mechanisms) are not defined at the level of species or organisms (e.g. iso- vs. anisohydric species) but instead within tree compartments (needles/branches vs. roots). Different mechanisms are not only interacting (McDowell *et al.* 2011) but also occur concurrently in different plant compartments. These findings need to be examined in more detail with both field experiments and observational studies in natural ecosystems.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Schematic representation of the experimental design and example of an individual sample tree.
Fig. S2. Schematic representation of the whole-tree chambers.
Fig. S3. Schematic view of push-rod dendrometer.
Fig. S4. Typical condition of root systems in the three treatments at the end of the experiment.
Table S1. Leaf area and biomass of structural/functional units of trees in the whole-tree chambers.

9.3. Chapter 3: You can't starve while being thirsty

Hartmann H., Ziegler W., Kolle O. & Trumbore S. (2013) Thirst beats hunger – declining hydration during drought prevents carbon starvation in Norway spruce saplings. *New Phytologist*, **200**, 340-349.

New Phytologist

Research

Thirst beats hunger – declining hydration during drought prevents carbon starvation in Norway spruce saplings

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Summary

- Drought-induced tree mortality results from an interaction of several mechanisms. Plant water and carbon relations are interdependent and assessments of their individual contributions are difficult. Because drought always affects both plant hydration and carbon assimilation, it is challenging to disentangle their concomitant effects on carbon balance and carbon translocation. Here, we report results of a manipulation experiment specifically designed to separate drought effects on carbon and water relations from those on carbon translocation.
- In a glasshouse experiment, we manipulated the carbon balance of Norway spruce saplings exposed to either drought or carbon starvation (CO_2 withdrawal), or both treatments, and compared the dynamics of carbon exchange, allocation and storage in different tissues.
- Drought killed trees much faster than did carbon starvation. Storage C pools were not depleted at death for droughted trees as they were for starved, well-watered trees. Hence drought has a significant detrimental effect on a plant's ability to utilize stored carbon.
- Unless they can be transported to where they are needed, sufficient carbon reserves alone will not assure survival of a drought except under specific conditions, such as moderate drought, or in species that maintain plant water relations required for carbon re-mobilization.

Introduction

Increasing occurrences of drought- and heat-induced tree and forest mortality have incited scientific discussions about how exactly drought kills trees (McDowell & Sevanto, 2010; Sala *et al.*, 2010). Drought affects both tree hydraulics and carbon balance because trees – like all vascular plants – respond to decreasing soil water availability and/or declines in leaf turgor with reductions in stomatal conductance, thereby reducing carbon assimilation rates (Brodribb & McAdam, 2011). In species with a conservative water-use strategy (i.e. isohydric or strong stomatal control), the reduction in carbon assimilation associated with stomatal closure occurs early during drought and may force trees into a negative carbon balance as conditions persist (McDowell *et al.*, 2008). Stored carbon may then be mobilized to meet metabolic needs – until reserves are depleted and trees die from carbon starvation (McDowell *et al.*, 2008). However, since prolonged droughts also cause declines in plant water potential, even in anisohydric species (with weak stomatal control) (Mitchell *et al.*, 2013), phloem functioning will probably be negatively affected (Hölttä *et al.*, 2009). This can in turn impede mobilization and translocation of stored carbon from source (storage) tissues to sink tissues (Sala *et al.*, 2010) and hence may prevent trees from both using and depleting their carbon reserves (Hartmann *et al.*, 2013).

Carbon storage pools increase in tissues of many plant species as a short-term response to drought (Muller *et al.*, 2011), although it still needs to be elucidated whether this increase is the result of reduced carbon translocation from sources to sinks or whether it results from an active control of carbon storage pool size (Sala *et al.*, 2012). Investigating whether storage pool control is active requires the assessment of genetic coordination of metabolic processes (Smith & Stitt, 2007) and may be very difficult to carry out in structurally and functionally complex perennial species such as trees. Similarly, *in situ* assessments of phloem functioning (transport) are currently limited to small trees and not practical under field conditions (Helfter *et al.*, 2007) or can only be inferred from measurements of phloem shrinking in woody stems (Sevanto *et al.*, 2011). Phloem transport can be investigated indirectly with tracers (e.g. ^{13}C pulse labeling) (Ruehr *et al.*, 2009), but the time window in which the tracer can be chased may not extend more than a few weeks (Högberg *et al.*, 2008), not long enough for monitoring drought-induced tree mortality, especially if assimilation is curtailed during drought conditions.

A different indirect approach is to simulate drought-induced reductions in carbon assimilation (negative net carbon balance) while maintaining plant water potential required for phloem functioning. Comparing carbon use, survival time and dynamics of stored nonstructural carbohydrate (NSC) pools between simulated and real drought allows inference to be made about of the role played by carbon translocation during drought. Simulating drought-induced reductions in carbon assimilation can be achieved by reducing the energy necessary for the light reactions during photosynthesis with shading (S. Sevanto *et al.*, unpublished) or by reducing ambient CO_2 concentrations below the carbon compensation point (net daily carbon assimilation < daily

respiration). Both methods force trees into a negative carbon balance and may cause carbon starvation if treatment is prolonged. However, the latter method may be advantageous, in that it manipulates directly the factor of interest, CO_2 availability, without creating other nonnatural conditions (i.e. continuous darkness).

In this paper, we report high-resolution data from a drought manipulation experiment performed under near-ambient and low-CO_2 conditions. For the current study, we chose Norway spruce (*Picea abies*) because of its sensitivity to climate change and its economic importance (Hlásny *et al.*, 2011a,b). The distributional range of Norway spruce is expected to shift during the upcoming decades, in part because of direct impacts from climate change (drought and heat spells) as well as indirect impacts from changes in pest dynamics (Dutilleul *et al.*, 2000). Because Norway spruce closes stomata early during drought and is relatively vulnerable to cavitation (Bréda *et al.*, 2006), it may be considered a rather isohydric species. We continuously monitored the carbon balance of Norway spruce saplings during induced lethal drought and carbon starvation and regularly assessed carbon pool loading. We hypothesized that: hydraulic limitations will kill droughted trees quickly, regardless of CO_2 concentration; limited carbon transport as a result of declining plant hydration will cause trees to die without being able to mobilize C stores; and low CO_2 concentrations (below compensation point) will also kill trees, but will take longer to do so because stored C can be mobilized throughout the plant. Hence C stores at death will be highest in the drought/high-CO_2 plants and roughly equal to the drought/low-CO_2 plants, while they will be lowest in the watered/low-CO_2 plants. Furthermore, watered/high-CO_2 plants will not die, their carbon storage loading being representative for nonstressed conditions.

Material and Methods

Growth chambers

We built a glasshouse facility of 12 glass growth chambers (80 cm high × 75 cm long × 45 cm wide, *c.* 250 l volume), allowing control of atmospheric [CO_2]. Each chamber was subdivided into above- (*c.* 200 l volume) and below-ground compartments (made of PVC-U, *c.* 50 l volume). Both above- and below-ground chambers had an open bottom, and an airtight seal was accomplished by covering the glasshouse table with closed-cell rubber foam mats of ethylene propylene diene monomer (EPDM), which acted as a gasket to create an airtight seal. Chambers were flushed in parallel at a rate of 25 l min^{-1} (above ground) or 5 l min^{-1} (below ground), with plastic tubing inserted through the glasshouse table serving as air inlet and outlet pipes. Incoming air entered at the bottom, while outgoing air left the chamber through a pipe opening at the top (Fig. 1).

In each chamber four 4-yr old half-sibling Norway spruce (*P. abies* (L.) H Karst.) saplings (*c.* 75 cm high) were grown with their pots in the below-ground compartment. Trees were grouped so as to homogenize biomass across chambers as best as possible using visual assessment. The pots contained a carbon-free 2 : 1 vermiculate : sand mixture (fertilized with Manna® Wuxal Super 8-8-6 with microelements and a slow-release conifer fertilizer Substral® Osmocote 11-8-17; Wilhelm Haug GmbH & Co. KG, Düsseldorf, Germany, and Scotts Celaflor GmbH, Mainz, Germany). As there was no organic matter initially in the growth substrate to contribute to CO_2 fluxes by decomposition, we interpret carbon efflux from below-ground chambers as purely tree-derived. During installation, tree canopies were guided through a *c.* 4-cm-wide opening which was then sealed around the trunk with a 3-cm-thick EPDM closed-cell rubber plug. Gas tightness between compartments was checked by flushing chambers with air of strongly contrasting [CO_2] (i.e. 0 vs 400 ppm). An absence of deviations in measured [CO_2] from the preset [CO_2] indicated the absence of air exchange between chamber compartments or with ambient air.

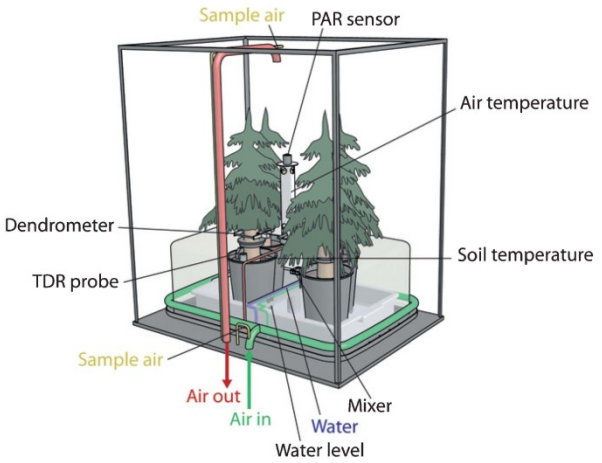

Fig. 1 Schematic view of one of the 12 growth chambers showing the ventilation system and the set of sensors. Chambers dimensions are 45 cm × 75 cm × 80 cm (W × B × H). PAR, photoysnthetically active radiation; TDR, time domain reflectometer.

Treatments

Controlling the atmospheric [CO_2] was achieved by first removing all CO_2 from ambient air with a molecular sieve and then mixing CO_2 from a pressurized bottle back into the CO_2-free air (Schnyder, 1992; Gamnitzer *et al.*, 2009; see Supporting Information Fig. S1). We chose 350 and 75 ppm as predefined concentrations of [CO_2] for the inlet air (IN) in control and carbon starvation treatments, respectively. The latter was determined in a preliminary study by progressively reducing [CO_2] until the carbon balance of the trees was negative (daily cumulative sum of assimilated carbon < daily cumulative sum of respired carbon). Other sink activities (e.g. growth, storage) were not considered in defining starvation values of [CO_2]. During the experiment, when droughted trees had already died, we reduced the [CO_2] of the starvation treatment to 40 ppm and later to 20 ppm to increase the starving effect. These concentrations are within the range of conditions under which carbon starvation has been observed (Gerhart & Ward, 2010).

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Drought was applied as a cross-treatment yielding four treatment groups: watered and high [CO_2] ($W+CO_2$); watered and low [CO_2] ($W-CO_2$); drought and high [CO_2] ($D+CO_2$); and drought and low [CO_2] ($D-CO_2$). Watered trees were given 200 ml of water each week and droughted trees were given 50 ml wk^{-1}. We did not apply total drought conditions, because a preliminary study showed that, without additional watering, mortality occurred after only *c.* 5 wk, making it difficult to detect carbon dynamics during this short period. Treatments were arranged on the glasshouse table in three replicates in a randomized design. Treatments were carried out until death occurred in droughted/starved trees. Tree death was determined by the presence of the following three indicators: zero carbon/water fluxes; complete wilting of foliage; and cambial necrosis (bark removal).

Chamber and environmental measurements

In each above-ground chamber, we measured air temperature (°C) and photon flux density (μmol m^{-2} s^{-1}) of photoysnthetically active radiation (PAR) with custom-made high-stability silicon photovoltaic detectors equipped with acrylic diffusers. On one tree per chamber we measured soil temperature and installed a custom-made dendrometer (see Fig. S1). Average air temperature varied between 24 and 26°C during the day and was *c.* 20°C at night, while the soil temperature varied between 24 and 25°C during the day and between 20 and 22°C at night (data not shown). Owing to controlled temperature, water vapor and light conditions, vapor pressure deficit and daily photon flux integral were quite constant with overall means of 3.52 kPa (± 0.39 kPa) and 4.64 mol m^{-2} d^{-1} (± 0.72 mol m^{-2} d^{-1}), respectively, throughout the experiment.

Volumetric soil water content was assessed with TDR (time domain reflectometer) sensors (CS645, 7.5 cm, three-rod probes with a TDR 100 connected to a SDMX50-series multiplexer, Campbell Scientific Inc., Logan, UT, USA) in two chambers per treatment. Volumetric soil water content at time *i* (SW_i) was then converted to relative extractable soil water at time *i* (REW_i) using the following equation:

$$REW_i = \frac{SW_i - SW_{min}}{SW_{max} - SW_{min}} \quad \text{Eqn 1}$$

where SW_{max} and SW_{min} are the volumetric soil water content before the start of the experiment and the minimum volumetric soil water content after several wk of drought, respectively. All sensor readings were recorded with a Campbell CR23X (temperature, PAR, dendrometer) or a Campbell CR10X (TDR) micrologger. Although droughted trees were given small amounts of water each week (a quarter of control trees), the relative water content of the substrate declined rapidly to a minimum in late July (Fig. 2).

Tree water status

Because of the small stature of the trees, measurements of branch hydraulic conductivity were not feasible. This would have required taking large branch sections and may have imposed an additional severe stress on the trees. We therefore collected small branch sections (branch and needles) regularly and determined their FW. We then oven-dried them at 70°C for 48 h and weighed them again. The difference between FW and DW approximates tissue water content (WC) which, when expressed as a ratio over the DW, yielded an estimate of the relative water content of tree tissues:

$$WC = \frac{FW - DW}{DW} \quad \text{Eqn 2}$$

Canopy gas exchange and root respiration

We measured [CO_2] and [H_2O] concurrently in above- and below-ground chambers with a Picarro 2131-*i* and 2101-*i*, respectively (Picarro Inc., Santa Clara, CA, USA). Within 1 h, all 12 below- and above-ground chambers were measured in 5 min

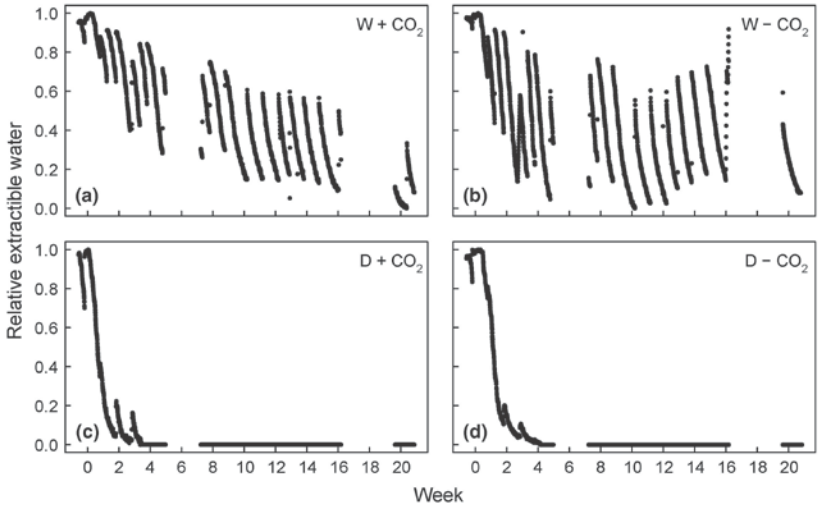

Fig. 2 Relative extractable soil water content of the four treatments: (a) watered and high [CO_2] ($W+CO_2$); (b) watered and low [CO_2] ($W-CO_2$); (c) drought and high [CO_2] ($D+CO_2$); and (d) drought and low [CO_2] ($D-CO_2$). Note that TDR (time domain reflectometer) readings were missing during early August and at the end of October because of a logger failure. Also, from the end of July onwards, the low soil moisture content produced noisy TDR estimates of soil water content in droughted trees (c, d) and these were replaced with the last reliable reading. TDR measurements were carried out in one pot per treatment and in one replicate only because of limited hardware availability.

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intervals using a custom-built valve switching device controlled by a Campbell CR1000 micrologger to rotate the sample gas stream. Within each 5 min interval, another logger-controlled manifold switched from incoming (IN) air to outgoing (OUT) air and we used a core period of 30 s of stable measurements in each 2.5 min measuring interval to compute an average. Instantaneous canopy gas exchange, root respiration and canopy transpiration of the trees were then defined as:

$$[CO_2 \text{ or } H_2O]_{trees} = [CO_2 \text{ or } H_2O]_{OUT} - [CO_2 \text{ or } H_2O]_{IN} \quad \text{Eqn 3}$$

These were considered constant for the whole 1 h cycle and were converted to hourly carbon and water flux (C_j or H_2O_j) at time j using the following equation:

$$C_j \text{or } H_2O_j(\text{g h}^{-1}) = \Delta[CO_2 \text{ or } H_2O]_{chamber}(\mu\text{mol mol}^{-1}) * \frac{VFR(\text{l min}^{-1}) * 60}{22.4(\text{l mol}^{-1})} * 10^{-6} * MW(\text{g mol}^{-1}) \quad \text{Eqn 4}$$

where $\Delta[CO_2 \text{ or } H_2O]$ was the difference in $[CO_2]$ between IN and OUT at time j for a given chamber, VFR was the volumetric flow rate of air going through the above- and below-ground chambers (25 and 5 l min^{-1}, respectively) and MW was the weighted molecular weight of carbon mol^{-1} CO_2 or the molecular weight of water (18.02 g mol^{-1}).

We computed the whole-chamber carbon balance on day i ($Ccum_i$) as the differences in carbon assimilation and respiration. To do so, we summed the carbon fluxes (above- and below-ground) at hour j on a daily basis and then accumulated these over the duration of the experiment (n d):

$$C_{cum_i} = \sum_{i=1}^{n} \sum_{j=0}^{23} {}_A C_j - {}_B C_j \quad \text{Eqn 5}$$

where the subscripts A and B in C_j denote above- and below-ground, respectively.

NSC concentrations

We analyzed glucose, fructose, sucrose and starch concentrations in leaf, branch and root tissue as the major physiologically important carbon storage compounds. Because repeatedly opening below-ground chambers during the experiment may have imposed a major disturbance to the tree root system, we sampled root tissues only before and at the end of the experiment. Branch and needle samples were collected once every second week during the first 2 months and then about every third week. We cut the samples with a sharp branch cutter, froze them immediately by immersion in liquid nitrogen and kept them on dry ice until they were placed in a freezer at −80°C for longer storage. For NSC extraction, frozen samples were vacuum freeze-dried for 72 h and milled with a ball mill (Retsch® MM200, Haan, Germany) to fine powder.

Water-soluble sugars After grinding, 50 mg of the samples were added to 1 ml of distilled water. The mixture was vortexed, incubated for 10 min at 65°C in a thermomixer and then centrifuged for 15 min at 2300 ***g***. The supernatant was removed with a pipette and stored on ice and the procedure was repeated twice. The supernatants were pooled and stored frozen at −20°C for later measurement (Raessler *et al.*, 2010).

Starch The same amount of ground sample (50 mg) was added to 0.35 ml distilled water, vortexed for 1 min and treated for 10 min in a thermomixer at 65°C. For starch hydrolysis we then added 0.5 ml of 33% perchloric acid and let it incubate in an orbital shaker for 20 min. After centrifuging at 14 300 ***g*** for 6 min, the supernatant was removed with a pipette and the procedure repeated on the remaining pellet (Raessler *et al.*, 2010). The supernatants from the two extractions were pooled and stored frozen at −20°C for later measurement.

NSC concentration measurements Sugar and starch extracts were diluted (1 : 20 and 1 : 55, respectively) before measurement with high-pressure liquid chromatography pulsed amperometric detection (HPLC-PAD) on a Dionex ICS 3000 ion chromatography system equipped with an autosampler (Raessler *et al.*, 2010). Starch concentrations were then computed as the differences in glucose concentration in the hydrolyzed extract minus the glucose and half of the sucrose concentration in the water-soluble sugar extract multiplied by a conversion factor of 0.9 (Sullivan, 1935).

Statistical analysis

We compared treatment means for each time step with an ANOVA (response ~ f(treatment)) followed by Tukey's honest significance test (Tukey's HSD, $\alpha < 0.05$) after checking the assumption of heteroscedasticity across groups with a Levene test (Morton & Forsythe, 1974). Tukey's HSD test results are only reported when ANOVA was significant and variances were homoscedastic. The temporal trend in NSC concentrations from the beginning to the end of the experiment was statistically assessed with repeated-measures ANOVA, after checking for sphericity with Mauchly's test (Mauchly, 1940). A significant ($P < 0.05$) treatment × date interaction indicated that observed differences between groups developed over the duration of the experiment. All analyses were carried out with R (v. 2.13.0, R Foundation for Statistical Computing, 2011).

Results

Stem diameter did not increase in any of the treatments over the duration of the experiment (Fig. 3). However, droughted trees showed the most pronounced shrinkage (Fig. 3c,d) and CO_2-deprived trees showed the highest variation between watering events (Fig. 3b,d). All droughted trees died within a 2 wk period, independent of CO_2 concentrations. These trees were dead after *c.* 13 wk while W − CO_2 trees died after only *c.* 20 wk and W + CO_2 trees survived until the end of the experiment (data not

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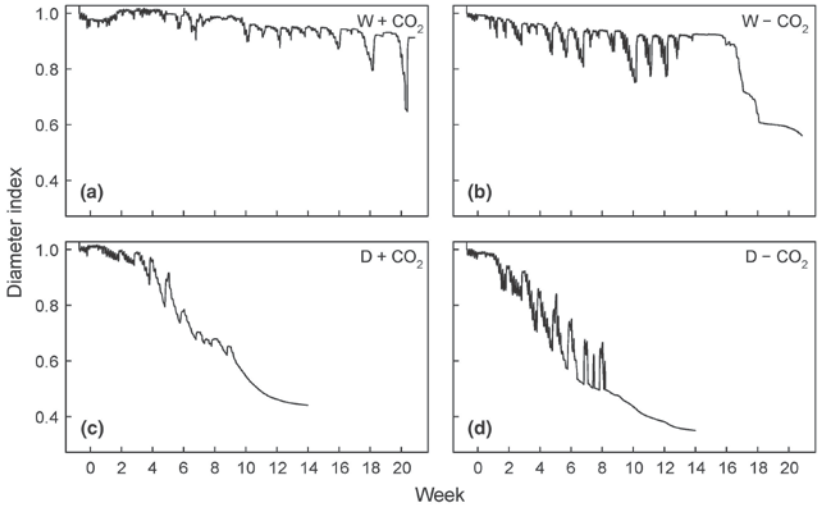

Fig. 3 Stem diameter variation of the four treatments – (a) watered and high [CO_2] ($W + CO_2$); (b) watered and low [CO_2] ($W - CO_2$); (c) drought and high [CO_2] ($D + CO_2$); and (d) drought and low [CO_2] ($D - CO_2$) – expressed as the change in diameter from the initial condition (beginning of the experiment) in young Norway spruce (*Picea abies*). Note the steep decline in $W - CO_2$ at the end of October.

shown). Relative water content was similar across treatments for a given tissue type at the beginning of the experiment (Table 1). Water content declined in all tissues of all treatments during the experiment, perhaps indicating seasonal tissue maturation. However, all tissues in droughted and CO_2-starved trees were below 10% of relative water content at death, except for root tissues in $W - CO_2$ trees (Table 1).

The carbon starvation treatment prevented carbon assimilation almost immediately (Fig. 4a). Canopy net carbon exchange approached zero in $D - CO_2$ trees after *c.* 1 wk, while $W - CO_2$ trees maintained low carbon assimilation fluxes until early September. Drought alone ($D + CO_2$) reduced carbon assimilation progressively and prevented carbon uptake from *c.* 10 wk onwards. Droughted trees started wilting during the second half of September and their canopy died within 2 wk, whether CO_2-deprived or not, while the canopy of trees in the $W - CO_2$ treatment survived much longer, almost until the end of November as indicated by maintained night-time respiration rates (Fig. 4b). Root respiration was similar among the three treatments until mid-August, and then declined rapidly in droughted trees but remained high in $W - CO_2$ (Fig. 4c). This apparent persistence of respiration in $W - CO_2$ trees towards the end of November (when foliage had wilted, needles had fallen and night-time above-ground respiration and transpiration had ceased) could reflect a shift from autotrophic (root) to heterotrophic respiration, that is, microbes feeding on senescent root matter in these wet soils. Canopy transpiration was highest in $W + CO_2$ trees and lowest in $D + CO_2$ and $D - CO_2$ trees. From mid-September onwards, both drought treatments stopped transpiring. Transpiration in $W + CO_2$ trees responded prominently to the watering events, while such a response vanished in $W - CO_2$ trees from early October onwards (Fig. 4d).

$W + CO_2$ trees assimilated on average three times, and $D + CO_2$ trees *c.* 1.6 times, more carbon than required for respiration, while $W - CO_2$ and $D - CO_2$ trees assimilated on average only 33 and 7%, respectively, of respiration demand (Fig. 5a). Water-use efficiency (WUE) was higher in $D + CO_2$ than in $W + CO_2$ trees, especially during early drought. On average, trees in high-CO_2 treatments assimilated 3.0×10^{-2} or 3.3×10^{-1} mg carbon, respectively, for each g of water transpired. This increase was mainly the result of highly varying WUE values during the period of severe water stress in August. Under low-CO_2 conditions, trees assimilated only 0.31×10^{-1} mg ($W - CO_2$) or 0.12×10^{-1} mg of carbon g^{-1} of water transpired ($D - CO_2$) (Fig. 5a). Carbon dynamics during drought showed three distinct phases: carbon surplus (assimilation/respiration (A/R) > 1) with high WUE; carbon deficiency (A/R < 1) with declining WUE; and (3) carbon limitation (A/R *c.* 1, but very low A) and increasing WUE (Fig. 5).

Table 1 Change in relative tissue water content (water content over DW, g g^{-1}) of the four treatments during the experiment in Norway spruce (*Picea abies*)

		Weeks of experiment			
Sample	Treatment	0	7	13	20
Needles	$W + CO_2$	1.89	1.96	1.41	1.04
	$W - CO_2$	1.90	2.30	1.22	0.04
	$D + CO_2$	2.04	1.88	0.02	
	$D - CO_2$	1.86	1.73	0.04	
Branches	$W + CO_2$	1.26	2.01	0.64	0.55
	$W - CO_2$	1.30	1.46	0.45	0.09
	$D + CO_2$	1.62	1.42	0.03	
	$D - CO_2$	1.04	1.56	0.04	
Roots	$W + CO_2$	3.03			0.45
	$W - CO_2$	3.21			0.49
	$D + CO_2$	2.89		0.09	
	$D - CO_2$	3.14		0.10	

$W + CO_2$, watered and high [CO_2]; $W - CO_2$, watered and low [CO_2]; $D + CO_2$, drought and high [CO_2]; $D - CO_2$, drought and low [CO_2]. Please note that root tissues were sampled only before and at the end of the experiment. Droughted trees were dead at week 13, while $W + CO_2$ trees died during week 20.

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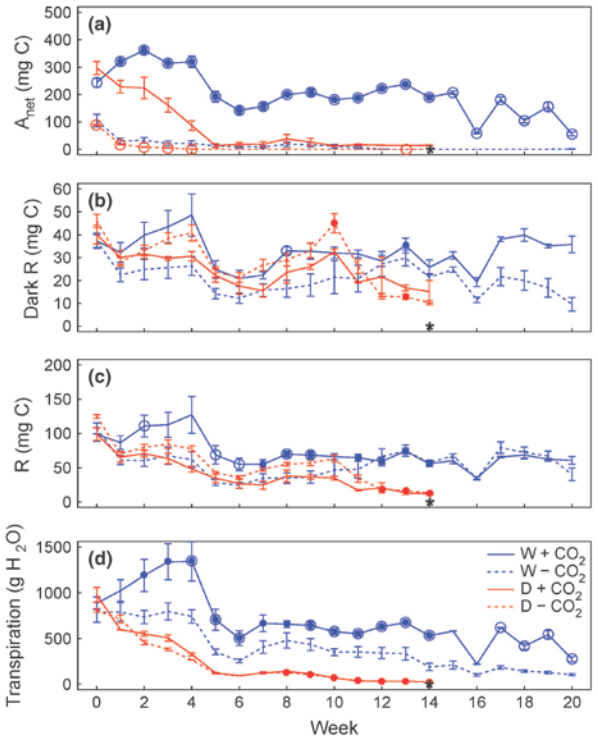

Fig. 4 Weekly averages of daily carbon assimilation, *A* (a), dark (nighttime) respiration (*R*) in leaves (b), total *R* (c) and transpiration for the four treatments in young Norway spruce (*Picea abies*): watered and high [CO_2] (W + CO_2, blue line); watered and low [CO_2] (W − CO_2, blue dashed line); (c) drought and high [CO_2] (D + CO_2, red line); and (d) drought and low [CO_2] (D − CO_2, red dashed line). Error bars are ± 1 SE. Occurrences of significant differences between treatments are indicated by symbols. Circles show significant differences ($P < 0.05$, Tukey's honest significance test (HSD) following significant ANOVA) between carbon concentrations within a given drought treatment, and dots indicate significant differences between drought intensities within a given CO_2 treatment. The asterisk marks the date when the chambers with D + CO_2 and D − CO_2 were disassembled after death of the trees.

The cumulative carbon gain of W + CO_2 continuously increased during the experiment and trees of D + CO_2 died before their cumulative carbon balance became negative, that is, they did not respire all carbon assimilated during the experiment (Fig. 6). Both W − CO_2 and D − CO_2 lost carbon from the beginning of the starvation treatment until death, but the rate of loss slowed in the last weeks before death (Fig. 6).

Drought had different effects on NSC concentrations, depending on carbon availability. In general, starch and sucrose accumulated in needles and branches of the D + CO_2 trees, while glucose and fructose concentrations decreased (Fig. 7). By contrast, droughted and starved trees (D − CO_2) showed decreases in starch and sucrose concentrations in branches but not in needles (Fig. 7). Starved trees generally had the lowest NSC concentrations and/or the steepest decline in all tissues and the depletion was stronger, almost complete, in most tissues of W − CO_2 trees. Contrary to above-ground tissues, all measured NSC compounds in roots showed a seasonal decline. However, this decline in root NSC was

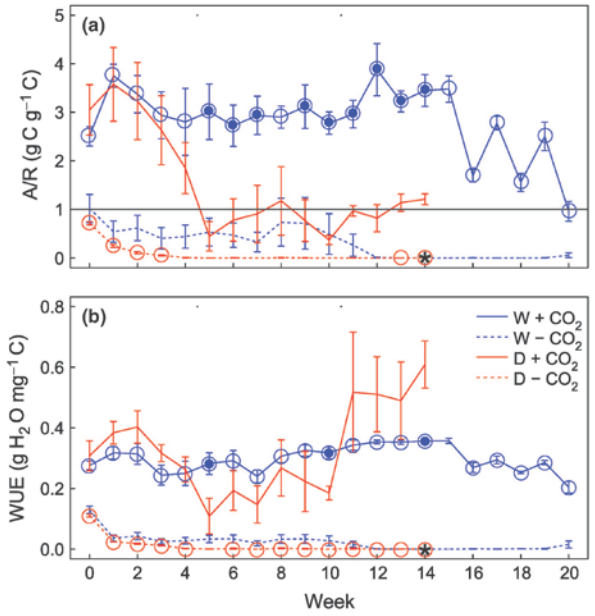

Fig. 5 (a) Ratio of weekly assimilation (*A*) over weekly respiration (*R*, ± 1 SE) (a) and daily water-use efficiency (WUE, ± 1 SE) for the four treatments in young Norway spruce (*Picea abies*): watered and high [CO_2] (W + CO_2, blue line); watered and low [CO_2] (W − CO_2, blue dashed line); (c) drought and high [CO_2] (D + CO_2, red line); and (d) drought and low [CO_2] (D − CO_2, red dashed line). The horizontal line in the upper panel indicates where assimilation equals respiration in upper panel; arrows indicate the different phases of carbon dynamics (see text). Occurrences of significant differences between treatments are indicated by symbols. Circles show significant differences ($P < 0.05$, Tukey's honest significance test (HSD) following significant ANOVA) between carbon concentrations within a given drought treatment, and dots indicate significant differences between drought intensities within a given CO_2 treatment. The asterisk marks the date when the chambers with D + CO_2 and D − CO_2 were disassembled after death of the trees.

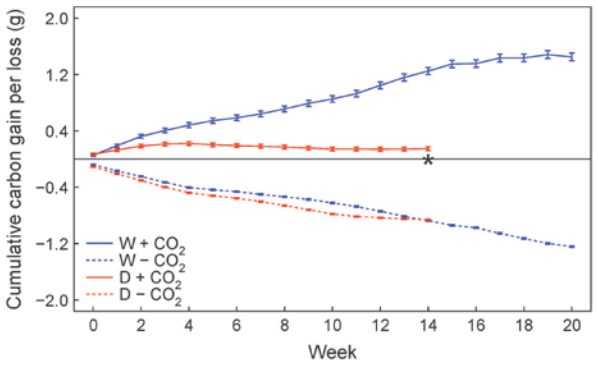

Fig. 6 Cumulative carbon gain/loss (± 1 propagated SD) for the four treatments in young Norway spruce (*Picea abies*): watered and high [CO_2] (W + CO_2, blue line); watered and low [CO_2] (W − CO_2, blue dashed line); (c) drought and high [CO_2] (D + CO_2, red line); and (d) drought and low [CO_2] (D − CO_2, red dashed line). The horizontal line indicates a zero cumulative carbon balance, that is, all carbon gained during the experiment would be consumed by respiration. Note that the low-CO_2 treatment caused a continuous negative carbon balance throughout the experiment. The asterisk marks the date when the chambers with D + CO_2 and D − CO_2 were disassembled after death of the trees.

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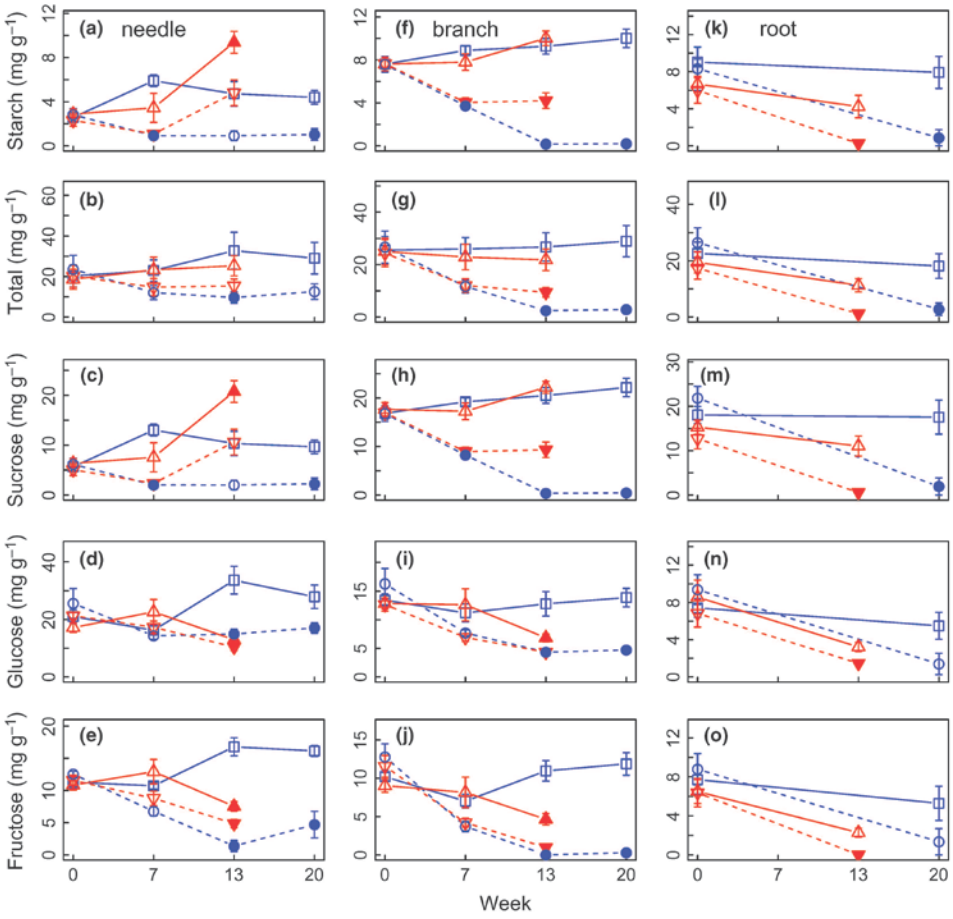

Fig. 7 Nonstructural carbohydrate (NSC) concentrations (from top to bottom, in mg g^{-1} of dry biomass ± 1 SE: starch, total mobile, sucrose, glucose, and fructose) in needles (a–e), branches (f–j) and roots (k–o) of young Norway spruce (*Picea abies*). Closed symbols indicate significant differences ($P < 0.05$, Tukey's honest significance test (HSD) following significant ANOVA) between droughted and/or starved trees (watered and low [CO_2] (W – CO_2, blue dashed line), drought and high [CO_2] (D + CO_2, red line), drought and low [CO_2] (D – CO_2, red dashed line)) from the control (watered and high [CO_2] (W + CO_2, blue line)). Closed symbols for dates with only two groups (week 20 for W + CO_2, W – CO_2 in all tissues, week 13 for D + CO_2 and D – CO_2 for roots) indicate significant differences ($P < 0.05$, Tukey's HSD following significant ANOVA) between these two groups only.

significant in all treatments but not in control trees (repeated-measures ANOVA, $P < 0.05$, data not shown) and drought combined with starvation (D – CO_2) reduced NSC more than starvation only, again contrary to above-ground tissues (Fig. 7).

Discussion

Major findings

The C-starvation treatment in our experiment forced trees into a negative carbon balance. These trees had to rely on stored carbon to survive and our results indicate that the hydration status (watered vs drought) had a strong influence on carbon storage remobilization and use, as well as on survival. Trees grown under carbon deficiency but with sufficient water supply survived longer than droughted trees. After *c.* 13 wk, droughted trees died, whereas carbon-deprived trees survived for *c.* 20 wk. NSC concentrations were generally lower in all tissues of watered, CO_2-deprived trees than in droughted trees, whether starved or not, indicating that the use of stored carbon was inhibited by drought-induced declines in hydration. Hence our study is the first to successfully demonstrate that the ability to move C reserves within the tree helps it survive persistent drought.

Carbon dynamics

We observed three distinct phases of carbon dynamics during drought (Fig. 5a): carbon surplus, carbon deficit and carbon limitation. During the first period, carbon assimilation was not yet strongly affected by drought and exceeded respiration. The second period was characterized by a tradeoff between carbon gain and water loss. Here trees required more water per unit C fixed to maintain assimilation at high rates (Fig. 5b). During the last period, respiration decreased with vanishing assimilation and was

seemingly limited by carbon supply. The apparent increase in A/R and WUE may be explained by very low carbon and water fluxes rather than by physiological recovery.

In contrast to observations in many different species and plant types (Adams *et al.*, 2009, 2013), we did not find more rapid declines in respiration than in carbon assimilation during drought. The droughted trees maintained assimilation rates at control values for *c.* 2 wk before these declined and approached zero after *c.* 4 wk. Although respiration also declined to some extent, it never declined as dramatically as assimilation rates (Fig. 4b,c). The rapid decline in respiration during early drought usually results from a turgor-mediated decrease in growth rates (Muller *et al.*, 2011), but this apparently did not occur in our trees. We started the experiment only after the longitudinal growth flush had already been accomplished and trees did not show any secondary growth (Fig. 3), probably because of the relatively low light intensities. Hence the trees in our study did not experience a sudden decline in carbon requirement from rapid turgor-mediated growth reduction, which may explain why carbon pools did not increase during early drought.

Our findings thus challenge the conceptual framework of carbon dynamics during drought, which predicts that growth respiration declines faster than assimilation, leading to an initial increase in carbon surplus and hence to an accumulation of NSC (McDowell, 2011; McDowell *et al.*, 2011). This concept is appropriate when drought stress occurs during a period of strong sink activity (e.g. early season longitudinal growth, high secondary growth rates), but may not hold as a general model for plant response to drought. The timing of drought and the phenology of the affected species can thus strongly influence carbon dynamics during drought.

NSC dynamics

Our findings underscore the importance of translocation in carbon dynamics during drought (Sala *et al.*, 2010) and emphasize that availability rather than mere carbon pool size determines the ability to draw on stored carbohydrates during drought (Sala *et al.*, 2012). Droughted starved trees were not able to redistribute available carbon from sources (above-ground tissues) to sinks (roots) in order to counteract carbohydrate depletion. Analogously, Mitchell *et al.* (2013) showed that tree species with a conservative water-use strategy, that is, species controlling water loss with early stomatal closure, survived longer and had more strongly depleted carbohydrate pools in leaves, stems and roots (only starch declined in roots) than 'water spenders', which died sooner and with less reduced NSC pools. In contrast to Mitchell *et al.* (2013), we were able to maintain plant hydration at functional levels for carbon translocation during storage dependency within the same species. Hence our results are not affected by species-specific storage use behavior (Piper, 2011) as a potential driver of the observed differences.

It is surprising that above-ground tissues of droughted trees showed increases in both sucrose and starch concentration in the absence of an apparent drought-related decline in sink strength. Drought stress is known to enhance starch degradation and increases sucrose synthesis under conditions of strong sink activity, such as grain filling in rice (Yang *et al.*, 2001). Although the continuous respiration demand of heterotrophic tissues, mainly roots, was a strong sink activity in the droughted trees, we did not observe an increase in starch degradation (i.e. lower starch concentration). Sucrose was seemingly synthesized as the main compound for long-distance carbohydrate transport (Holbrook & Zwieniecki, 2005), but declining hydration may have impeded its export to sinks and hence may have led to this accumulation. The cause of starch accumulation and degradation in perennial species like trees is still not well understood (Stitt & Zeeman, 2012), but the observed accumulation in above-ground tissues of droughted trees may be the result of declines in enzymatic activity for starch degradation (Sala *et al.*, 2010).

Even more surprising is the fact that starch and sucrose concentration also increased, after an initial decline, in needles of droughted and starved trees towards the end of the experiment (Fig. 7). These trees were not assimilating any carbon during this period (Fig. 4) and the increase in starch and sucrose may have been actively allocated. NSC accumulation during drought stress is not uncommon but may result from decreased sink activity (Muller *et al.*, 2011) and hence be a passive process. In our study, sink activity relative to carbon availability did not decrease during that period (Fig. 5b,c), making such a passive accumulation unlikely. Also surprising is the fact that starch and sucrose and not the monosaccharides accumulated in needles. Increases in glucose and fructose as means for osmoregulation (Dichio *et al.*, 2009) have been observed in leaves of *Malus domestica* (Borkh.) (Wang & Stutte, 1992) as well as in shoots and roots *of Pinus banksiana* (Lamb.) and *Picea glauca* ([Moench] Voss) (Koppenaal *et al.*, 1991). However, starch is osmotically inactive and the conversion of sucrose into glucose/fructose would be osmotically more efficient. A recent study documented that glucose and fructose concentrations increased in droughted Norway spruce saplings at the expense of sucrose and starch (Hartmann *et al.*, 2013), but others have also found NSC reductions in foliar tissues during drought (Adams *et al.*, 2013). In our study, the decreasing water content in needles and branches may explain why both sucrose and starch were not depleted. Conversion of starch to glucose or of sucrose to glucose/fructose requires the presence of water for hydrolysis during invertase and amylase catalytic reactions (Stitt & Zeeman, 2012). However, this does not explain the observed increase in both compounds during advanced drought and further research is necessary to elucidate these issues.

In contrast to above-ground tissues, the concentration of all NSC species declined in roots during drought. Similar results were shown in an earlier drought study and were explained by the uncoupling of the root system from above-ground tissues, seemingly as a result of carbon translocation failure (Hartmann *et al.*, 2013). As a consequence, roots became entirely dependent on local carbohydrates, and continued relatively good hydration in roots (compared with above-ground tissues) may have allowed them to remobilize any locally stored compounds. By contrast, in above-ground tissues exposed to dry air, tissue desiccation was probably faster than in roots, thus preventing carbohydrate mobilization and use. Our data show that water content was higher in roots than in above-ground

tissues, even at tree death (Table 1), and may have been higher all along during the experiment, thereby setting more adequate conditions for carbon mobilization and use.

Causes of tree mortality

Drought-induced tree mortality is thought to be mediated by hydraulic failure, carbon starvation or impeded carbon translocation (McDowell & Sevanto, 2010; Sala *et al.*, 2010). In this 'hydraulic framework' (McDowell *et al.*, 2008), hydraulic failure is defined as irreversible catastrophic xylem cavitation interrupting water transport to the canopy, carbon starvation as the decrease of available carbon below metabolic requirements, and impeded carbon translocation as the inability to move carbon from sources (photosynthetic tissues, storage tissues) to sinks (heterotrophic tissues). These definitions have been generally accepted by the scientific community concerned with drought-induced tree mortality, although they are not very descriptive of the mechanisms involved. For example, for the trees in the W – CO_2 treatment it was probably not irreversible catastrophic xylem cavitation but rather declining water content that impeded cellular functioning until tissue senescence and – maybe – tree death. Here we argue that more mechanistic definitions of drought effects on water and carbon relations in trees are needed.

Physiological drought can be defined as the discrepancy between water demand and supply causing hydraulic limitations to plant development and survival (Passioura, 1996). For individual plants, water deficit during drought imposes hydraulic limitations on cellular carbon metabolism (respiration and growth), carbon balance (carbon acquisition, storage and remobilization) and carbon translocation (phloem functioning). Severe hydraulic limitations may induce a failure of these processes. They are mutually dependent; for example, decreased carbon acquisition and transport will reduce carbon availability for maintaining cellular metabolism. Similarly, reduced cellular functioning will impede carbon acquisition, remobilization and translocation within the plant. Hence, this more mechanistic view precludes the view of three mutually exclusive tree mortality mechanisms, and instead emphasizes how these mechanisms are interdependent (McDowell *et al.*, 2011).

While our experiment was not designed strictly to test the validity of mortality mechanisms, our results give a detailed picture of the carbon metabolism during lethal drought and, as such, allow careful inferences to be drawn on mortality mechanisms. Given the very low water content in needle and branch tissue of droughted trees, we assume that stems and branches of these trees experienced extensive cavitation during advanced drought. The fact that droughted trees, regardless of CO_2 supply, died earlier than starved trees and that only the droughted high-CO_2 trees maintained available NSC pools at death indicates that hydraulic failure of cellular metabolism was probably the ultimate cause of mortality in droughted trees. On the other hand, both droughted and nondroughted starved trees depleted their carbon storage reserves at similar rates during the early part of the experiment (weeks 0–7), making carbon limitation the main stress factor during this period, while water limitations became important only afterwards. Carbon limitation on phloem functioning was apparently not the cause of death, because the amount of NSC varied for the different CO_2 treatments when death occurred in droughted trees. Although carbon starvation does not necessarily require carbon pools to be completely depleted (Sala *et al.*, 2012) and minimum requirements for maintenance of hydraulic and phloem functioning may not be a fixed constant (McDowell *et al.*, 2011), one would expect the same 'lethal' amounts of NSC if carbon limitation alone was the cause of mortality. Our results corroborate findings by S. Sevanto *et al.* (unpublished), who showed that both hydraulic failure and carbon starvation may act in parallel during drought-induced tree mortality and that the relative dominance of different mortality mechanisms ultimately determines the cause of death.

On the other hand, trees in the watered low-CO_2 treatment probably died of starvation. Concentrations of all NSC were very low, almost zero, in all tissues at death, apparently causing cell metabolism to collapse. This breakdown may have occurred at the end of October, as indicated by a sudden collapse of stem diameter in these trees (Fig. 3). Throughout the whole experiment, both low-CO_2 treatments showed a much greater response of stem diameter to watering events than trees in the high-CO_2 treatment (Fig. 3). Reduced phloem NSC concentration may have impeded maintenance of phloem turgor with declining hydration, causing phloem to shrink (Zweifel *et al.*, 2000; Sevanto *et al.*, 2011). In the watered low-CO_2 treatment, the depletion of NSC may have caused an osmotic collapse of phloem turgor and phloem functioning, observed elsewhere as a precursor of drought-induced tree mortality (S. Sevanto *et al.*, unpublished). The very low NSC we observed in the watered low-CO_2 treatment also indicates that minimum carbohydrate requirements for hydraulic and phloem functioning can be very small in young Norway spruce trees.

Implications for drought-induced tree mortality

This experimental study provides mechanistic insights into links between carbon and water dynamics during drought. However, these insights must be carefully extrapolated with respect to drought-induced tree mortality in natural ecosystems. For example, it has been hypothesized that isohydric species (tighter stomatal control) are potentially more vulnerable to carbon starvation than anisohydric species, especially during moderate but prolonged drought (McDowell *et al.*, 2008). Our findings support this hypothesis by demonstrating the importance of translocation for storage use. Only trees grown with adequate hydration to maintain phloem functioning were able to strongly (almost completely) reduce carbon pools in our study. Severe water limitations had a stronger and more prominent effect on tree survival than carbon supply alone.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Schematic representation of the technical setup for controlling [CO_2].

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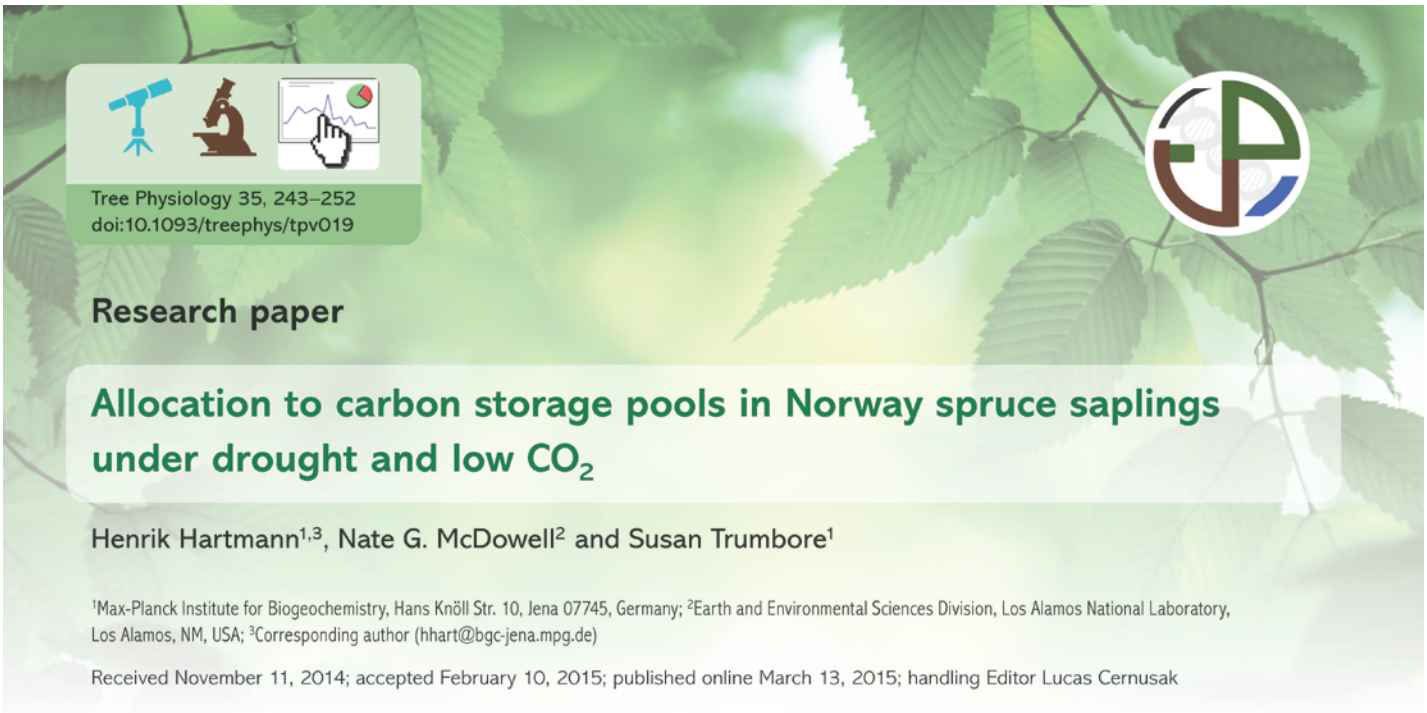
9.4. Chapter 4: Saving your last penny – carbon storage during starvation

Hartmann H., McDowell N.G. & Trumbore S. (2015) Allocation to carbon storage pools in Norway spruce saplings under drought and low CO_2. *Tree Physiology*, **35**, 243-252.



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Research paper

Allocation to carbon storage pools in Norway spruce saplings under drought and low CO_2

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Non-structural carbohydrates (NSCs) are critical to maintain plant metabolism under stressful environmental conditions, but we do not fully understand how NSC allocation and utilization from storage varies with stress. While it has become established that storage allocation is unlikely to be a mere overflow process, very little empirical evidence has been produced to support this view, at least not for trees. Here we present the results of an intensively monitored experimental manipulation of whole-tree carbon (C) balance (young *Picea abies* (L.) H Karst.) using reduced atmospheric [CO_2] and drought to reduce C sources. We measured specific C storage pools (glucose, fructose, sucrose, starch) over 21 weeks and converted concentration measurement into fluxes into and out of the storage pool. Continuous labeling (^{13}C) allowed us to track C allocation to biomass and non-structural C pools. Net C fluxes into the storage pool occurred mainly when the C balance was positive. Storage pools increased during periods of positive C gain and were reduced under negative C gain. ^{13}C data showed that C was allocated to storage pools independent of the net flux and even under severe C limitation. Allocation to below-ground tissues was strongest in control trees followed by trees experiencing drought followed by those grown under low [CO_2]. Our data suggest that NSC storage has, under the conditions of our experimental manipulation (e.g., strong progressive drought, no above-ground growth), a high allocation priority and cannot be considered an overflow process. While these results also suggest active storage allocation, definitive proof of active plant control of storage in woody plants requires studies involving molecular tools.

Keywords: carbohydrates, carbon starvation, carbon storage control, stress physiology, tree mortality.

Introduction

Trees are among the largest, longest-lived organisms on earth (Petit and Hampe 2006). Their long life span requires that trees survive stressful environmental conditions and attack by pathogens and insects, often multiple times within their lifespan (Gutschick and BassiriRad 2003). To minimize loss of biomass and to maintain life-sustaining functions, trees must store resources (Chapin et al. 1990). Despite a long history of research on how plants survive stressful conditions, fundamental processes underlying how exactly trees regulate carbon storage remain highly debated (Sala et al. 2010).

A tree's mass provides considerable capacity for storage of resources such as water, nutrients and carbon that can be drawn upon as needed to survive periods of low resource acquisition (Bloom et al. 1985, Hoch et al. 2003). Non-structural carbohydrates (NSCs) may accumulate in live tissues of stems, branches and roots during periods when carbon (C) gain exceeds use (Körner 2003) and the amount of C stored in carbohydrates may equal more than half of the requirement for annual stem growth or for several canopy refoliations (Hoch et al. 2003). Non-structural carbohydrates can be retained in mature tree tissues for decades (Carbone et al. 2013, Richardson et al. 2013) and carbon dioxide respired in tree stems may be several years to more than a decade old (Muhr et al. 2013), indicating the use of stored NSCs for respiration. It is well established that NSCs are critical to supply energy to fuel growth, respiration and other functions (see review

in McDowell et al. 2011), and their depletion results in cessation of metabolism and mortality (e.g., Marshall and Waring 1985, Marshall 1986). However, in comparison to other resources such as nutrients and water, and carbon fluxes such as photosynthesis, growth and respiration, there is relatively less understanding about storage and utilization of NSC in woody plants (Sala et al. 2010, Stitt and Zeeman 2012, Dietze et al. 2014).

Research on the mechanisms of recent drought-induced forest mortality has raised several questions about the function and regulation of carbon storage. Accounts of increased NSC content during drought suggest that drought-induced declines in sink activity (i.e., growth) can outweigh declines in carbon uptake if photosynthesis continues during drought (Muller et al. 2011). There is no common pattern in how carbohydrate levels vary during lethal drought; in numerous cases declines occurred (Galiano et al. 2011, Galvez et al. 2013, Poyatos et al. 2013), but increases in NSC concentration have also been observed prior to death (Galvez et al. 2011, Anderegg et al. 2012). Manipulations of tree C balance via defoliation substantially reduced NSC concentration in pine (Li et al. 2002) but not in poplar (Anderegg and Callaway 2012), while in oak allocation to starch was increased by defoliation and at the expense of growth (Wiley et al. 2013). The lack of a clear pattern has led to questions regarding the usefulness of NSC concentration measurements alone, i.e., without information about the fluxes, as indicators for the carbon balance (Ryan 2011). Such an approach requires going beyond measures of NSC concentration for determining C balance at the level of the whole-plant or organ/C reservoir in order to elucidate the role of C storage and its regulatory mechanisms in trees (Dietze et al. 2014). Very few studies have manipulated and assessed the whole-tree C balance so far (but see Hartmann et al. 2013*a*, Zhao et al. 2013) and we are not aware of any study that directly relates measured net C balance to NSC storage fluxes (but see Klein and Hoch 2015 for a top-down scaling approach).

Here, we present a highly controlled experimental approach in which we monitored the C balance of tree saplings with manipulations that limited carbon availability by imposing drought or lowering the atmospheric CO_2 content. We monitored the net plant C balance, as well as the amount of C in storage pools, and used a continuous $\delta^{13}C$ label to trace C flow into growth and NSC following the start of the treatment. We integrated NSC concentration measurements over whole trees and converted these into fluxes over the experimental period. Our objective was to test whether allocation to storage reserves occurred only when the net plant C balance is positive (i.e., allocation to storage is an 'overflow' process).

Materials and methods

Experimental design

The study was carried out in July through November 2012 in the greenhouse of the Max-Planck Institute for Biogeochemistry in Jena, Germany. Temperature in the greenhouse was maintained at ~22 °C during the day and ~13 °C at night, natural sunlight was augmented with greenhouse lighting (400 W Gro-Lux®, Osram Sylvania Ltd, Danvers, MA, USA) yielding an average of ~5 mol PAR m^{-2} day^{-1} (measured). Under normal daylight conditions, light flux density levels were beyond light saturation (~400 µmol PAR m^{-2} day^{-1}, measured on similar seedlings).

We placed 12 individually ventilated glass chambers (45 cm wide × 75 cm long × 80 cm high) in one of the greenhouse bays. Daily average temperatures in the glass chambers deviated from greenhouse temperatures by several degrees (usually between 21 and 24 °C during days, and 12 and –16 °C during nights) with a seasonal average of 22.5 °C (day) and 13.5 °C (night). In each glass chamber, we placed four small Norway spruce saplings (*Picea abies* (L.) H Karst., ~75 cm high) in separate 2.5 l pots. Because changes in biomass are difficult or even impossible to measure at the required resolution for the purpose of our study (i.e., partitioning C balance), we started the experiment only after longitudinal growth had ceased and lignification of newly grown branch biomass was completed. During this period all chambers were flushed with the same air and all trees were given equal amounts (200 ml) of water once per week.

Trees were grown in a 2 : 1 vermiculate-sand mixture (i.e., with no native soil organic matter) and were supplied with nutrients (Manna® Wuxal Super 8-8-6 with microelements and a slow-release conifer fertilizer Substral® Osmocote 11-8-17; Wilhelm Haug GmbH & Co. KG, Düsseldorf, Germany and Scotts Celaflor GmbH, Mainz, Germany). Chambers were subdivided into above- and below-ground chambers (flush rate 25 and 5 l min^{-1}, respectively) allowing separate measurements of root respiration and above-ground net gas exchange (see Hartmann et al. 2013*a* for more information). Flow rates were set to achieve a measurable draw-down of CO_2 in the chamber while avoiding any limitation to carboxylation and/or ^{13}C-enrichment of the chamber air by photosynthesis.

Treatments

Treatments were initiated on 1 July 2012. From this point onwards, one-third of the chambers were supplied with air of reduced [CO_2] by first removing CO_2 using a molecular sieve (Schnyder 1992, Gamnitzer et al. 2009). Depending on the specified treatment, CO_2 from a pressurized tank was then added to the CO_2-free air at concentrations of either 350 or 40 ppm, depending on treatment. The high concentration was meant to simulate current ambient [CO_2], i.e., concentrations >350 ppm, but these levels could not be achieved due to the limited capacity of our mass flow controller. The lower concentration (40 ppm) was established in a pre-experiment as a treatment causing a permanent negative daily carbon balance. The concentration was continuously assessed and re-adjusted with a computer-controlled mass flow controller (see Figure S1 available as Supplementary Data at *Tree Physiology* Online). In the pre-treatment

period, trees were grown in ambient air with a $\delta^{13}C$ of approximately −9‰ (on VPD scale), during the experiment the CO_2 supplied to the CO_2-free air had a $\delta^{13}C$ of approximately −42‰.

Normally irrigated trees were given 200 ml of water once a week, and trees in the drought treatment were given only 50 ml at the same time. We therefore had three treatments: (i) 200 ml water per week and 350 ppm [CO_2] (Ambient-C), (ii) 200 ml water per week and 40 ppm [CO_2] (Low-C) and (iii) 50 ml water per week and 350 ppm [CO_2] (Drought). There were three glass chambers per treatment. Trees in the drought treatment died after ~14 weeks, while trees in the Low-CO_2 treatment survived until Week 21, at which time Ambient-C trees were still alive and healthy-looking. Tree death was determined by complete foliage browning, near-zero respiration and cambial necrosis in branches and stems. Trees in the drought treatment showed very low relative tissue water content at the end of the experiment (Table S1 available as Supplementary Data at *Tree Physiology* Online). We concluded the experiment after trees in the Low-C treatment had died.

Measurements of carbon fluxes

Weekly net carbon gain We measured above-ground net carbon exchange and root respiration as the difference between [CO_2] of air entering and air leaving the chambers. To do so, the in- and outlet air stream of each chamber was sampled for 2.5 min each with a Picarro® 2131-*i* (above-ground) and 2101-*i* (below-ground) before switching to the next chamber. The rotation between chambers was achieved with a logger-controlled valve switching unit (Campbell Scientific® CR 1000 micrologger, Campbell Scientific Inc., Logan, UT, USA, see Figure S1 available as Supplementary Data at *Tree Physiology* Online), completing a whole cycle within 1 h.

Each 5-min measurement cycle was converted to hourly carbon flux (C) at time j using the following equation:

$$C_j(\text{g h}^{-1}) = \Delta[CO_2]_j(\mu\text{mol mol}^{-1}) \times \frac{\text{VFR (l min}^{-1}) \times 60\text{ (min)}}{22.4\text{ (l mol}^{-1})} \times 10^{-6} \times \text{MW (g mol}^{-1}) \quad (1)$$

where $\Delta[CO_2]_j$ is the difference in [CO_2] between inlet and outlet air stream at time j for a given chamber, VFR the normalized volumetric flow rate of air going through the above- and below-ground chamber (25 and 5 l min^{-1}, respectively) and MW the molecular weight of carbon per mole of CO_2.

We computed the whole-chamber (above- and below-ground) net carbon gain at week i (mg C per week, NCG_i) as the differences in carbon assimilation and respiration. To do so, we summed hourly carbon fluxes (above- and below-ground) on a daily basis and over week i (Day 1–7).

$$NCG_i = \sum_{d=1}^{7}\sum_{j=0}^{23} {}_A C_{ij} - \sum_{j=0}^{23} {}_B C_{ij} \quad (2)$$

where the subscript A and B in C_{ij} denotes above- and below-ground, respectively.

NSC measurements Carbon storage was estimated by assessing tissue-specific concentrations of NSC and by upscaling to total NSC content with biomass measurements. We measured soluble sugars (SS), glucose (Glu), fructose (Fru) and sucrose (Suc) as the main mobile compounds and starch (Star) as the main non-mobile NSC compound in needles, branches and roots. Because repeatedly opening the below-ground chambers may have severely disturbed the tree root systems we only collected root samples when trees were placed in the chambers and at the end of the experiment. Branch and needles were sampled on average every 2.5 weeks using a sharp branch cutter. Samples were frozen immediately by immersion in liquid nitrogen and then placed in a −80 °C freezer for longer storage.

For NSC extraction, frozen samples were vacuum freeze-dried for 72 h and milled with a ball mill (Retsch® MM200, Haan, Germany) to a fine, consistent powder. To extract Glu, Fru and Suc we added 50 mg of ground sample to 1-ml distilled water. The mixture was vortexed, incubated for 10 min at 65 °C in a thermomixer (1050 rpm) and then centrifuged for 15 min at 2300*g*. The supernatant was removed with a pipette, stored on ice and the procedure was repeated twice. The supernatants were pooled and stored frozen at −20 °C for later measurement. For starch analysis, 50 mg of ground sample was added to 0.35-ml distilled water, vortexed for 1 min and treated for 10 min in a thermomixer at 65 °C (1050 rpm). For starch hydrolysis we then added 0.5 ml of 33% perchloric acid and let it incubate in an orbital shaker for 20 min. After centrifuging at 14,300*g* for 6 min, the supernatant was removed with a pipette and the procedure repeated on the remaining pellet. The supernatants from the two extractions were pooled and stored frozen at −20 °C for later measurement.

Sugar and starch extracts were diluted (1 : 20 and 1 : 55, respectively) before measurement with high-pressure liquid chromatography pulsed amperometric detection (HPLC–PAD) on a Dionex® ICS 3000 ion chromatography system equipped with an autosampler (Raessler et al. 2010). Starch concentrations were then computed as the differences in Glu concentration in the hydrolyzed extract minus the Glu and half of the Suc concentration in the water-SS extract multiplied by a conversion factor of 0.9 (Sullivan 1935).

Biomass measurement and whole-tree carbon storage flux estimation At the end of the experiment, we harvested and dried all the trees and measured dry biomass of each tissue type (needles, branches and stems, roots). Biomass samples taken for NSC measurements were also dried and weighed and these were added to the final biomass estimates.

Tissue-specific NSC (NCC_i) was estimated by multiplying tissue-specific NSC concentration at period i with tissue mass at period i (accounting for biomass reduction from sampling) and

by multiplying the tissue-specific NSC content by the mass proportion of carbon in NSC (0.4 for SS, 0.42 for Star). We obtained whole-tree NCC by summing over all tissues. Because of the small stature of the studied trees, stems and branches were quite similar in size and we treated them as one category.

Fluxes to and from storage (S_i) were estimated with the following equation:

$$S_i = NCC_i - NCC_{i+1} \quad (3)$$

where NCC_i is the non-structural carbon content at period *i*. Hence, storage fluxes were the change in NCC from one period to the following. Please note that this definition of storage also includes transitory pools (i.e., SS) that are translocated prior to utilization. However, such pools can be depleted during stressful periods (Hartmann et al. 2013*a*) and hence may provide similar functionality as immobile compounds like starch. Potential changes in biomass (e.g., fine root turnover, allocation to secondary metabolites, lignin deposition) were not assessed in our study. Because above-ground longitudinal growth had ceased before the start of the experiment and because secondary growth did not occur (Hartmann et al. 2013*a*), potential changes in biomass can be considered minor. However, as we were unable to account for changes in root biomass during the experiment period, we estimated allocation to a 'residual pool' (B_i, comprising mainly root biomass increases) by assuming mass balance (sensu, McDowell 2011):

$$B_i = NCG_i - S_i \quad (4)$$

Error propagation Error of measurements were propagated throughout the experimental period for computed (e.g., storage flux) or cumulated variables using standard error propagation rules.

Allocation to storage and biomass using $\delta^{13}C$ as indicator

During the experiment trees were assimilating CO_2 with a very different isotopic composition (−42‰) compared with the ambient air of the pre-experimental period (−9‰). Knowing the two different sources, one can compute the fractions of each source in plant carbon pools (i.e., mobile sugars and starch, biomass) using a mixing model (Dawson et al. 2002). This approach requires either assuming constant fractionation factors across treatments (not realistic in our experiment) or measuring isotopic discrimination during the experiment (difficult to measure in situ and online). We therefore use the $\Delta\delta$ notation as an indicator for such proportions, where

$$\Delta\delta^{13}C_i = \delta^{13}C_i - \delta^{13}C_{start} \quad (5)$$

where $\Delta\delta^{13}C_i$ is the difference in $\delta^{13}C$ of a particular pool (e.g., NSC, biomass) at period *i* minus $\delta^{13}C$ at the beginning of the experiment ($\delta^{13}C_{start}$). Since the CO_2 source used during the experiment had a much lower $\delta^{13}C$ than the ambient air trees were growing in prior to the experiment, negative values indicated the strength of incorporation of 'newly assimilated' (i.e., during the experiment) carbon into the pool. Decreases in fractionation during drought may cause an upward shift in $\delta^{13}C$ of leaf metabolites (approximately +6‰, Duranceau et al. 1999, Ghashghaie et al. 2001) but such a shift would not offset the approximately −33‰ shift in the source signal. We measured $\delta^{13}C$ of the SS pool as an indicator for allocation to NSC and of the remaining pellet (which includes also starch and lipids but mainly, i.e., >95%, structural biomass, data not shown) as an indicator for allocation to structural biomass.

Results

Net carbon assimilation

Trees growing at 350 ppm [CO_2] assimilated more carbon than they respired throughout most of the entire experimental period (Figure 1). The seasonal decline in assimilation may be attributed to decreasing daytime length from July until November that was not compensated by the artificial greenhouse lighting. Droughted trees showed a sharp decline in net carbon gain during the first 4 weeks and a net carbon loss from Week 5 onwards. Trees in the Low-C treatment showed no positive net carbon gain at all during the experiment (Figure 1).

Carbohydrate pool measurements

Generally, concentrations of starch were substantially lower than of SS in all tissues and treatments. NSC pools (both starch and SS) increased in needles of Ambient-C trees during the experiment. This trend was similar in Drought trees except for a decline prior to their death (Figure 2). Branch NSC showed a similar

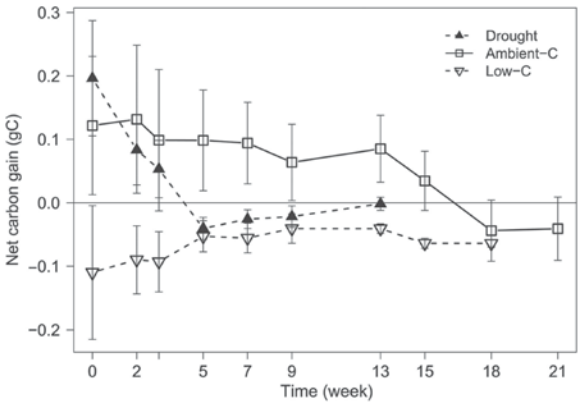

Figure 1. Net weekly carbon gain (g C, ±1 SE) during the experimental period. Droughted trees (filled triangles) died during Week 14, trees in the Low-C (inverted triangles) treatment during Week 20 while trees in Ambient-C (squares) survived the experiment. Data are shown only for weeks for which NSC measurements were taken.

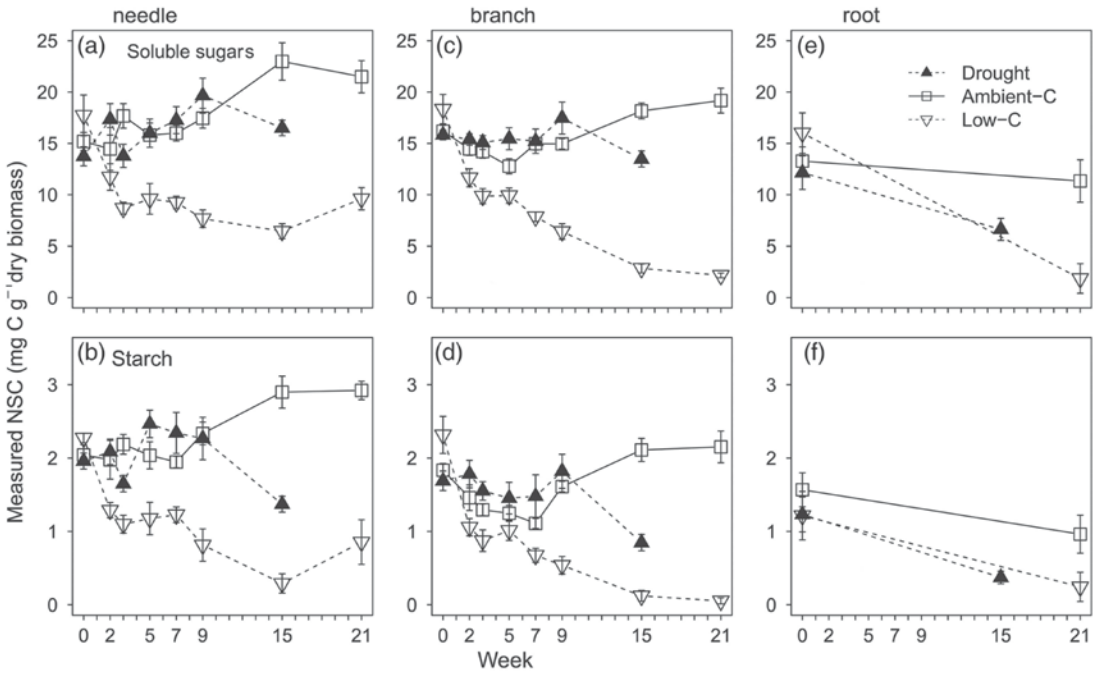

Figure 2. Weekly concentrations (mg C g^{-1} dry biomass, ±1 SE) of SS (a, c and e) and starch (b, d and f) in needles (a and b), branches (c and d) and roots (e and f) (Drought: filled triangles, Ambient-C: squares, Low-C: inverted triangles). Note that the last measurements in Drought and Low-C trees were at final harvest after their death (Weeks 12 and 20, respectively).

Table 1. Averages (g) and standard deviation (SD) of tissue and total dry biomass of sample trees as well as B_{cum} (estimated residual biomass cumulated over experimental period) and the percentage proportion of B_{cum} over total biomass.

	Needle		Branch		Root		Total		B_{cum}		B_{cum}/total
Treatment	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	(%)
Ambient-C	31.21	2.35	46.41	5.83	30.17	1.80	107.79	6.54	0.38	0.49	0.35
Low-C	32.37	4.28	46.27	6.67	21.03	2.91	99.67	8.44	1.00	0.27	1.00
Drought	37.34	5.50	51.89	5.48	30.33	7.92	119.56	11.09	0.52	0.42	0.43

seasonal trend to needle NSC but both SS and starch declined almost to zero in Low-C trees. Root NSC declined in all treatments, but most severely in Low-C trees (Figure 2).

Biomass and residual of mass balance

There were no significant differences in tissue or in total biomass of sample trees across treatments. The within-treatment variability was greatest in needle, root and total biomass of Drought and Low-C trees (Table 1).

As noted previously, while we assume there is no biomass change above-ground, we cannot assess below-ground change; hence we have estimated the residual of the mass balance as the difference between measured plant C mass balance (from gas exchange) and the changes in C reserves. Due to multiple error propagation these estimates are highly uncertain and show very small positive values, ranging from 0.38 g (±0.49 g, Ambient-C) to 1.00 g of carbon (±0.27 g, Low-C). Weighted on biomass, this residual growth makes up between 0.35% (Ambient-C) and 1.00% (Low-C) of the total biomass (Table 1).

Changes in carbon storage

Overall changes in whole-tree SS and starch were small compared with variability between trees within a given treatment. For Drought and Ambient-C treatments, storage pools remained essentially constant during the first few weeks of treatment, and the only period with substantial storage/remobilization occurred during Week 9. Trees in the Low-C treatment were losing carbon from SS and starch throughout the experiment (Figure 3).

Allocation to storage and biomass

Trees at Ambient-C allocated more of the C fixed after the start of the experiment to storage compounds and to biomass than trees in the other treatments (Figure 4). Relative allocation (per unit of biomass) of newly assimilated carbon was greater in roots

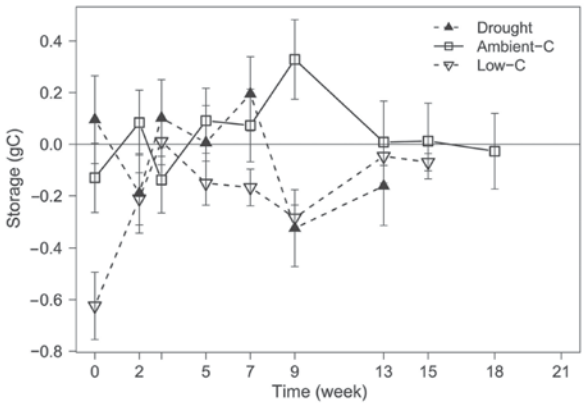

Figure 3. Net weekly storage fluxes (g C, ±1 SE) during the experimental period (Drought: filled triangles, Ambient-C: squares, Low-C: inverted triangles). Positive values indicate allocation to storage, negative values storage mobilization. Data are shown only for weeks before final harvest.

than in above-ground tissues in all treatments. Positive $\Delta\delta^{13}C$ values in branch NSC of Drought and Low-C trees indicate mobilization of stored, heavier compounds like starch. There was no substantial carbon allocation to above-ground storage or biomass in Drought and Low-C trees, while $\Delta\delta^{13}C$ of root NSC and biomass in Drought trees show indication for allocation of newly assimilated carbon. A small fraction of newly assimilated carbon was incorporated into root biomass in Low-C trees (Figure 4).

During the experimental period, the strong declines in storage pools in the Low-C treatment were greater than net C losses measured from gas exchange and hence the residual pool in this treatment was greatest across treatments (Figure 5).

Discussion

Plant allocation to storage remains a debated issue (Sala et al. 2012) and is plausibly controlled by active regulation within

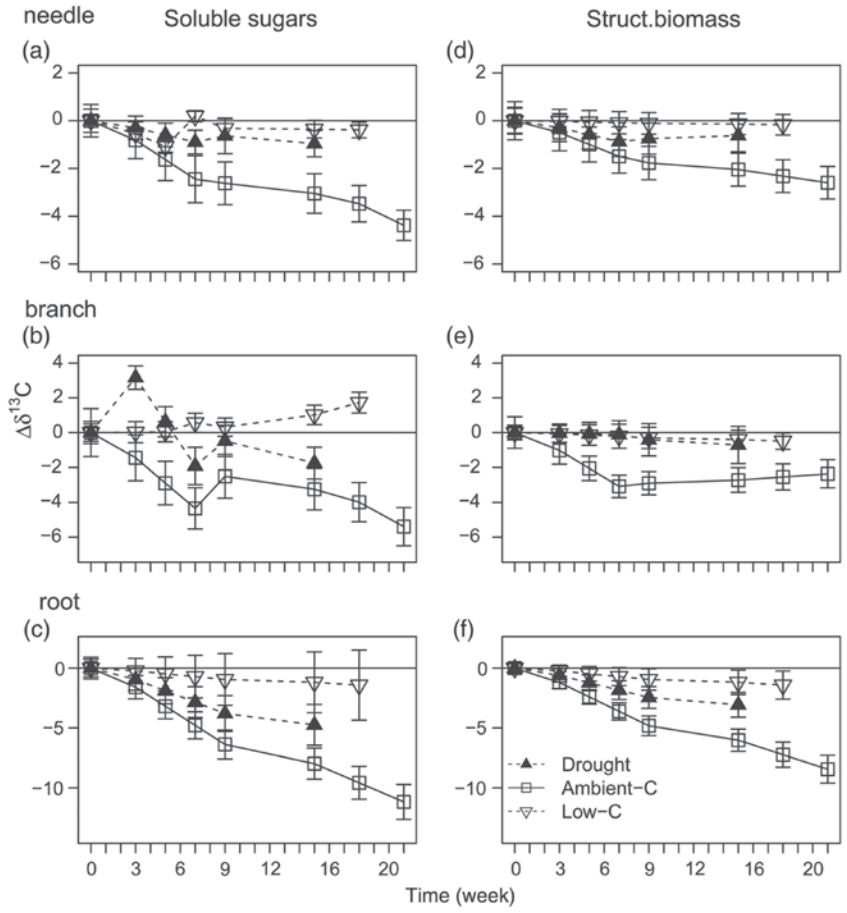

Figure 4. Weekly $\Delta\delta^{13}C$ (‰, ±1 SE) of SS (a, b and c) and in the remaining pellet following the extraction (structural biomass + starch, d, e and f) in needles (a and d), branches (b and e) and roots (c and f) (Drought: filled triangles, Ambient-C: squares, Low-C: inverted triangles). Negative values indicate incorporation of carbon assimilated during the experimental period in a given pool, positive values can indicate allocation of previously stored compounds.

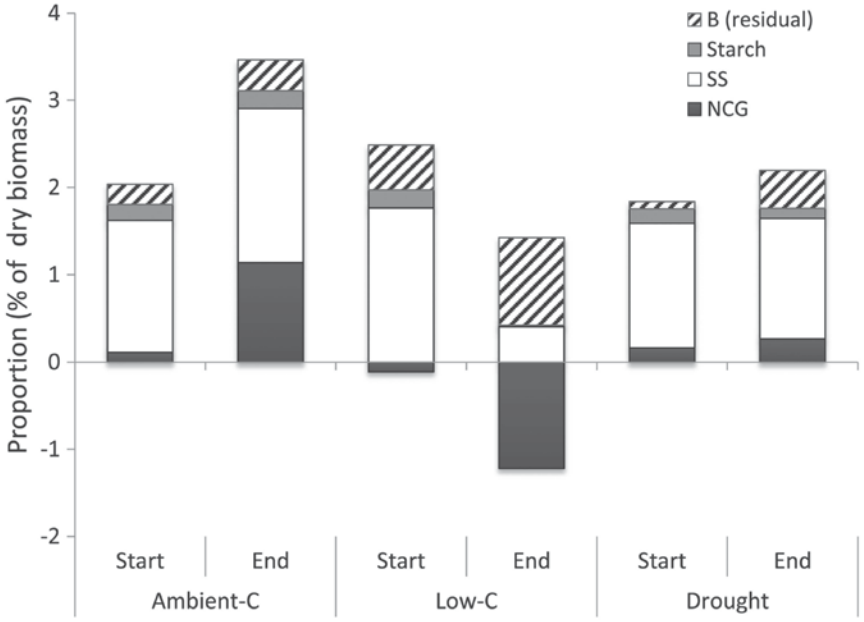

Figure 5. Summary figure showing components of the carbon balance (as % of dry biomass) between beginning (first week of experiment, Start) and end (End) of the experiment. Net C gain (NCG, Eq. (2)) and residual (*B*, Eq. (4)) were cumulated throughout the experiment period.

biophysical constraints (Dietze et al. 2014). By combining measures of whole-plant mass balance with measurements of carbon storage pools (SS and starch) at the whole-plant and organ level and use of a stable carbon isotope label to identify the fate of pre- versus post-treatment C, our experiment allows us to draw inferences on storage allocation of newly assimilated carbon. While we are aware that measurements of carbohydrates are highly uncertain (A.G. Quentin et al., submitted) likely making our pool estimates and computed fluxes inaccurate (i.e., show true values), we are confident that our methods are precise enough to yield reliable qualitative estimates of flux direction, i.e., storage allocation or mobilization.

Continuous losses in SS and starch pools in the Low-C treatment indicated a net depletion of storage pools. While the stable carbon isotope data corroborate storage depletion in above-ground tissues, root $\Delta\delta^{13}C$ data suggest that both the root SS pool and the root starch/biomass contained small proportions of newly assimilated carbon. Given the very low carbon availability and the consistently negative carbon balance in the Low-C treatment, these trees had little carbon to allocate to storage (but apparently did) and rather remobilized existing pools for survival. The observed allocation to storage under these conditions indicates a high storage allocation priority consistent with active storage regulation. Similar to our findings, manipulations of carbon availability via defoliation showed a relative increase in storage pools (compared with growth) in half-defoliated trees, suggesting that storage allocation was independent of carbon availability and potentially under active control (Wiley et al. 2013).

Droughted trees showed a substantial net increase in the storage pool at the whole-plant level after entering negative net carbon balance between Weeks 5 and 7, followed by rather strong storage remobilization (Figure 3). As in the Low-C treatment, $\Delta\delta^{13}C$ changes support a continuous incorporation of newly assimilated carbon into SS and starch/biomass, at least in below-ground tissues. This is particularly interesting because previous investigations on changes in carbohydrate concentrations in the same species showed that reduced hydration may prevent translocation of above-ground carbon storage to the root system and cause a decoupling of above- and below-ground tissues (Hartmann et al. 2013*a*). The isotopic data here suggest that newly assimilated carbon was transported into the root system even during later phases of the drought treatment and hence do not corroborate reduced translocation. Drought causes a strong increase in $\delta^{13}C$ of SS in above-ground tissues (Hartmann et al. 2013*b*) and such an increase may have partially offset the observed $\Delta\delta^{13}C$ signal in needle and branch storage pools of droughted trees. Positive $\Delta\delta^{13}C$ in SS of branches in the early phase of the experiment corroborate this idea and, if so, droughted trees may have also been allocating carbon to storage pools in above-ground tissues. Klein et al. (2014) showed that drought-stressed trees maintained storage pool size by dramatically decreasing growth rates and interpreted the decrease in growth as a regulatory mechanism to maintain a positive C balance (Klein et al. 2014). A similar mechanism may have acted in our trees, at least in below-ground tissues.

Trees in the Ambient-C treatment maintained a net positive C balance through most of the experimental period. Newly assimilated C was incorporated into SS and starch/biomass throughout the experiment, with most pronounced incorporation of new C into the root system, even though net fluxes into storage were not always positive. Declines of $\Delta\delta^{13}C$ during these periods could also occur if C from pre-treatment storage reserves were preferentially used for C sinks (like respiration) that removed 'old', i.e., heavier, C from the plant system.

Our mass balance approach relies on the assumption that all components are accurately assessed. While measurements of $[CO_2]$ with sophisticated technology and verified against calibration gases of known concentration can be considered accurate, carbohydrate concentrations cannot be accurately assessed due to a lack of standards (A.G. Quentin et al., submitted). This implies that the absolute concentrations presented here (Figure 2) are potentially wrong. However, a large international study of carbohydrate assessments across 31 laboratories (including our own) also revealed a high intra-laboratory precision (repeatability) (A.G. Quentin et al., submitted). This means that our differential measurements (between periods) and resulting flux estimations are valid and accurate and hence the partitioning of available carbon into different pools based on mass balance (i.e., residual pool) is also accurate. Allocation to this residual pool is greatest in the Low-C and Drought treatment (strongest change Pre vs Post in Figure 5) suggesting either below-ground biomass production or stress-induced synthesis of other compounds. Drought and shading studies have shown increases of several amino acids (Cyr et al. 1990, Vance and Zaerr 1990, Busing and Mailly 2004, Ditmarová et al. 2010) that may alleviate physiological stress during drought (Ashraf and Foolad 2007) or act as nitrogen storage compounds during C limitation (Miflin and Lea 1977, Llácer et al. 2008). Future research on storage processes should include assessments on such secondary metabolites but also other storage compounds like lipids as potential competing sinks in the C balance. In our study, lipid synthesis was unlikely to be the sink accounting for the residual pool (see Table S2 available as Supplementary Data at *Tree Physiology* Online) but we have no data allowing us to exclude allocation to amino acids.

While it could be argued that our $\Delta\delta^{13}C$ data merely indicate mixing of newly assimilated C into a transient carbohydrate pool (Keel et al. 2007) and not allocation to a storage pool, we agree that this may be the case for SS but not for starch/biomass, which must be synthesized. Starch synthesis can be triggered by high carbohydrate supply to reduce osmolyte accumulation (Koch 1996) and could be considered a protective mechanism rather than storage allocation under high carbohydrate availability. Given the very low SS concentrations in tissues of Low-C trees such a process would be very unlikely and the incorporation of newly assimilated carbon into root starch/biomass appears to be active allocation.

The difference between active and passive allocation to storage is likely anchored in the regulation of growth and storage: (i) passive allocation to storage may occur when plants up-regulate growth but carbon supply is sufficiently large to allow storage allocation (concurrent flux to growth and to storage), (ii) 'quasi-active' allocation to storage may be achieved via down-regulation of growth, which allows diversion of carbon to storage (flux to storage under reduced flux to growth) and (iii) active allocation would occur via direct up-regulation of allocation to storage independent of growth (Dietze et al. 2014). Trees in our study allocated newly assimilated C into storage pools even during periods when they experienced a negative net C balance. Such a behavior would be very unlikely if storage was a mere overflow process and we have good reason to refute passive storage as a sole acting mechanism. Our data do not allow us to distinguish whether the observed allocation to storage was directly up-regulated or via indirectly down-regulation of growth (Wiley and Helliker 2012), especially since we deliberately avoided the period of strong above-ground growth during the experiment. Further advances in this domain clearly depend on the application of genetic and biochemical tools in investigations on trees, similar to what has been carried out in studies on *Arabidopsis* (e.g., Smith and Stitt 2007, Stitt and Zeeman 2012). Such investigations have become feasible by recent advances in genome sequencing of common tree species like eucalypts, spruce or cottonwood (Hogberg et al. 2001, Tuskan et al. 2006, Myburg et al. 2011) and can identify whether direct or indirect up-regulation of storage occurs even under C limitation. To better define C limitation, a more complete assessment of the competing C pools is required, including structural biomass changes, secondary metabolites, alternative storage compounds like lipids and proteins, defense compounds, volatile organic substances and also C sinks like root exudates and biotic interactions (herbivory, symbiotic exchanges). Maybe most important, further research is required to develop more accurate assessments of carbohydrates, the most abundant storage compound family, in plant tissues (A.G. Quentin et al., submitted).

Supplementary data

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

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Conflict of interest

None declared.

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9.5. Chapter 5: You can't starve while being thirsty – part II

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High temperature causes negative whole-plant carbon balance under mild drought

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Summary

- Theoretically, progressive drought can force trees into negative carbon (C) balance by reducing stomatal conductance to prevent water loss, which also decreases C assimilation. At higher temperatures, negative C balance should be initiated at higher soil moisture because of increased respiratory demand and earlier stomatal closure. Few data are available on how these theoretical relationships integrate over the whole plant.
- We exposed *Thuja occidentalis* to progressive drought under three temperature conditions (15, 25, and 35°C), and measured C and water fluxes using a whole-tree chamber design.
- High transpiration rates at higher temperatures led to a rapid decline in soil moisture. During the progressive drought, soil moisture-driven changes in photosynthesis had a greater impact on the whole-plant C balance than respiration. The soil moisture content at which whole-plant C balance became negative increased with temperature, mainly as a result of higher respiration rates and an earlier onset of stomatal closure under a warmer condition.
- Our results suggest that the effect of drought on whole-plant C balance is highly temperature-dependent. High temperature causes a negative C balance even under mild drought and may increase the risk of C starvation.

Introduction

Forests represent large global stores of carbon (C) and account for the largest proportion of annual C exchange with the atmosphere (Phillips *et al.*, 1998; Malhi *et al.*, 1999; Le Quere *et al.*, 2008; Pan *et al.*, 2011; Ballantyne *et al.*, 2012). The C balance of forest ecosystems is sensitive to climate change, which is influenced, for example, by increased growing season duration (Walther *et al.*, 2002), changes in tree growth rates (Clark *et al.*, 2003; Cole *et al.*, 2009) and increased plant respiration and photosynthesis (Piao *et al.*, 2008). There are different means of evaluating the C budget of an ecosystem, such as the widely used eddy covariance technique (Baldocchi, 2003). However, forest ecosystems are made up of individual trees that can respond differently to environmental conditions (e.g. isohydric and anisohydric species; see Tardieu & Simonneau, 1998). Hence, explaining changes in net forest C gain or loss depends on improved understanding of C fluxes at the level of individual trees.

The C balance of an individual plant is the difference between photosynthesis (C input) and respiration (C output). The sum of daytime CO_2 uptake from photosynthesis minus the sum of 24 h CO_2 release through respiration, referred to as the whole-plant daily C balance, is a meaningful indicator of C accumulation within plants (McCree, 1986). Under favorable conditions, a plant takes up more C than it consumes on a daily basis (C surplus) and the excess C is used for growth and synthesis of defense compounds or is stored in the form of nonstructural carbohydrates for future use, resulting in biomass increase (McCree, 1986; Estiarte & Peñuelas, 1999).

Environmental stresses can alter both photosynthetic and respiratory rates and therefore affect the plant C budget in the short term. The point of zero net C assimilation for the whole plant (i.e. when photosynthetic C uptake equals respiratory C loss on a 24 h basis) is a useful concept to investigate the whole-plant C balance under environmental stresses, and is defined here as the whole-plant C compensation point (CCP).

Under progressive drought, plant growth can be reduced at first (McDowell, 2011) and, as water stress progresses, photosynthesis will be curtailed when stomatal conductance is reduced to prevent water loss through transpiration (Bates & Hall, 1982; Schulze, 1986; McDowell *et al.*, 2008a). However, maintenance respiration may not decline as fast as photosynthesis and the resulting C deficit forces plants to consume stored carbohydrates for respiration (McDowell, 2011). If this C deficit persists, plants will suffer from C starvation as carbohydrates are depleted (Sayer & Haywood, 2006; McDowell, 2011).

At elevated temperatures, enhanced transpiration can hasten the closure of stomata under water stress to prevent water loss (Schulze *et al.*, 1973), simultaneously advancing the decline of photosynthesis. In addition, plants usually present higher maintenance respiration at higher temperatures (Atkin *et al.*, 2000; Atkin & Tjoelker, 2003), which, combined with reductions in photosynthesis, could lead to a rapid depletion of C storage pools (McDowell, 2011). Some authors suggest that, under elevated

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temperature and drought, plants could be threatened by, or even die from, C starvation rather than hydraulic failure (McDowell *et al.*, 2008a; Sala *et al.*, 2010; Hartmann, 2011; McDowell, 2011). In support of this hypothesis, Adams *et al.* (2009) provided evidence that under drought stress, leaf-level respiratory C consumption by pinyon pines was significantly higher at elevated temperature (+ 4°C) than under ambient temperature. However, difficulties of scaling leaf-level observations of gas exchange to the whole plant did not allow quantification of the net C balance of these trees, owing to the mismatch between leaf-level CO_2 exchange measurements and actual biomass responses (Evans & Dunstone, 1970; Wardlaw, 1990). Responses of plants to drought and high temperature have been mostly studied at the tissue level, while changes in whole-plant C balance have rarely been studied (but for studies in some crops, see McCree, 1986; Miller *et al.*, 2001). To our knowledge, the combined effects of elevated temperature and drought on the whole-plant CCP have not been assessed.

In this study, we carried out a water stress experiment on *Thuja occidentalis* L. at three different temperatures (15, 25 and 35°C), and focused on tracking variations of the components (i.e. photosynthesis and respiration) of whole-plant daily C balance as soil moisture declined. *T. occidentalis* is a drought-tolerant evergreen coniferous species in the Cupressaceae family (Collier & Boyer, 1989). It grows in a wide range of moisture conditions, from swamps to cliff edges, making it an interesting choice for drought studies (Beals, 1965; Collier & Boyer, 1989; Kelly & Larson, 2003; Harlow *et al.*, 2005). The average temperature of its natural habitat usually ranges from −12 to −4°C in winter and from 16 to 25°C in summer (Fowells, 1965). Our hypothesis was that trees would be forced into a negative C balance at higher soil moisture content when grown under high temperature (> 30°C) because of higher respiratory demand combined with reduced photosynthesis driven by earlier stomatal closure. At lower temperatures, trees should survive longer and reach the CCP at lower soil moisture contents. The results improve our understanding of the effects of environmental drivers on the whole-plant C balance, with implications for the mechanisms underlying drought-induced tree mortality.

Materials and Methods

Materials

Four-year-old trees of *T. occidentalis* L. (half-siblings) purchased from a regional nursery were transferred to plastic pots (14.5 cm diameter × 16 cm high) and kept well watered outdoors for *c.* 10 months. The trees were planted in a C-free mixture of vermiculate and sand (volumetric ratio 2 : 1) without any added organic matter. This ensured that CO_2 released from the soil was solely derived from roots in our short-term experiment. Nutrients were supplied with a single application of instant fertilizer (Manna Wuxal Super 8-8-6 with microelements; Wilhelm Haug GmbH & Co. KG, Düsseldorf, Germany), combined with a slow-release conifer fertilizer (Substral Osmocote 11-8-17; Scotts Celaflor GmbH, Mainz, Germany). In July, 2012, nine healthy individuals (height from stem base = 58 ± 3 cm, diameter at stem base = 1.0 ± 0.2 cm) were randomly chosen and transferred into airtight cylindrical transparent chambers (17 cm diameter × 80 cm high, made of methyl methacrylate resin) connected to a measurement system to determine CO_2 and H_2O exchange. The chambers were placed in a climate chamber to control temperature and light. A plastic lid (acrylonitrile butadiene styrene resin) covered each pot and was used to separate the chamber into above- and below-ground compartments. The tree stem passed through center of the cover and was fitted with an airtight seal.

Experimental design

Three trees were randomly assigned to each of the three different temperatures (15, 25 and 35°C). A cycle of 12 h daylight, which was supplied by halogen lamps with a constant photosynthetically active radiation of 390 ± 10 µmol m^{-2} s^{-1} (the approximate light saturation point of *T. occidentalis*, Matthessears & Larson, 1991) measured inside the chambers at the top, was followed by 12 h of darkness. Air and soil temperatures within the chambers were kept constant during the experiment in each temperature treatment. As lighting had a heating effect on the temperature inside the chambers, the temperature outside the chambers was down-regulated 3–4°C during the daytime to maintain constant temperature inside the chambers. The growth substrate was watered to achieve field capacity (volumetric water content *c.* 50%) at the beginning (data from the first day at 25°C were missing as a result of technical failure), and pots were then left to dry with no further moisture addition until the end of the experiment. Trees were kept well watered and under their corresponding temperature treatment for 2 d before the experiment started to let them acclimate to the experimental conditions. Because of limited room in the growth chamber, the three temperature treatments were conducted in three separate trials and lasted for 34, 30 and 11 d, respectively. Trees from the different trials were not significantly different in size (height and basal stem diameter) or phenology at the start, to ensure comparability among trials. The experiment ended when transpiration approached zero (i.e. below 0.03 mol H_2O d^{-1}). At the end, the foliage of all the trees was brown and dry.

Ambient air with constant moisture ([CO_2], 400 ± 20 ppm; $\delta^{13}C$-CO_2, −9.2 ± 0.5‰; and vapor pressure deficit (VPD), 1.02, 1.90 and 3.94 kPa for 15, 25 and 35°C, respectively) was continuously pumped through above- and below-ground compartments of the chambers at constant rates (above ground, 5.5, 6.5 and 9.5 l min^{-1}; below ground, 1.0, 1.0 and 1.5 l min^{-1} for 15, 25 and 35°C, respectively), which were controlled by needle valves outside the growth chamber. The difference in flow rates between temperatures was to ensure that the difference in the CO_2 and H_2O concentrations between incoming and outgoing air was within 60 ppm and 20‰, respectively. CO_2 and H_2O concentrations of the air entering and leaving each compartment were measured separately with a LI-6262 (Li-Cor Inc., Lincoln, NE, USA) once every hour for 6 min and 40 s, switching from one compartment to the next automatically with electromagnetic

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valves. Data were collected with a CR1000 data logger (Campbell Scientific Inc., Logan, UT, USA). Hourly CO_2 and H_2O fluxes (μmol h^{-1}) in each compartment were calculated by

$$F_S = \frac{[S]_o - [S]_e}{22.4} \cdot \mathrm{FR} \cdot 60, \qquad \text{Eqn 1}$$

where $[S]_o$ and $[S]_e$ are the CO_2 (or H_2O) concentrations (ppm) of the air leaving and entering each compartment, respectively; FR is the air flow rate (l min^{-1}) through each compartment; and 22.4 is the molar volume at standard temperature and pressure (l mol^{-1}). The hourly fluxes were then summed to obtain a daily value. The daytime (12 h) CO_2 flux from the above-ground compartment was defined as net photosynthesis (P_n) and the night-time (12 h) above-ground CO_2 flux was defined as above-ground respiration. Root respiration was the summed 24 h below-ground CO_2 flux. The sum of root and above-ground respiration on a daily basis is referred to as total respiration. The above-ground H_2O flux was defined as transpiration and was divided into night-time (E_n) and daytime (E_d) components. Water-use efficiency (WUE) was calculated as P_n divided by the daytime transpiration (E_d) on a daily basis. Stomatal conductance (G_s, mol d^{-1}) was estimated by (Jarvis & McNaughton, 1986; Whitehead, 1998; McDowell *et al.*, 2008b)

$$G_S = \frac{E_d}{\mathrm{VPD}} \qquad \text{Eqn 2}$$

During CO_2 assimilation, a reduction in discrimination against ^{13}C is considered to be a physiological indicator of drought stress (Farquhar *et al.*, 1989). To reveal the variation of ^{13}C discrimination as soil water content declines and drought stress increases, discrete air samples were taken from the air flow entering and leaving above-ground compartments every 1–3 d at a fixed time (i.e. 2 h after the light was switched on), and were analyzed for ^{13}C composition in reference to Pee Dee Belemnite ($\delta^{13}C$) with a Delta$^+$ XL Isotope Ratio Mass Spectrometry (IRMS, ThermoFinnigan, Bremen, Germany). $\delta^{13}C_p$ indicates here the ^{13}C composition of the CO_2 assimilated by trees, computed from the mass balance of CO_2 and $^{13}CO_2$ of the air entering and leaving the chamber:

$$\delta^{13}C_p = \frac{[CO_2]_o \cdot \delta_o - [CO_2]_e \cdot \delta_e}{[CO_2]_o - [CO_2]_e} \qquad \text{Eqn 3}$$

where δ_o and δ_e represent ^{13}C composition of the CO_2 in the air leaving and entering the chamber, respectively. We assumed the shift in $\delta^{13}C_p$ mainly reflects photosynthetic fractionation.

A dendrometer (self-assembled with an 8 mm potentiometric linear transducer supplied by Megatron Elektronik AG & Co., Putzbrunn, Germany) was installed on the stem of one tree in each trial to measure stem diameter variation in response to drought as a proxy for water capacitance (Zweifel *et al.*, 2005). We report here the difference in stem diameter (μm) at a given soil moisture from initial diameter. Soil moisture and air/soil temperature were monitored using a ThetaProbe ML2x (Delta-T Devices Ltd, Burwell, Cambridge, UK) and a 100 kΩ NTC Thermistor EC95 (Thermometrics Corporation, USA), respectively.

Statistics

The stress point (SP) was defined as the first day that transpiration showed a significant decline from the average value of the previous days (*t*-test, $P < 0.05$), and the mean soil moisture value (± SD) at the SP of the three individuals was compared among the three temperature treatments. For the period before the SP, it is assumed that no water stress was imposed and differences in respiration and photosynthesis rates reflected the influence of temperature only. The average values of each variable (i.e. P_n, respiration, transpiration, WUE, net C gain, ratio of respiration to P_n and $\delta^{13}C_p$) before the SP were computed and compared among the three temperature treatments. One-way ANOVA followed by a Holm–Šidák test ($P < 0.05$) was applied to identify instances with statistically significant differences. For the period after the SP, we applied mixed-effects modeling to investigate how soil moisture and temperature influenced the C balance of the plants using net C gain, P_n, or total respiration as dependent variables. Interactions of soil volumetric water content (or G_s) and temperature were included as fixed effects and temperature as random effects to obtain separate intercepts and slopes for each temperature (see Supporting Information, Table S1, for a summary of the models). By modeling the temperature effect on the components of C gain, we aimed to separate the temperature effects on the whole-plant C balance from those of P_n (via stomatal conductance) and respiration.

To determine the CCP under each temperature treatment, a sigmoidal model was fitted to the daily net CO_2 gain as a function of soil moisture, from the starting point to the lowest point:

$$y = a + \frac{b}{1 + e^{(c - dx)}} \qquad \text{Eqn 4}$$

where y is the daily net CO_2 gain, x is the soil moisture and a–d are regression parameters. The point (measured as soil moisture) where net CO_2 gain equals zero was defined as the CCP. All statistics were carried out with R, version 2.15.0 (R Development Core Team, 2012) and the package 'lme4' (Bates *et al.*, 2012) was used in the mixed-effects modeling.

Results

Progressive drought

P_n and respiration showed similar patterns of response for all three temperature treatments as the soil dried out (Fig. 1a–c). P_n declined sharply after the SP and continued to decrease to values below the respiration rates. Respiration also decreased after the SP but not as fast as P_n. After the CCP, respiration rates showed a rapid decline, eventually reaching values close to zero. Daily net C gain began to decline at the SP and became negative after CCP (Fig. 1d). As total respiration declined to near zero at the end of

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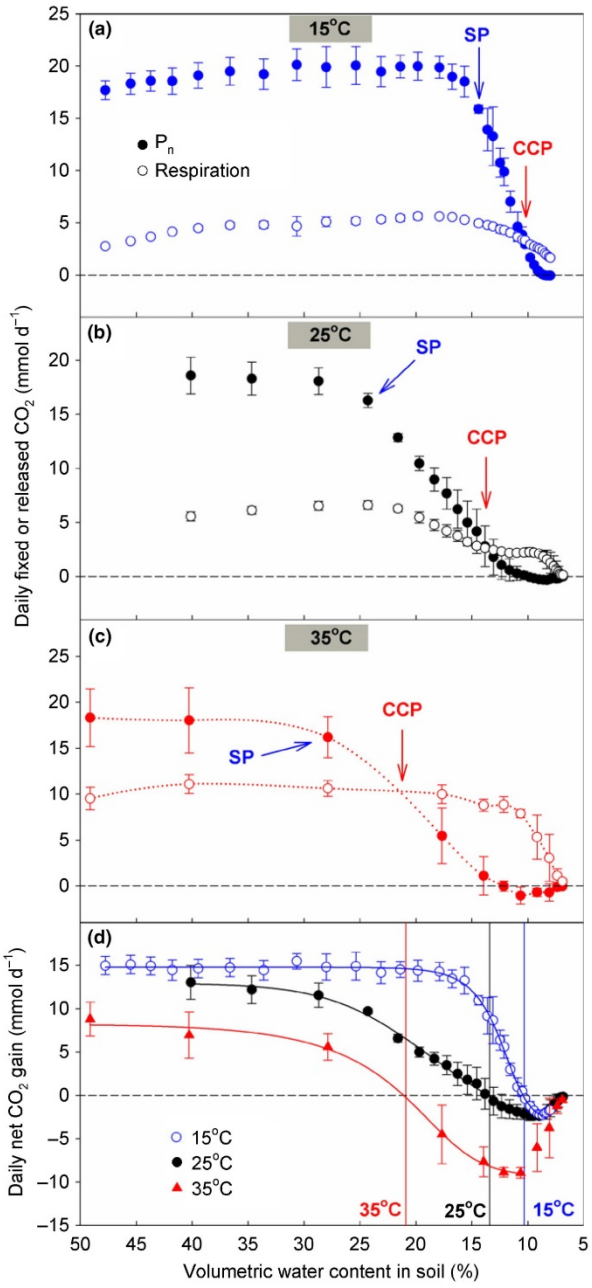

Fig. 1 Net photosynthesis (P_n), respiration (a–c) and net carbon gain (d) of *Thuja occidentalis* as a function of soil moisture under three temperature treatments (15°C, blue; 25°C, black; 35°C, red). In panels (a)–(c), closed circles represent P_n and open circles represent respiration. Blue and red arrows indicate where we defined the stress point (SP) and the carbon compensation point (CCP), respectively. Smooth (dotted) lines were added in panel (c) to improve visualization because fewer data points were available for the 35°C treatment. In (d) sigmoidal curves (see the Materials and Methods section) were used to fit the data points to estimate the zero point of daily net CO_2 gain (i.e. CCP, denoted by vertical lines). Bars represent ± 1 SD (three replicate trees) and bars not visible are within the limits of the symbols.

the experiment, daily C gain increased again and reached approximately zero. As predicted, CCP was shifted by temperature and occurred at higher soil moisture contents (10.3, 13.4 and 20.4%, $F = 31.49$, $P < 0.001$) as the temperature increased (15, 25, and 35°C, respectively). In particular, 20% soil moisture was still wet enough for trees at 15°C to maintain normal photosynthesis and transpiration, while trees at 35°C with the same soil moisture content were already in net C deficit. P_n and total respiration at the CCP had already dropped to low rates at 15 and 25°C, while rates at 35°C remained high, suggesting that only mild stress was imposed at this point. Based on total respiration rates at the end of the experiment, trees at 25 and 35°C were dead (zero respiration) after 29 and 11 d, respectively, while trees at 15°C were still alive after 34 d (total respiration = 1.65 ± 0.18 mmol CO_2 d^{-1}) when we ended the experiment.

Cumulative net C gain (Fig. 2) showed different patterns among the three temperature treatments. Trees grown at 15°C accumulated much more C than trees grown at 25 or 35°C. At the end of the experiment, trees grown at 15 and 25°C still had not used up the equivalent amount of C accumulated during the experiment. However, trees grown at 35°C had already consumed, within 6 d, as much C as was accumulated during the experiment and seemingly relied on stored C for respiration after that.

After the onset of the SP (Fig. 3a), P_n declined as a result of decreasing daytime G_s (Fig. 3b). The higher temperature led to increased transpiration rates and hastened soil drying. Night-time transpiration rates remained low (not exceeding 1 mol H_2O d^{-1}) at 15 and 25°C (Fig. 3c). However, at 35°C night-time transpiration was much greater (even higher than daytime transpiration at 15 and 25°C). Night-time G_s showed a similar pattern for the three temperatures but was slightly lower at 25°C (Fig. 3d).

Water-use efficiency did not drop at the SP, and only dropped after the CCP (Fig. 4a) because P_n and daytime transpiration

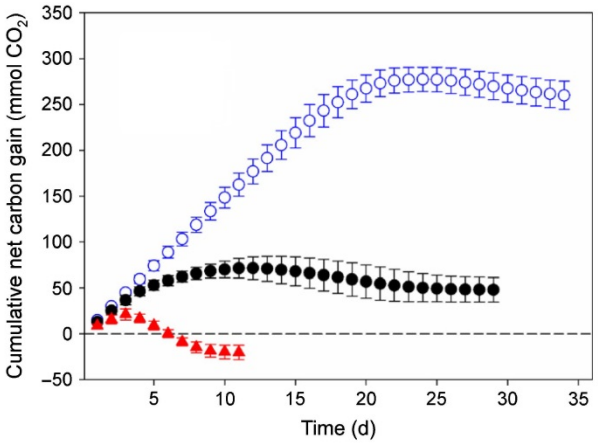

Fig. 2 Daily cumulative net carbon gains of *Thuja occidentalis* for the three temperatures over the experimental periods, which are 34, 28 and 11 d, respectively, for 15°C (blue circles), 25°C (black circles) and 35°C (red triangles). Bars represent ± 1 SD (three replicate trees) and bars not visible are within the limits of the symbols.

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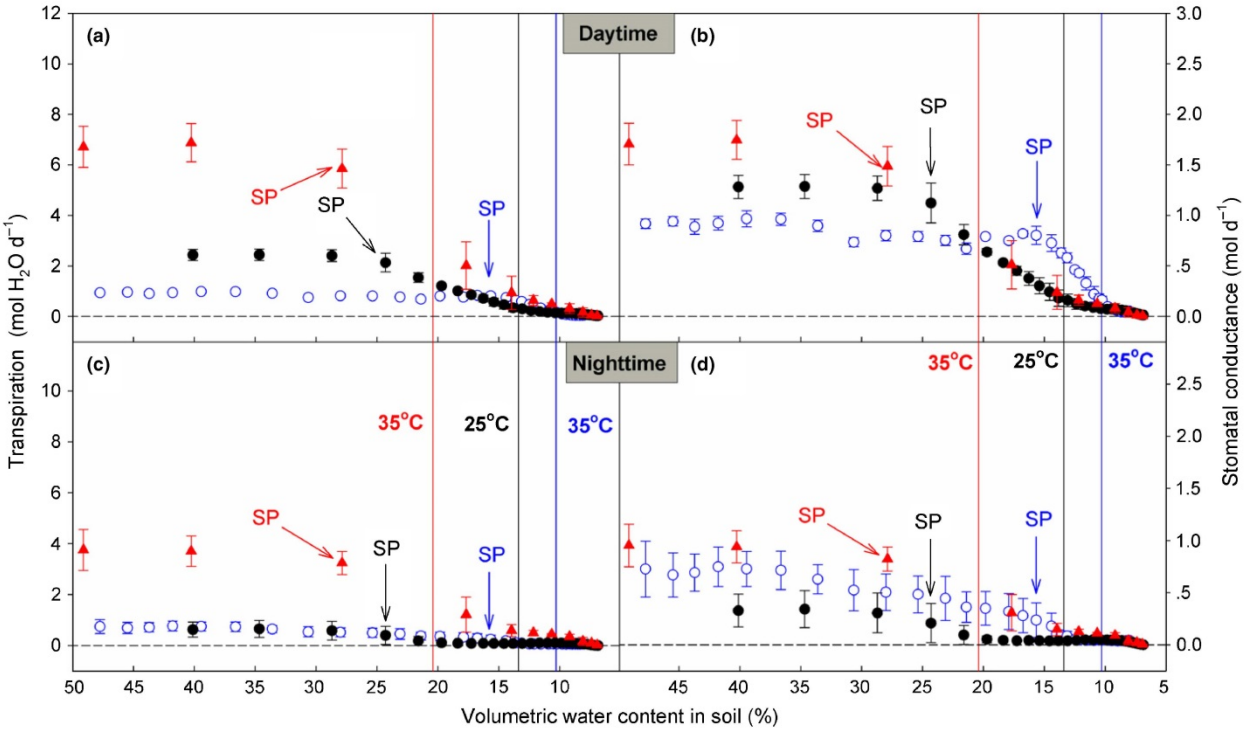

Fig. 3 Daytime (above) and night-time (below) transpiration (a, c) and stomatal conductance (G_s) (b, d) of *Thuja occidentalis* at different soil moisture content for three temperature treatments. Arrows indicate the stress point (SP) at three temperatures and vertical lines denote the carbon compensation point (CCP) (15°C, blue circles; 25°C, black circles; 35°C, red triangles). Bars represent ± 1 SD (three replicate trees) and bars not visible are within the limits of the symbols.

decreased proportionally after SP. After CCP, P_n declined faster than transpiration, leading to the decrease in WUE. Stems of the trees started to shrink after the SP (Fig. 4b), indicating reductions in stem water potential. The increased $\delta^{13}C_p$ after the SP (Fig. 4c) indicated that stomatal conductance was limiting intercellular CO_2 concentration during C assimilation and induced a decline in isotopic discrimination. Similar to former studies (Farquhar & Richards, 1984; Henderson *et al.*, 1998), $\delta^{13}C_p$ is well correlated with WUE at the three temperatures (Fig. S1).

Effect of temperature without drought stress

The SP, which indicates the onset of stomatal closure, occurred at lower soil moisture at 15°C than at 25 and 35°C (Fig. 5a). Before the SP, that is, before the onset of drought stress, temperature had a significant effect on the C balance. Respiration rates above and below ground increased with temperature and were significantly higher at 35°C (Fig. 5c,d), whereas P_n was not affected by temperature before the onset of drought stress ($F = 0.224$, $P = 0.805$, Fig. 5b). As a result, daily net C gain was significantly lower at 35°C than at 15°C (Fig. 5e). The ratio of total respiration to photosynthesis was *c.* 0.24 at 15°C but reached 0.33 and 0.60 at 25°C and 35°C, respectively (Fig. 5f). Night-time transpiration was much higher at 35°C (Fig. 5g). Daytime transpiration rates increased exponentially with temperature and were significantly different among temperature treatments (Fig. 5h). WUE dropped significantly as temperature increased and was five to seven times higher at 15°C than at 35°C (Fig. 5i). Similar to transpiration, night-time G_s was significantly lower at 15 and 25°C than at 35°C, while daytime G_s increased with temperature (Fig. 5j,k). $\delta^{13}C_p$ was significantly higher at 15 than at 35°C (Fig. 5l), indicating that CO_2 availability at the leaf level was not as limited at 35°C as it was at 15°C before the SP.

Interaction of drought stress and temperature

Temperature showed a significant effect on how the trees' C balance responded to declining soil moisture (Fig. 6). The P_n of trees grown at 15°C was more sensitive to soil moisture decline after drought stress developed than at higher temperatures (Fig. 6a), whereas response of total respiration to soil moisture was not significantly affected by temperature increase (Fig. 6b). P_n was more sensitive to soil moisture decline than respiration, making it more of a determinant of the tree C balance during progressive drought. P_n at lower temperatures was more sensitive to G_s than at higher temperatures (Fig. 6c), indicating that photosynthesis was more constrained by stomatal conductance at lower temperatures, which in turn made G_s more important for net C gain at 15°C (Fig. 6d).

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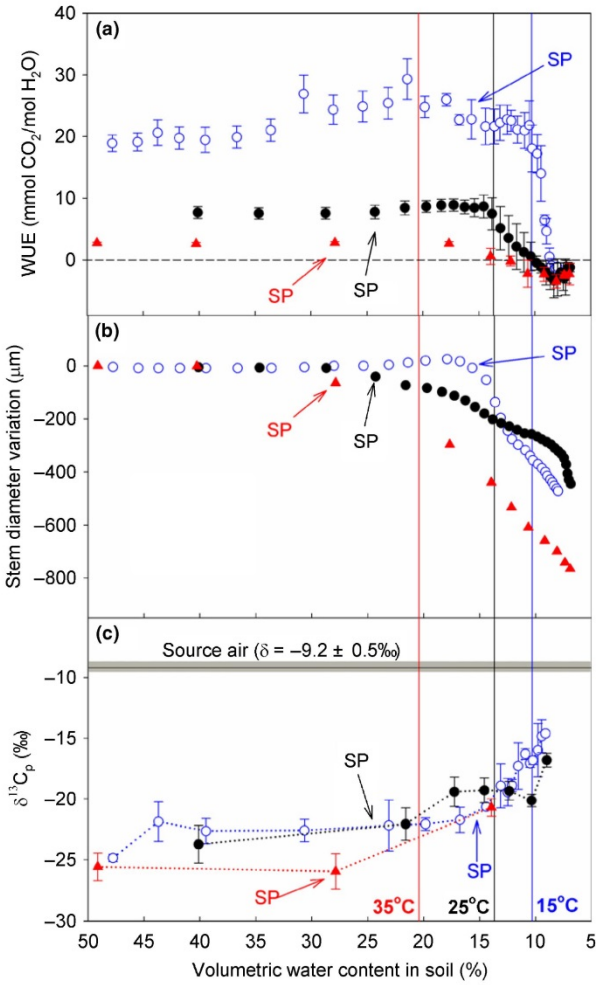

Fig. 4 Water-use efficiency (a), stem diameter (b) and $\delta^{13}C_p$ of assimilated CO_2 (c) of *Thuja occidentalis* as a function of soil moisture content. Arrows indicate the stress point (SP) under three temperature treatments (15°C, blue circles; 25°C, black circles; 35°C, red triangles), and vertical lines denote the carbon compensation point (CCP). (c) The horizontal line and gray area represent the ^{13}C signature ($\delta = -9.2 \pm 0.5‰$) of the source air entering the chambers. Bars represent ± 1 SD (three replicate trees) and bars not visible are within the limits of the symbols.

Discussion

Advance of the CCP at elevated temperature

This study revealed that changes in whole-plant C balance during drought stress are highly temperature-dependent. As hypothesized, C deficit occurred in trees at a high temperature (35°C) at a soil moisture content that may not be considered as drought for trees growing at a lower temperature (15°C). Former studies of whole-plant C balance in crops (McCree, 1986; Miller *et al.*, 2001) also suggest that whole-plant C gain is inhibited under water stress and high temperature, but these studies did not separate the temperature and drought effects nor did they determine whole-plant CCP. Temperature did not affect how plant respiration responded to the progressive drought after the SP (Fig. 6b), while sensitivity of P_n to soil moisture decline was lower at higher temperatures (25 and 35°C) (Fig. 6a), which itself ran counter to the advance of the CCP at higher temperatures. Therefore, considering initial values before the SP, the earlier occurrence of CCP caused by high temperature was mainly attributed to the higher initial respiration rates (Fig. 5c,d) and an earlier onset of stomatal closure (i.e. SP, Fig. 5a), but not initial P_n (Fig. 5b).

Previous studies in vascular plants have demonstrated that elevated temperatures increase plant maintenance respiration (Atkin *et al.*, 2000; Atkin & Tjoelker, 2003) and hasten stomatal closure as water stress develops (Schulze *et al.*, 1973). These results are consistent with the patterns of the processes that caused the CCP to occur at higher moisture content under elevated temperature. In addition, as temperature rises beyond the optimum point, plant photosynthesis can be reduced (Bernacchi *et al.*, 2001, 2002) and may further advance the CCP during drought/heat events. While the conditions imposed in our treatments may not realistically mirror anticipated patterns of climatic change, our results indicate that the combined effects of drought and elevated temperature have important implications for the C budget of vascular plants under a warmer and drier scenario.

Temperature effects on plant C balance without drought

Owing to technical constraints, we did not have well-watered trees as a control at each temperature in our experiment, which would have allowed us to separate the effects of temperature from those of declining soil moisture. Instead, we took the period before the SP at each trial as a baseline, assuming no major changes in plant physiology would occur during the experiment under strictly controlled conditions (i.e. without day-to-day variations in temperature and light conditions) if no water stress was imposed.

As temperatures increased from 15 to 35°C, net photosynthetic rates remained constant (Fig. 5b), while respiration increased with a Q_{10} (the rate of respiration change as temperature increases by 10°C) of *c.* 1.4. Stronger photosynthetic discrimination against ^{13}C occurred at 35 than at 15°C (Fig. 5l), similar to what has been observed in studies on shrubs under experimental warming (Welker *et al.*, 1993, 2004; Michelsen *et al.*, 1996). This can be explained by the high stomatal conductance at 35°C (Fig. 5k), which kept intercellular CO_2 concentration high and thereby enhanced C isotope discrimination (Farquhar *et al.*, 1982, 1989). In addition, during the imposed progressive drought, the slopes of P_n to G_s (Fig. 6c), reflecting the gradient between ambient and intercellular CO_2 concentration (c_a–c_i) (Farquhar & Sharkey, 1982), were lower at elevated temperatures. This suggests that intercellular CO_2 concentration was highest at 35°C for a given G_s value, potentially another reason for the large ^{13}C discrimination at 35°C.

High VPD is usually considered to have a negative effect on stomatal conductance (Gucci *et al.*, 1996; Wullschleger *et al.*, 2002), which in turn influences photosynthesis and C balance. Yet, elevated temperature can also widen the aperture of stomata at constant VPD (Schulze *et al.*, 1973). Our results show that

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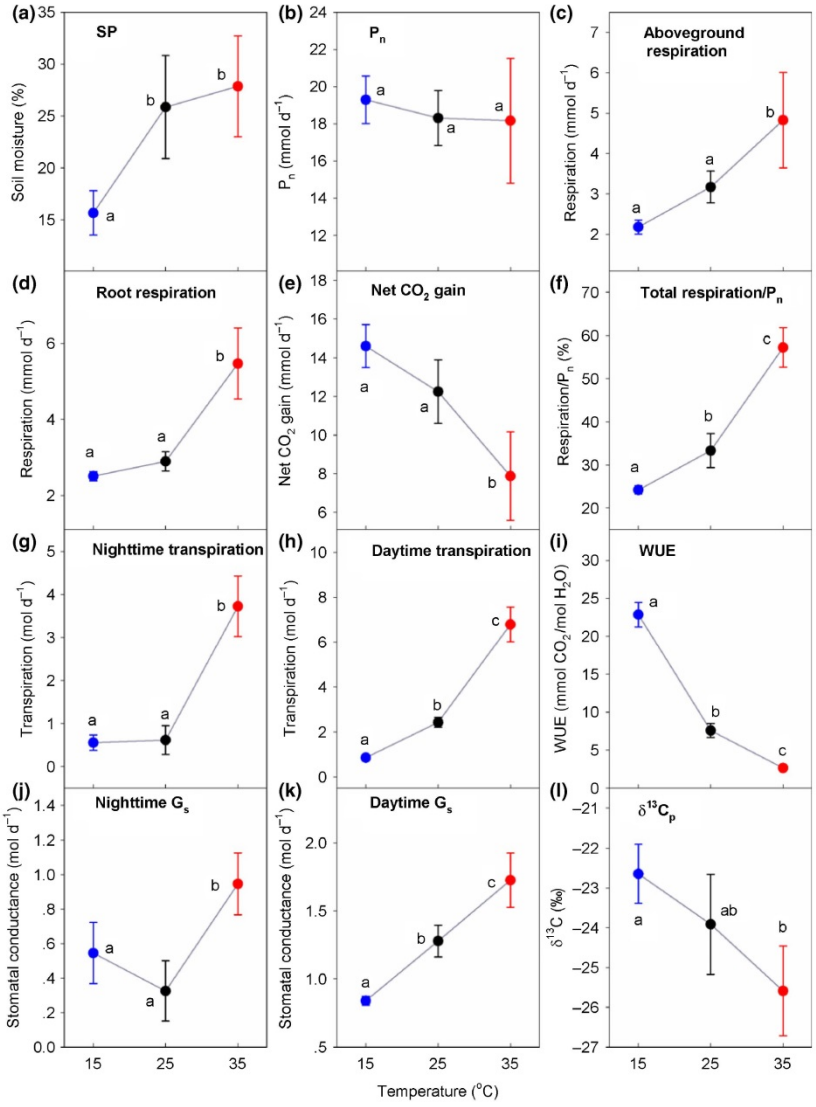

Fig. 5 The stress point (SP) and values of the other studied variables of *Thuja occidentalis* under nondrought conditions (i.e. average values before the SP) at the three temperatures (15°C, blue circles; 25°C, black circles; 35°C, red circles). Different lowercase letters denote significant differences between the three temperatures (Holm–Šidák test, $P < 0.05$). Bars represent ± 1 SD (three replicate trees) and bars not visible are within the limits of the symbols. P_n, net photosynthesis; G_s, stomatal conductance; WUE, water-use efficiency.

daytime G_s was higher under warmer conditions (Fig. 5k), indicating that direct temperature effects were much stronger than temperature effects via VPD. However, their individual contributions to changes in stomatal conductance as well as to the C balance need to be quantified in a specifically designed experiment.

Effects of temperature and drought on the plant C balance

During the progressive drought, P_n played a more important role than respiration in the C balance, because P_n was more sensitive than respiration to soil moisture decline at all temperatures (Fig. 6a,b). Temperature, including its effect on VPD, affected P_n through G_s (Fig. 6c), and, hence, the C balance (Fig. 6d). Respiration was primarily directly affected by temperature (Fig. 5c, d), rather than by temperature/drought interactions as indicated by parallel slopes of respiration over soil moisture (Fig. 6b).

We observed two distinct phases of drought stress response. In the first phase, P_n, respiration, transpiration, stem diameter and $\delta^{13}C_p$ all started to decline after the SP (Figs 1, 3, 4). However, WUE started to decrease only around the CCP, characterizing the second phase of the response (Fig. 4). Before reaching the CCP, stomatal closure reduced both water loss and CO_2 diffusion into leaves, thereby causing simultaneous reductions in P_n and transpiration. After the onset of the CCP, P_n decreased at a greater rate than transpiration, inducing a decline in WUE. One possible explanation is the nonlinear decrease of mesophyll conductance under drought conditions (Flexas *et al.*, 2002), which may impede CO_2 diffusion and induce a sudden decline in C fixation at the CCP. Another possibility is that, regardless of stomatal closure, there could be some metabolic impairment inhibiting CO_2 fixation when plants reach a negative C balance. The rapid decline of respiration after the CCP may also have

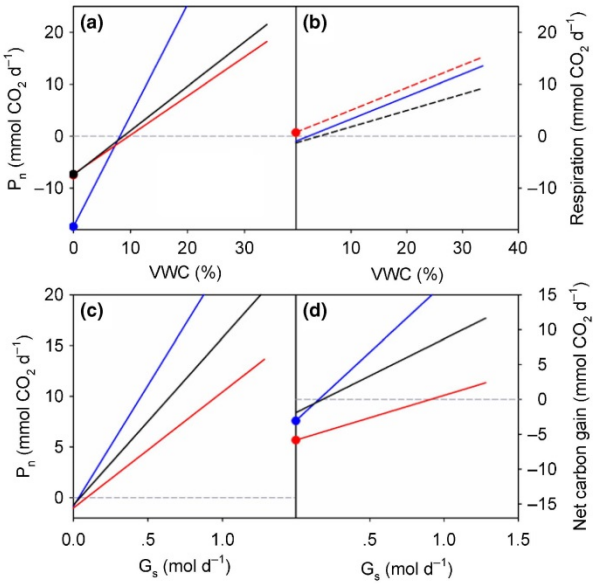

Fig. 6 Coefficients (intercepts and slopes) of mixed-effects models for net photosynthesis (P_n) (a, c), total respiration (b) and net carbon gain (d) of *Thuja occidentalis* in response to soil volumetric water content (VWC) or stomatal conductances (G_s) at the three temperatures (blue, 15°C; black, 25°C; red, 35°C). Closed circles on the *y*-axis indicate the intercepts (at 25 or 35°C) that are significantly different from zero (for 15°C) or from 15°C (for 25 and 35°C). Solid lines indicate slopes that are significantly different from zero (for 15°C) or from 15°C (for 25 and 35°C), and dashed lines indicate the insignificant slopes. The significance level is $P < 0.05$.

been induced by this (Fig. 1). Flexas & Medrano (2002) demonstrated that stomatal closure is the dominant limitation of photosynthesis under mild and moderate drought conditions, whereas, under severe drought, metabolic impairment (i.e. decreased ribulose-1,5-bisphosphate content) becomes the dominant limitation. Whether the CCP can actually indicate metabolic impairment needs to be further investigated. Given the usefulness of the CCP as an indicator of critical plant C and water relations, further exploration of the physiological processes and potential indicators of the CCP and how these vary with tree species may provide important insights.

Implications for mechanisms of drought-induced tree mortality

Our results suggest that high temperatures can increase the risk of C starvation by forcing trees to be C-deficient under relatively high soil moisture contents. McDowell *et al.* (2008a) suggested C starvation as a possible cause of drought-induced mortality, especially in warmer environments. In support of this point, Adams *et al.* (2009) demonstrated that higher temperatures increased cumulative respiration of plants at a leaf level under water stress. In our study, at the end of the 15 and 25°C trials, the amount of C that had been accumulated during the experimental period was still far from being depleted (Fig. 2). It seems that these trees were not C-limited at the end of the experiment, even though respiration rates had dropped to nearly zero. Given the rapid decline in their stem diameter at the end of the experiment (Fig. 4b), indicating a decrease of stem water content, hydraulic failure was the most likely main threat to tree survival at these temperatures. At 35°C, the amount of C respired by the trees exceeded what they had accumulated during the experimental period. At the time of their death, they had consumed up to 27 mmol (324 mg) more C than was accumulated during the experiment. However, given the stem diameter decline (Fig. 4b), hydraulic failure or an interaction between water stress and C deficiency (McDowell, 2011) was probably the cause of the death in these trees. Without sufficient data, we cannot determine the exact mortality mechanism. Still, it is obvious that plants experiencing higher temperatures accumulate less C and are more likely to become C-limited under the same soil moisture conditions.

Conclusion

With our whole-plant chamber design, we demonstrated that high temperature shifts the CCP of plants to higher soil moisture values. It can be inferred that a warming climate may cause physiological drought (i.e. C deficit) even when no meteorological or severe drought occurs, and may consequently threaten the survival of plants in more regions. Similarly, summer drought may impose a greater threat to plants than droughts during colder periods.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Correlations between $\delta^{13}C_p$ and WUE at the three temperatures.

Table S1 Summary of the mixed-effects models with temperature (15, 25 and 35°C) as the random effect

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9.6. Chapter 6: The dry and the wet drought

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Research

Rapid report

Pinus sylvestris switches respiration substrates under shading but not during drought

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Key words: carbon starvation, hydration, respiratory quotient, respiratory substrates, stress physiology, tree ecophysiology, tree mortality.

Summary

- Reduced carbon (C) assimilation during prolonged drought forces trees to rely on stored C to maintain vital processes like respiration. It has been shown, however, that the use of carbohydrates, a major C storage pool and apparently the main respiratory substrate in plants, strongly declines with decreasing plant hydration. Yet no empirical evidence has been produced to what degree other C storage compounds like lipids and proteins may fuel respiration during drought.
- We exposed young scots pine trees to C limitation using either drought or shading and assessed respiratory substrate use by monitoring the respiratory quotient, $\delta^{13}C$ of respired CO_2 and concentrations of the major storage compounds, that is, carbohydrates, lipids and amino acids.
- Only shaded trees shifted from carbohydrate-dominated to lipid-dominated respiration and showed progressive carbohydrate depletion. In drought trees, the fraction of carbohydrates used in respiration did not decline but respiration rates were strongly reduced. The lower consumption and potentially allocation from other organs may have caused initial carbohydrate content to remain constant during the experiment.
- Our results suggest that respiratory substrates other than carbohydrates are used under carbohydrate limitation but not during drought. Thus, respiratory substrate shift cannot provide an efficient means to counterbalance C limitation under natural drought.

Introduction

Increased tree and forest mortality in response to heat and drought have been observed around the globe (Allen *et al.*, 2010) and are likely to increase even further since climate predictions foresee more frequent and more severe heat and drought events during the upcoming decades (IPCC, 2013). Yet, the scientific understanding of the physiological processes and mechanisms responsible for drought-induced tree death is still incomplete (Zeppel *et al.*, 2011; McDowell *et al.*, 2013; Hartmann *et al.*, 2015) with tree carbon (C) relations providing the greatest uncertainties (McDowell, 2011). When avoiding desiccation by stomatal closure, plants also reduce CO_2 influx into the leaf, causing inhibition of photosynthesis and hence of C assimilation (Farquhar & Sharkey, 1982). Thus, trees will eventually induce the use of stored C reserves to fuel essential metabolic activities like respiration (Chapin *et al.*, 1990; McDowell & Sevanto, 2010).

So far, most studies on C dynamics during drought have focused on carbohydrates, which represent the largest C storage fraction (Hoch *et al.*, 2003), and apparently provide the main respiratory substrate in plants (Plaxton & Podestá, 2006; Araujo *et al.*, 2011). However, declining hydration slows the production of new carbohydrates and hampers the mobilization and use of stored carbohydrates during drought (Hartmann *et al.*, 2013a; Sevanto *et al.*, 2014). Trees also store lipids and proteins as C reserves (Sauter & van Cleve, 1994; Hoch *et al.*, 2003) and their role as respiratory substrates has been documented in cell cultures, excised tissues or shaded herbaceous plants under carbohydrate limitation (Jacobson *et al.*, 1970; Journet *et al.*, 1986; Brouquisse *et al.*, 1991; Dieuaide-Noubhani *et al.*, 1997; Devaux *et al.*, 2003; Tcherkez

et al., 2003; Morkunas *et al.*, 2012). However, for complex perennial organisms like trees empirical support for shifts in substrate use from carbohydrates to lipids and/or proteins is nonexistent but would help elucidate one of the many uncertainties in drought induced mortality mechanisms (McDowell, 2011).

We investigated shifts in respiratory substrates in one of the most widespread and economically important tree species, scots pine (*Pinus sylvestris*) (Sabaté *et al.*, 2002; Martinez-Vilalta *et al.*, 2009). Pine stores substantial amounts of lipids (Höll, 1997; Hoch *et al.*, 2003) but whether lipids function as substrate for respiration is unknown. Respiratory consumption of individual substrates is usually inferred from net changes in substrate concentration over time (Brouquisse *et al.*, 1991) but since putative respiration substrates may also be used for other metabolic processes (e.g. fatty acid synthesis, osmoregulation) such an approach is questionable. Here we present an online investigation of the respiratory quotient (RQ, CO_2 released per O_2 consumed), an indirect but sensitive measure of the energy source consumed by respiration. The RQ depends on a substrate's degree of oxygenation (Tcherkez *et al.*, 2003; Lambers *et al.*, 2005): a pure combustion of relatively oxygen rich compounds like carbohydrates yields RQ values *c.* 1.0. For more reduced compounds like proteins or lipids, a complete breakdown requires more oxygen and produces RQ values of *c.* 0.81 and 0.7, respectively, while intermediate values indicate a mixture of different respiratory substrates.

RQ measurements are sparse in plant physiology and technically challenging because of the relatively small changes of oxygen concentrations caused by respiration (several hundred parts per million) on the large background of ambient air (*c.* 20–21 vol.% O_2) compared to the relatively large changes of CO_2 in ambient air (*c.* 0.036 vol.% CO_2) (S. Hanf *et al.*, unpublished). In this study we used cavity-enhanced Raman multi-gas spectrometry (CERS), a novel measurement technique to continuously and nondestructively monitor real time respiratory gas fluxes in plants (Keiner *et al.*, 2013, 2014). CERS allows analyzing multiple gases at once and provides a suitable precision for both O_2 and CO_2 measurements. The devices used in our study are state of the art photonic technology, so far commercially unavailable, and we were able to use two for our study. For more details on the devices see Frosch *et al.* (2013) and Keiner *et al.* (2014).

We also assessed the stable C isotope signature of respired CO_2. Isotope measurements are inexpensive and a powerful tool to trace C fluxes within plants (Dawson *et al.*, 2002). Fractionation processes during photosynthesis (Farquhar *et al.*, 1989) and subsequent metabolic downstream processes, normally enzymatic discrimination against the heavy C isotope ^{13}C (Badeck *et al.*, 2005), result in distinct $\delta^{13}C$ signatures of different C compound classes (lipids $\ll$ proteins $<$ carbohydrates, Gleixner *et al.*, 1993; Bowling *et al.*, 2008). While $\delta^{13}C$ of leaf carbohydrates mirror mainly fractionation from CO_2 diffusion into leaves and enzymatic discrimination during carboxylation (*c.* −24‰), post-photosynthetic fractionation processes further deplete proteins and lipids leading to values of *c.* −27‰ and −33‰, respectively (Tcherkez *et al.*, 2003; Badeck *et al.*, 2005). Whenever a compound is converted to CO_2 during respiration its inherent and specific C isotope signature is transmitted to the evolving CO_2 (Ghashghaie *et al.*, 2001) and may serve as a coarse proxy for substrate identity. While there can be considerable diel variability in $\delta^{13}C$ of respired CO_2 in all plant tissues (Werner & Gessler, 2011) leaf-respired CO_2 is usually enriched by *c.* 6–11‰ compared to leaf carbohydrates (Ocheltree & Marshall, 2004; Tcherkez *et al.*, 2004; Barbour *et al.*, 2007). Whether this enrichment varies with substrate is, to our knowledge, unknown.

To assess shifts between different C compounds used during respiration we invoked whole-tree C limitation by either drought or shading. Drought not only reduces C assimilation but concurrent declining hydration also hinders translocation and utilization of C reserves, at least of the carbohydrate fraction (Hartmann *et al.*, 2013b). Shading, however, prevents C assimilation via reduced light energy input while maintaining favorable water balance for C translocation (Sevanto *et al.*, 2014). The comparison of both treatments allows partitioning the effects of reduced C availability and of declining hydration on substrate changes in respiration.

We predicted carbohydrate-dominated respiration (RQ value *c.* 1) for all trees before treatment and for control trees throughout the experimental period. For drought trees we expected a sudden decline in carbohydrate use during drought due to hydration-mediated impeded mobilization with a concurrent sudden increase in lipid/protein metabolism (indicated by a steep decline in RQ and $\delta^{13}C$ and declining substrate content). For shaded trees, we expected a gradual mobilization and ensuing depletion of carbohydrates and hence a more gradual shift to lipid/protein metabolism with a gradual decline in RQ and $\delta^{13}C$.

Materials and Methods

Plants and treatments

Four Scots pine (*Pinus sylvestris* L.) trees (80–100 cm high, 25 l pots) were randomly assigned to each of the three following treatments:

- control: full light (photon flux density (PFD) lower canopy – upper canopy, 162–730 μmol s^{-2} m^{-2}); daily irrigation to field capacity, *c.* 1 l d^{-1},
- drought: full light (PFD lower canopy – upper canopy, 162–730 μmol s^{-2} m^{-2}); daily irrigation *c.* 25% of control for 18 d, followed by 3 d with re-watering,
- shading: no light (complete darkness) for 18 d with daily irrigation to field capacity, followed by 3 d of return to lighted conditions.

Control and shading treatments were started in mid-April 2014, drought treatment in mid-May (see Supporting Information Methods S1 for tree conditioning). Soil moisture (volumetric water content, %) was monitored with a time domain reflectometer (TDR) sensor (CS645, 7.5 cm, three-rod probes with a TDR 100, Campbell Scientific Inc., Logan, UT, USA) for each individual tree.

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Rapid report Research 3

Experimental setup: gas analysis, respiration rates, RQ, $\delta^{13}C$ and photosynthesis rate

The gas measuring system was installed on one 10–15 cm long, intact, live branch (Fig. 1). CO_2 production and O_2 consumption from dark respiration were measured automatically during the experiment as described in S. Hanf *et al.* (unpublished; see also Methods S1).

Branch sections were inserted and tightly packed in measurement chambers. To prevent a self-shading effect in the light treatments (i.e. control and drought), branch sections were enclosed in the darkened measuring chambers only for measurements and no longer than half a day at a time. Chamber installation required needle removal at the sealing surface around the branch and in order to prevent recurrent branch damage on the same tree we not only rotated trees but also used different branches within individuals in the light treatments. Because self-shading was not an issue in shaded trees, this procedure was not required and we kept the same branch in the chamber throughout the experiment to avoid unnecessary needle removal.

Respiration rates were calculated from the slope of a linear fit of the increases in $[CO_2]$ and declines in $[O_2]$. The ratio of both rates yielded the RQ (reported as daily averages and their standard deviations of repeated measurements during the day) according to the following equation:

$$RQ = \frac{\Delta[CO_2]}{-\Delta[O_2]} \qquad \text{Eqn 1}$$

For each day one respiratory CO_2 sample (see Methods S1) was taken *c.* 11 h after daytime start and analyzed for $\delta^{13}C$ using a stable isotope ratio mass spectrometer (DELTA$^+$XL, Finnigan MAT, Bremen, Germany), reported relative to the Vienna Pee Dee Belemnite (VPDB):

$$\delta^{13}C(‰) = \left[\frac{\left[\frac{^{13}C}{^{12}C}\right]_{sample}}{\left[\frac{^{13}C}{^{12}C}\right]_{VPDB}} - 1\right] \times 1000 \qquad \text{Eqn 2}$$

Photosynthesis rates as a source of carbohydrates were measured once a day and for 1 h, starting from day 5, in drought and control trees using a Licor® 6400 photosynthesis system (LI-COR, Bad Homburg, Germany) and area of the needle sections enclosed in the leaf chamber was measured using an area meter (Li 3100A, Li-Cor, Bad Homburg, Germany).

Tissue sampling and processing

Needle and branch tissues were sampled regularly, always *c.* 11 h after daytime start. Immediately after harvesting, samples were frozen by immersion in liquid nitrogen. The fresh weight (FW) of the tissues was determined gravimetrically and samples were vacuum freeze-dried for 96 h. Dry weight (DW) was weighted before samples were ground, needles and branches individually, to fine powder using a ball mill (Retsch 400, Retsch GmbH, Haan, Germany).

Tree water status

Tissue water content (WC) was estimated as difference between FW and DW expressed as a ratio of DW.

$$WC = \frac{FW - DW}{DW} \qquad \text{Eqn 3}$$

Putative respiration substrates

Extraction and measurement of carbohydrates, lipids and free amino acids Following hot water extraction (50 mg ground plant

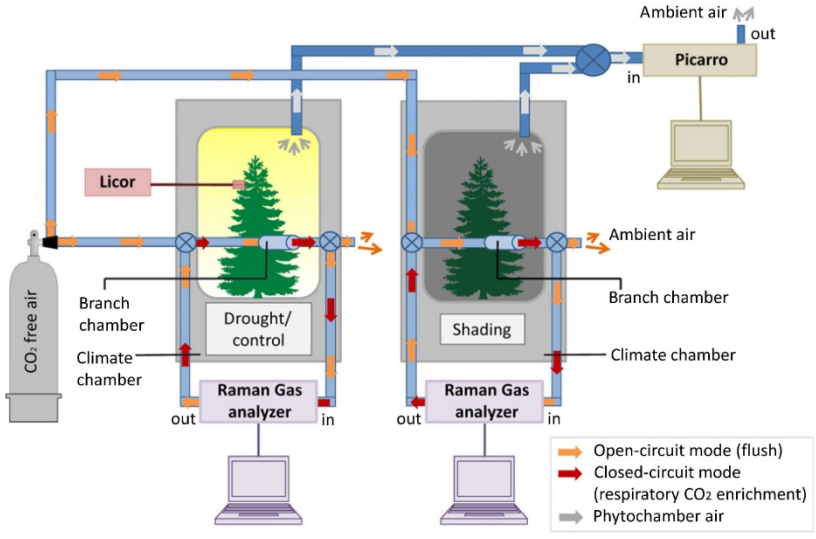

Fig. 1 Schematic view of respiratory gas analysis system for control/drought and dark treatment carried out in climate-controlled chambers (York Industriekälte, Mannheim, Germany) (day : night cycle 16 h : 8 h, temperature cycle 25°C : 18°C, relative air humidity 30%). Each Raman gas analyzer was connected with a darkened, airtight chamber enclosing a branch and either flushed with CO_2-free synthetic air in open-circuit mode (orange arrows) or run in close-circuit mode for measuring dark respiration (red arrows). Switching between modes was performed with an automated magnetic valve system. Photosynthesis rates were measured daily using a LiCor® 6400 photosynthesis system (Li-cor, Bad Homburg, Germany). Isotopic composition of the climate chamber air was monitored with a Picarro cavity ring-down spectrometer (Picarro, Santa Clara, CA, USA).

material, 3 ml double-distilled water) and perchloric acid (PCA) hydrolyses (10 mg ground plant material, 0.7 ml bi-distilled water, 1 ml PCA) soluble sugar and starch concentrations were measured in diluted extracts (1 : 20, 1 : 55, respectively) with high performance liquid chromatography (HPLC) coupled with a pulsed amperometric detection (PAD) using a Dionex® ICS 3000 ion chromatography system equipped with an autosampler (Thermo Fisher GmbH, Idstein, Germany) (Raessler *et al.*, 2010) as described in Hartmann *et al.* (2013b, see also Methods S1).

Storage lipids (triglycerol) were measured according to Eggstein & Kuhlmann (1974) described in Hoch *et al.* (2002). After saponification of lipids (cleavage of fatty acids from glycerol) the enzymatic conversion of glycerol to glycerol-3-phosphate was measured photometrically with a micro plate reader (Infinite M200, Tecan, Salzburg, Austria) using glycerol standards for calibration of total glycerol concentration (see also Methods S1).

An aliquot of the hot water extract of soluble sugars was diluted (1 : 10, v/v) in water containing a ^{13}C, ^{15}N labelled amino acid standard (Isotec, Miamisburg, OH, USA). Free amino acids in the diluted extracts were directly analyzed by LC-MS/MS as described in Docimo *et al.* (2012) with the modification that an API5000 mass spectrometer (Applied Biosystems, Darmstadt, Germany) was used (see also Methods S1).

Starch and lipid extraction for $\delta^{13}C$ measurements Starch extraction for C isotope analysis was done by enzymatic hydrolysis of starch according to Wanek *et al.* (2001) modified by Göttlicher *et al.* (2006). After removing soluble sugars and gelatinization of starch, starch was hydrolyzed by purified alpha-amylase before a filtration step removed the enzyme (see also Methods S1).

Total lipids were extracted by accelerated solvent extraction (ASE-200, Dionex Corp., Sunnydale, CA, USA) using 500 mg ground plant material in a dichloromethane/methanol solution (9 : 1) at 137.5 bar and 100°C. The heating phase lasted 5 min and was followed by a static extraction phase of 15 min. This procedure was repeated and extracts were combined.

Measurement of $\delta^{13}C$ of substrates Aliquots from the hot water extracts of soluble sugars, from enzymatic starch extracts and ASE extracts of lipids, respectively, were transferred into tin cups, dried and measured with a Finnigan MAT DeltaPlus XL EA-IRMS (ThermoFinnigan GmbH, Bremen, Germany), combined with an autosampler (Koppenaal *et al.*, 1991). Measurement error for $\delta^{13}C$ was determined by measurements of a laboratory internal standard (NBS 22 −30.03‰ on VPDB scale) (Coplen *et al.*, 2006).

Although amino acids could not be separated from hot water extracts for separate $\delta^{13}C$ measurement, we assumed that their small proportion in the extract (*c.* 12%) and their only slightly different $\delta^{13}C$ (0.6‰ to soluble sugars, Bowling *et al.*, 2008) did not substantially influence our estimate of soluble sugar $\delta^{13}C$.

Data analysis

Since gas measurements were performed on branch sections including needles and branches, individual substrate concentrations of needles and branches were converted to tissue-specific substrate contents (multiplied by tissue biomass). Needles accounted, on average, for *c.* 80% of the total biomass in the branch chambers (data not shown). Tissue-specific substrate contents were summed to calculate the total substrate content of the branch section and then divided by the total biomass, yielding tissue-weighted average concentrations (per gram of dry biomass contained in the chamber). Soluble sugars, starch, lipid and amino acid concentrations were summed to provide an estimate of total available substrate concentration and the proportion of each individual substrate in this overall pool was calculated (see Supporting Information Table S1 for tissue-specific substrate concentrations of needles and branches).

Daily treatment means of substrate proportions were compared with analysis of variance (ANOVA) after testing for heteroscedasticity (Levene test) across treatments. Following significant ANOVA test ($P < 0.05$) Tukey's honestly significant difference (HSD) test ($\alpha < 0.05$) was applied to assess differences between specific treatments. Significant results of Tukey's HSD test were only considered when variances were homoscedastic. Statistical analysis was carried out using R (v. 2.13.1, 2014, R Foundation for Statistical Computing, Vienna, Austria).

Results

Soil moisture, relative tissue water content and photosynthetic rates

Drought treatment caused soil moisture to drop rapidly to significantly lower values (*c.* 10% vol.) than in the shade and control treatment (*c.* 40–50% vol., Fig. 2a). Tissue water content in needles and branches (averaged for tissue proportions in the measuring chambers) increased by 14% in control and 27% in shade trees compared to the start of the experiment, but decreased by 25% in drought trees during the treatment (data not shown).

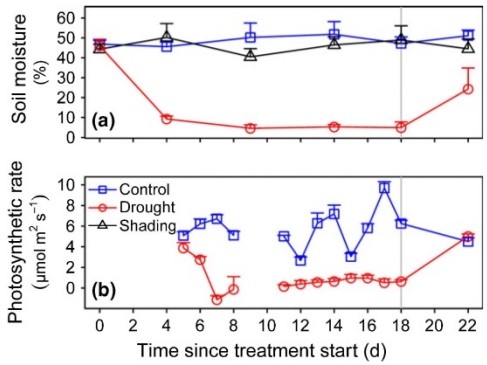

Fig. 2 (a) Daily average (in % of soil vol., ±1 SD) relative soil moisture in control (blue line, squares), drought (red line, circles) and shading (black line, triangles) treatments on *Pinus sylvestris*. Drought and shading were started at day 1 and ended at day 18, indicated by the grey line. (b) One-hour average specific photosynthesis rates (in μmol CO_2 m^{-2} s^{-1}, ±1 SD) of control (blue line, squares) and drought (red line, circles) trees. Photosynthesis was not measured in shaded trees (because 'residual' photosynthesis in the dark is impossible) and data are missing for days 9 and 10.

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Despite a decrease in plant hydration, low photosynthesis rates were sustained in drought trees (*c.* 10% of average rates in control trees) throughout the experiment, and returned to initial values 3 d after re-watering (Fig. 2b).

Normalized respiration rate, RQ and $\delta^{13}CO_2$ of respired CO_2 and of putative substrates

Respiration rates are reported as normalized to initial rates and were more variable in treatments with light exposure, that is, drought and control (Fig. 3a). This variability was likely caused by the rotation between different trees in these treatments and, to a small degree, by light-enhanced dark respiration upon darkening (Barbour *et al.*, 2007; Gessler *et al.*, 2009; please see Methods S1). While respiration rates in the control treatment declined just slightly throughout the experiment, drought caused a rapid and strong while shading a gradual but substantial decrease in respiration rates. Respiration rates of drought trees were lower than in shaded trees from day 2 through day 5 (Fig. 3a). The RQ of control trees remained stable and oscillated *c.* 0.88 (Fig. 3b). In the drought treatment the RQ was higher than in control trees halfway through the experiment but also with a more or less stationary trend. By contrast, the RQ in shaded trees was lower than in control and drought trees from day 4 onwards (Fig. 3b) and decreased progressively from an initial value of *c.* 0.86 to a minimum at the end of the experiment of *c.* 0.77. After treatment release RQ values of all treatments were similar again (0.90–0.92). The $\delta^{13}C$ signature of respired CO_2 did not show any clear pattern in control and drought trees and only in shaded trees showed a clear decline from initial values. This decline lasted until day 7 and then remained stable until the end of the experiment (Fig. 3c). Although $\delta^{13}C$ of respired CO_2 can show a substantial diurnal variation due to changing allocation of C to metabolic pathways (Werner & Gessler, 2011) the clear long-term pattern in shaded trees most likely originates from shifts in respiration substrate. The obtained $\delta^{13}C$ values of putative substrates at the beginning of the experiment were highest in starch (−26.61‰, SD = ±1.43, $n = 9$), lower in soluble sugars (−30.29‰, SD = ±0.64, $n = 12$) and lowest in lipids (−33.08‰, SD = ±0.63, $n = 9$).

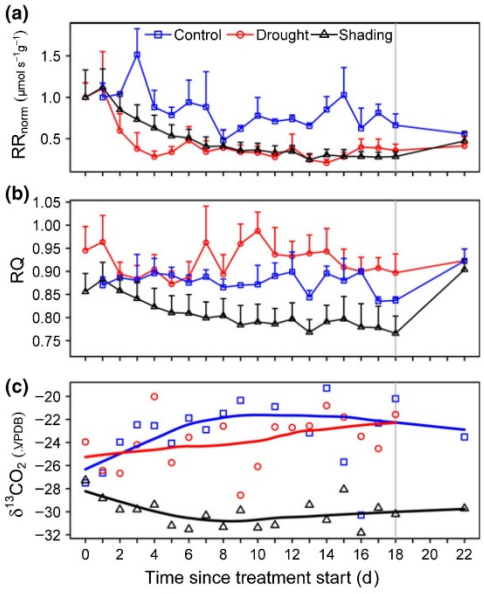

Fig. 3 (a) Daily average normalized respiration rates (RR_{norm}; dimensionless, +1 SD as indicator for measurement error), (b) average respiratory quotient (RQ) ($\Delta CO_2/\Delta O_2$, +1 SD as indicator for measurement error) and (c) $\delta^{13}C$ (‰) of a closed-cycle measurement 11 h after beginning of the daytime period in control (blue line, squares), drought (red line, circles) and shading (black line, triangle) treatment on *Pinus sylvestris*. Drought and shading were started at day 1 and ended at day 18, indicated by the grey line. Trend lines in (c) are derived from loess using a smoothness parameter of 2/3 and a first degree polynomial. Note that $\delta^{13}C$ was measured only once every day precluding the computation of standard deviations (VPDB, Vienna Pee Dee Belemnite).

Proportions of putative respiratory substrates in total pool

In all treatments, carbohydrates initially made up the largest fraction of the total available substrate pool followed by lipids and free amino acids (Fig. 4). Their abundance remained relatively stable in control and drought trees during the experiment (Fig. 4, upper and mid panels). By contrast, the proportion of carbohydrate decreased sharply in shaded trees, with an increase in the proportion of the lipid and amino acid fraction of available substrates. This trend was reversed after treatment release (Fig. 4f). These dynamics are driven mainly by needle concentrations and were most prominent in soluble sugars of shaded trees which sharply declined during the experiment (Fig. 4c).

Discussion

Major findings

The application of CERS allowed us to closely investigate metabolic activity of intact trees with high temporal resolution and yielded insights into respiratory substrate usage (Keiner *et al.*, 2013, 2014) during drought and shading. Our results show that tree respiration was fuelled by a mixture of substrates, comprising carbohydrates, lipids and likely amino acids. The most abundant substrate in plant tissues apparently also contributes most to respiration. Drought may have hampered the use of carbohydrates but did not result in a shift to more reduced, alternative substrates. In response, respiration rates strongly decreased. By contrast, shading resulted in a gradual decline of carbohydrates with a concurrent shift to more lipid-dominated respiration but respiration rates also declined.

Sugar levels may modulate C metabolism during drought

Carbohydrate content remained high in drought trees throughout the experiment. This may be due to impeded use of carbohydrates from declining plant hydration (Hartmann *et al.*, 2013a,b) or by allocation of carbohydrates from other tissues and plant parts to the branch section. Labeling studies have shown that phloem transport is slowed by drought (Ruehr *et al.*, 2009). Moreover, declining

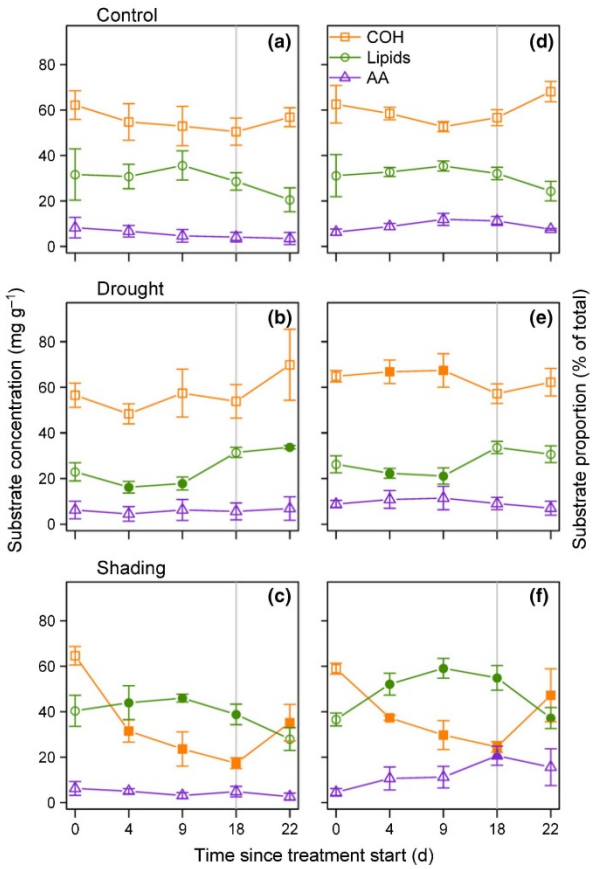

Fig. 4 Average weighted substrate concentrations (a–c, in mg g^{-1} dry biomass) and substrate proportions (d–f, in % of total) of carbohydrates (COH), lipids and free amino acids (AA) (%, ±1 SD; $n = 4$ for COH and lipids; $n = 3$ for AA) in control (a, d), drought (b, e) and shade (c, f) treated *Pinus sylvestris*. Drought and shading were started at day 1 and ended at day 18, indicated by the grey line. Closed symbols indicate significant differences in treatments drought and shading ($P < 0.05$, significant ANOVA followed by Tukey's honest significance test) from control.

water potential during drought increases phloem sap viscosity and has a negative impact on phloem transport velocities (Hölttä *et al.*, 2009). It is hence questionable whether allocation, a process dependent on phloem functioning, allowed the maintenance of carbohydrate levels in the drought but not in the shading treatment.

The concomitant reductions in respiration could be an active shift in carbohydrate allocation from respiration to osmoregulation (Morgan, 1984; Sala *et al.*, 2012). However, a number of amino acids are also important for osmotic adjustment (Ashraf & Foolad, 2007) and accumulate during drought stress (Smirnoff, 1993; Cruz de Carvalho, 2008). Since we did not observe increased amino acid content in branch and needle tissues increased carbohydrate allocation to osmoregulation is doubtful and not supported by our data.

Substrate shifts from sugars to lipids and proteins have so far only been reported under sugar starvation (Brouquisse *et al.*, 1991; Dieuaide-Noubhani *et al.*, 1997; Borek *et al.*, 2001; To *et al.*, 2002) but not during drought, in accord with our results. Maintained levels of carbohydrates in drought trees may have prevented lipid and/or protein catabolism. Sugars are not only substrates in C metabolism but also act as primary messengers in hormone-like functions like signal transduction for modulating growth, development and stress responses (Rolland *et al.*, 2002, 2006). Because genes encoding for lipid and protein catabolism are down-regulated when sugar concentrations are high (Koch, 1996; Yu, 1999) the drought-induced impediment of carbohydrate and sugar use may have indirectly also prevented the use of alternative substrates and concomitant advantages. The oxidation of lipids can provide twice as much metabolic water (*c.* 1.1 g of water per gram of lipids) than the oxidation of carbohydrates (*c.* 0.5 g of water per gram of carbohydrates; Edney, 1977; Candlish, 1981) and may be advantageous during drought as a water recycling mechanism (Levitt, 1980).

Declining plant hydration and concomitant impeded carbohydrate and lipid use apparently caused a strong decline in metabolic activity, that is, respiration rates. To what extent this decline was source (i.e. substrate availability) or sink driven (i.e. reduced energy demand for growth or ion uptake during drought) remains uncertain but a decline in respiration due to substrate availability alone (i.e. pure source limitation) is unlikely (Atkin & Macherel, 2009). Reductions in respiration during drought have been reported elsewhere (Adams *et al.*, 2009; Hartmann *et al.*, 2013a) and were interpreted as responses of C utilization to declining water potential and decreasing phloem functioning (Atkin & Macherel, 2009; Sevanto *et al.*, 2014) rather than declining assimilation.

Shading facilitates the use of all substrate types

Constant hydration under shading apparently allowed trees to mobilize and continuously utilize the carbohydrate fraction of the substrate pool. The subsequent decrease of carbohydrate content may have signaled substrate scarcity and triggered an up-regulation of gene expression encoding lipid/protein catabolism (Koch, 1996). Our RQ and $\delta^{13}C$ measurements in shaded trees indicated a gradual shift from the initial carbohydrate-dominated substrate mix to an almost pure lipid metabolism in the advanced experimental stages. Similar results, that is, decreasing RQ and $\delta^{13}C$ indicating increasing fatty acid oxidation, have been observed in shaded French bean (Tcherkez *et al.*, 2003) and in isolated starved cell cultures or excised plant tissues (Brouquisse *et al.*, 1991; Aubert *et al.*, 1996). The RQ may also decline if some of the respired CO_2 is transported away from the site of respiration as occurs, for example, in the xylem of tree stems (Angert *et al.*, 2012). However, we measured RQ at distal parts of the trees, precluding substantial CO_2 transport against the transpiration stream. Low RQ values may also arise during the synthesis of more oxidized compounds (than carbohydrates) such as organic acids (Lambers *et al.*, 1996). Although increased gene expression for synthesis of organic acid like malate has been shown in response to shading-induced sugar starvation (Ismail *et al.*, 1997) starvation was truly not occurring in our shaded trees. The observed decline in RQ hence strongly suggests shifts in respiration substrates.

Unlike studies on isolated starved cell cultures or excised plant tissues, we did not observe substantial declines in lipid content in response to shading. Carbon allocation across plant organs in grasslands was not affected by shading (Bahn *et al.*, 2013) and such C relocation may obscure a local depletion of mobile C compounds like carbohydrates. Thus it is very unlikely that such compounds would be converted to lipids in the measured branch section. However, shaded trees also showed reduced metabolic activity indicated by declining respiration rates, although less pronounced than drought trees. Declines in respiration in response to shading have been observed elsewhere but were more gradual than in our experiment and were suggested to reflect maintained phloem functioning and accessibility of carbohydrates in shaded as opposed to drought trees (Sevanto *et al.*, 2014). The decreased energy requirements from rapidly declining respiration rates in our experiment may have been covered by small amounts of lipids (energetic yield 9.1 kcal g^{-1} vs 3.8 kcal g^{-1} of carbohydrates) and hence caused only a relatively small draw-down of lipid content which went undetected by our analyses.

The observed increases in free amino acid concentrations of shaded trees are indicative for protein degradation (Genix *et al.*, 1990) in response to carbohydrate starvation (Brouquisse *et al.*, 1991; Dieuaide-Noubhani *et al.*, 1997; Devaux *et al.*, 2003) and have been observed in shaded seedlings (Vance & Zaerr, 1990; Durzan, 2010). The measured low RQ values in shaded trees may therefore comprise signals from both lipid and protein catabolism.

Conclusion and outlook

Our study provides first results on the dynamics of respiratory substrate use in trees subjected to drought and C limitation and gives valuable insights into plant functioning and tree stress responses. Comparing responses of drought and shaded trees suggested that declining plant hydration prevented lipid consumption and is hence a key parameter in C storage use during drought stress. Taken together, our results question the role of stored C compounds other than carbohydrates as an energy buffer for tree survival during strong droughts. Further studies with sufficient replication are needed to corroborate our results but require more CERS devices or refined sampling designs. Since the underlying control mechanisms at the cellular level were also not addressed in this study several unresolved questions remain that require further investigation, including: how do changes in hydration influence the utilization of stored carbohydrates? Is there a threshold of hydration provoking such a response? Why is there no 'backup' mechanism allowing lipid catabolism when carbohydrate utilization is inhibited?

Moreover, further studies should take resource translocation across plant organs into account and should aim to solve the stoichiometry of the gas exchange as a function of the substrate changes within the measured branch section and at the whole-tree level. Comparison between different species may provide a physiological explanation for drought tolerance.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Tissue-specific (needles and branches) branch section concentrations of soluble sugars, starch, lipid, amino acids and total substrates

Methods S1 Preconditioning of trees, branch chamber system, extraction and measurement of water soluble sugars, starch, lipids and amino acids.

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9.7. Chapter 7: Sweet drought and fatty shade

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Research

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Rapid report

How fresh is maple syrup? Sugar maple trees mobilize carbon stored several years previously during early springtime sap-ascent

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Key words: bomb-radiocarbon approach, nonstructural carbon (NSC) pool, reserve carbon (C) mobilization, springtime sap production, sugar maple (*Acer saccharum*).

Summary

- While trees store substantial amounts of nonstructural carbon (NSC) for later use, storage regulation and mobilization of stored NSC in long-lived organisms like trees are still not well understood.
- At two different sites with sugar maple (*Acer saccharum*), we investigated ascending sap (sugar concentration, $\delta^{13}C$, $\Delta^{14}C$) as the mobilized component of stored stem NSC during early springtime. Using the bomb-spike radiocarbon approach we were able to estimate the average time elapsed since the mobilized carbon (C) was originally fixed from the atmosphere and to infer the turnover time of stem storage.
- Sites differed in concentration dynamics and overall $\delta^{13}C$, indicating different growing conditions. The absence of temporal trends for $\delta^{13}C$ and $\Delta^{14}C$ indicated sugar mobilization from a well-mixed pool with average $\Delta^{14}C$ consistent with a mean turnover time (TT) of three to five years for this pool, with only minor differences between the sites.
- Sugar maple trees hence appear well buffered against single or even several years of negative plant C balance from environmental stress such as drought or repeated defoliation by insects. Manipulative investigations (e.g. starvation via girdling) combined with $\Delta^{14}C$ measurements of this mobilized storage pool will provide further new insights into tree storage regulation and functioning.

Introduction

Trees can build up large pools of nonstructural carbon (NSC) in perennial tissues such as branches, stem and roots. Theoretically, these organs contain enough carbon (C) to re-foliate the whole canopy at least once (Würth *et al.*, 2005) or even up to four times (Hoch *et al.*, 2003). NSC pools are generally assumed to function as reserves for later use, but substantial amounts of NSC stored in inner sections of stems may simply be sequestered and inaccessible for mobilization (Sala *et al.*, 2012). The extent to which older NSC is mobilized for growth and metabolism during springtime (i.e. in the absence of a photosynthesizing canopy) remains unresolved even today (Dietze *et al.*, 2014).

Several recent investigations have used bomb-radiocarbon to estimate the mean ages of C in NSC reserves, in CO_2 derived from respiration, or in the formation of newly grown biomass after disturbance (Gaudinski *et al.*, 2001; Vargas *et al.*, 2009; Carbone *et al.*, 2013; Muhr *et al.*, 2013; Richardson *et al.*, 2013; Trumbore *et al.*, 2015). The amount of radiocarbon (^{14}C) in atmospheric CO_2 has declined since the mid 1960s as ^{14}C created during atmospheric nuclear weapon testing mixed into the biosphere and oceans, and was diluted by CO_2 from fossil fuel burning (Levin & Hesshaimer, 2000). During each growing season of the last 50 years, photosynthesized C was therefore marked with a distinct ^{14}C signature that reflects the year C was fixed from the atmosphere. Using this radiocarbon clock, NSC pools in red maple were found to be made up of C fixed seven to 14 years previously (Carbone *et al.*, 2013; Richardson *et al.*, 2013) while CO_2 emitted from stems of the same species in spring and before emergence of leaves had ^{14}C signatures indicating the C on average was one to two years old

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(Carbone *et al.*, 2013), providing evidence for the contribution of C from older NSC reserves to springtime metabolism. The CO_2 emitted from the stems of deciduous and evergreen oaks was found to be one to four years old through a whole year of measurements, and modeling indicated that these ages reflected a mix of contributions from recent and older NSC that mixed laterally into the stem (Trumbore *et al.*, 2015). Further support for metabolism of older NSC reserves comes from in-stem CO_2 in tropical trees that had radiocarbon signatures equivalent to average ages six and even for a few trees more than 20 years old (Muhr *et al.*, 2013).

Sugar maple trees (*Acer saccharum*) are known for mobilizing substantial amounts of stored NSC by transforming starch in xylem ray cells of stems into sugar during late winter and early spring before bud break and early leaf expansion (Gregory, 1982). This mobilization increases the sugar concentrations in the xylem sap to values between 1 and 6% during a time of year when no photosynthesis occurs (Larochelle *et al.*, 1998). Due to these high sugar concentrations, the harvesting of maple sap for the production of maple syrup has become the largest commercial use of temperate deciduous trees for food production worldwide, even though sugar maple sap is only commercially harvested in the south-eastern Canada and the north-eastern United States (Farrell, 2013). This early springtime sap production by sugar maple offers a great opportunity for investigating how far back trees can access and remobilize NSC reserve pools.

Here, we used the bomb-radiocarbon approach to estimate the mean time elapsed since C in ascending sap sugars was fixed from the atmosphere and to derive turnover times of stem NSC storage pools. Storage turnover times have been investigated in other studies (e.g. Keel *et al.*, 2007) that relied on continuous, multi-year ^{13}C labeling that is difficult to apply under field conditions, especially in mature trees. We used sugar maple because the sweet sap can be considered the mobilized (and hence metabolically relevant) portion of stem NSC storage, and it avoids some of the potential biases involved in extracting NSC from wood (Quentin *et al.*, 2015). We hypothesized the maple sap sugars being remobilized from NSC pools comprising C fixed during several previous growing seasons, thus resulting in sugar ^{14}C signatures higher than the sampling year's atmosphere CO_2 and a turnover time (TT) of several years.

Materials and Methods

Sampling

Sugar maple (*Acer saccharum* Marsh.) sap samples were obtained every 3–8 d between 31 March and 27 April 2014, from two different sites in Canada and from six trees per site. Both sites were located *c.* 40 km apart from each other in the Outaouais region in south-western Quebec. The Roger Lake site (S1; Latitude: 45.954173, Longitude: −74.862945) is a mixed deciduous-conifer forest stand some 10 km from the village of Lac-des-Plages. Sugar maple does not make > 30% of the basal area, while the rest is composed of balsam fir (*Abies balsamea* L.), white birch (*Betula papyrifera* Marsh.), yellow birch (*Betula alleghaniensis* Brit.) and Black Cherry (*Prunus Serotina* Ehrhart), and has not been used for commercially harvesting maple syrup for at least several decades at this site. The Epoque site (S2; Latitude: 45.692206, Longitude: −75.071786) is located in a 100-ha forest stand dominated by sugar maple (98% of sugar maple in canopy) south of the village of Saint-Andre-Avellin. This maple forest has continuously been used for harvesting sugar maple syrup since 1987. Trees on both sites were selected to be mature canopy trees (diameter/height: 45.5 ± 5.5 cm/21.4 ± 1.2 m [S1], 47.4 ± 5.1 cm/21.9 ± 1.7 m [S2], see Supporting Information Table S1) and free of disease, critical injuries or any obvious signs of dieback. Immediately after sampling, 5 ml of the sugar maple sap were sterile filtrated (Multoclear-13 PVDF 0.2 µm, Chromatographie Service GmbH, Langerwehe, Germany) to remove microorganisms and then stored in silanized chromatographic brown-glass vials (1.5 ml with screwcap, VWR International, Darmstadt, Germany) in the freezer until the end of the sampling period. Samples were then sent to the Max Planck Institute for Biogeochemistry in Jena for further analysis.

Measuring sugar concentrations

Soluble sugar concentrations were measured in diluted samples (1:800 *v/v*) of sugar maple sap using HPLC-PAD (high-precision liquid chromatography with pulsed amperometric detection) on a Dionex® ICS 3000 ion chromatography system equipped with an autosampler (Thermo Fisher GmbH, Idstein, Germany) (Raessler *et al.*, 2010).

Measuring $\delta^{13}C$

Aliquots of maple sap were transferred into tin cups, dried and measured with a Finnigan Delta Plus XL EA-IRMS (Thermo Finnigan GmbH, Bremen, Germany), equipped with an autosampler. Measurement error for $\delta^{13}C$ was determined using a laboratory internal standard (NBS 22: −30.03‰ on VPDB scale) (Coplen *et al.*, 2006) and was < 0.1‰.

Measuring $\Delta^{14}C$

We averaged mean growing season (May–September) values of ^{14}C in atmospheric CO_2 measured at Point Barrow, Alaska, as northern hemisphere background (i.e. reflecting unpolluted conditions). To check whether the ^{14}C of C fixed by local vegetation at our site accurately reflects these background conditions, we measured ^{14}C in plants (*Daucus carota* and *Lactuca sativa*) grown from seed at both sites at the beginning of the vegetation period of 2014. Both species are characterized by fast growth and very small seeds (i.e. little C derived from the seed itself) so we used the ^{14}C in newly grown leaf tissue to monitor the signature of the current atmospheric input. Monitoring plants were harvested at the end of the growing season, cleaned, dried, ground and then analyzed for ^{14}C. This comparison tests for the potential influence of local sources of CO_2 (mainly anthropogenic combustion of fossil fuels; Hsueh *et al.*, 2007), and to assess whether we can use the Point Barrow record to estimate the ^{14}C signature of C fixed by maple trees at our sites before 2014.

For radiocarbon measurements of maple sap sugars, aliquots equivalent to 0.7 mg C were pipetted into tin cups, air-dried, and then processed according to Steinhof *et al.* (2004). Radiocarbon data are expressed as $\Delta^{14}C$, which is the per mil deviation from the $^{14}C/^{12}C$ ratio of oxalic acid standard in 1950. The sample $^{14}C/^{12}C$ ratio has been corrected to a $\delta^{13}C$ value of −25‰ to account for any mass-dependent fractionation effects (Stuiver & Polach, 1977). Due to this correction, the $\Delta^{14}C$ is identical for atmospheric CO_2 and photosynthetic products fixed from it, allowing us to compare sugar $\Delta^{14}C$ values directly to the atmospheric $^{14}CO_2$ record.

The mean age of C in NSC can be estimated in two ways. First, we can compare the $\Delta^{14}C$ of NSC-C to the atmospheric curve directly. However, as NSC is a reservoir to which C is added and lost, a second approach is to estimate the TT of C in the NSC using a time-dependent, steady-state one-pool model as described in Gaudinski *et al.* (2001). As noted in Gaudinski *et al.* (2001), given the approximately linear decline of atmospheric ^{14}C, both approaches should yield similar results for C that is less than a decade old. The turnover time is hence not defined as the age of the carbon but rather as the time that is required to empty the entire carbon pool if inputs were to cease. As new production of NSC is seasonal, while sinks are year-round, the turnover time we derived is an approximation that averages across years and can not capture the faster dynamics of NSC reserves.

There is a seasonal cycle for atmospheric ^{14}C values, with lower winter and higher summer values. Because deciduous sugar maple does not fix much C outside the growing season, we averaged bi-weekly data for $^{14}CO_2$ cryogenically purified from air collected between May and September at Point Barrow, Alaska, to get a ^{14}C atmospheric record representative for the C uptake during the growing season of the years 2003–2013. For the model, we combined this record with the (monthly) summer northern hemispheric record as published by Hua *et al.* (2013) for the years 1950–2002.

Results

Sugar concentrations

The NSC concentrations of all samples varied between *c.* 1–4% (with an exceptional unique occurrence of 6%; Fig. 1). For the majority of the samples (*c.* 80%), the measurable sugar pool consisted of sucrose only. For the remaining 20%, some fructose and/or glucose were also found in the samples, but always in low concentrations, with sucrose still being the main sugar (> 85 wt%, see Table S1). The average NSC concentrations increased with time at S1, but decreased over the same period at S2. With xylem flux data unavailable it is unclear, however, if these trends in concentration represent, at the whole-tree level, substantial changes in the amount of sugar mobilized or merely dilution effects.

$\delta^{13}C$

The $\delta^{13}C$ of the sap sugars showed no temporal trend on either site (Fig. 2). The two sites showed significantly different average $\delta^{13}C$ signature, however, with S1 averaging −23.2 ± 0.6‰ (n = 36) and S2 −25.0 ± 0.4‰ (n = 29) (t-test, P < 0.01).

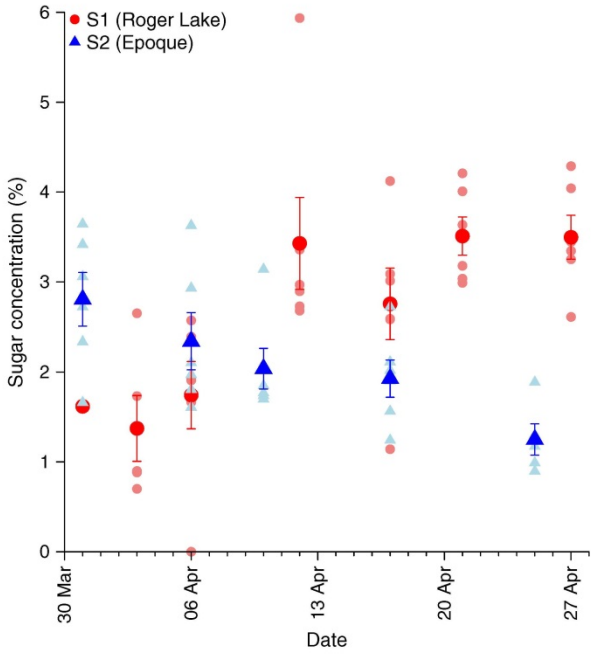

Fig. 1 Sugar concentrations of xylem sap samples of sugar maple (*Acer saccharum*) of the two sites (Roger Lake, S1 in reddish colors; Epoque, S2 in blueish colors) throughout the sampling period. Light-colored small symbols represent individual samples, the bigger symbols in saturated color represent mean values with respective standard errors.

Radiocarbon data

The monitoring plant samples had radiocarbon signatures averaging 22.6 ± 1.8‰ (standard deviation [SD], n = 4), which was not significantly different from the mean atmospheric value for the 2014 growing season at Point Barrow (21.0 ± 2.2‰, n = 10) (Fig. 3). Site pollution by local sources of anthropogenic fossil fuel combustion or by nuclear power plants is thus not affecting radiocarbon at these sites.

The $\Delta^{14}C$ was significantly above the atmospheric background for 64 of the 65 sugar samples by our working definition (i.e. values were greater by at least twice the mean precision of the measurement compared to the atmospheric background), indicating that a substantial amount of the C in these samples originated from pools assimilated in previous years (Fig. 3). Direct comparison with the mean growing season atmospheric CO_2 at Point Barrow shows that the sugar $\Delta^{14}C$ is close to atmospheric $\Delta^{14}C$ measured in the growing seasons of 2010 and 2011, that is, the C in NSC was derived from photosynthesis that occurred several years previously. Using the one pool model, the estimated TTs for the mobilized reserve C pool ranged between three and five years on average for S1 and four to five years for S2 (Fig. 4).

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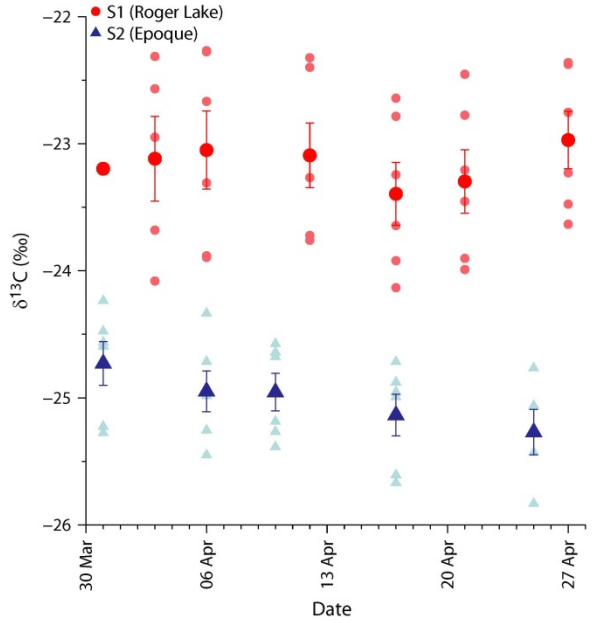

Fig. 2 $\delta^{13}C$ measurements of xylem sap samples of sugar maple (*Acer saccharum*) of the two sites (Roger Lake, S1 in reddish colors; Epoque, S2 in blueish colors) throughout the sampling period. Light-colored small symbols represent individual samples, the bigger symbols in saturated color represent mean values with respective standard errors.

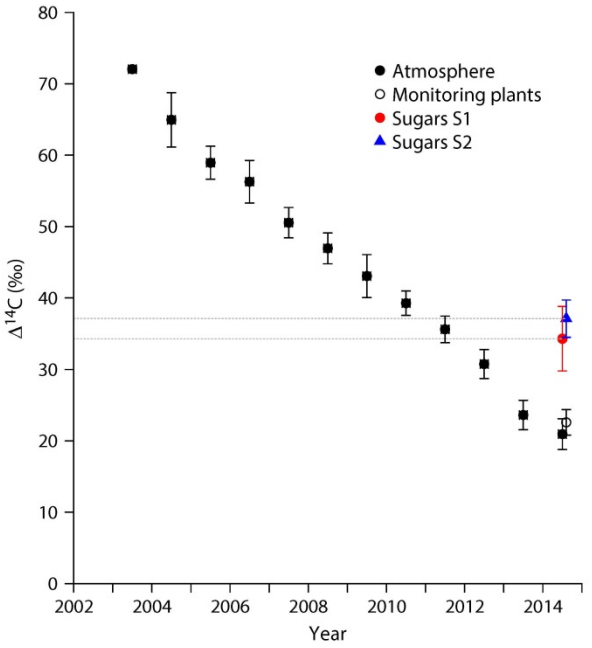

Fig. 3 Mean $\Delta^{14}C$ (± 1 standard deviation [SD]) data for growing season (15 May–15 September) atmospheric background measured at Point Barrow, Alaska between 2003 and 2014 (black circles), monitoring plant biomass harvested at the end of the growing season of 2014 at the sites (open circle), and for the xylem sap samples of sugar maple (*Acer saccharum*) from Roger Lake (S1; red circle) and Epoque (S2; blue triangle). Positioning of sugar data along the *x*-axis was slightly offset for visibility.

Discussion

Our study showed for the first time that sweet sap of sugar maple integrates sugars produced during several growing seasons and hence can serve as a straightforward tool to estimate NSC storage turnover rates. Other measures of NSC turnover have relied on continuous, multi-year ^{13}C labeling, a method that is not easily applied under field conditions. Moreover, the sugars in sweet sap represent the mobilized component of stem parenchyma NSC and hence are metabolically relevant. Given this and the additional temporal dimension provided by ^{14}C data our study contributes to the understanding of NSC storage regulation and use. The radiocarbon signatures of the sugars in the ascending sap were too high to originate from C assimilated during only the growing season before sampling (i.e. 2013; Fig. 4). Part of the sugar C thus has to originate from the tree's reserve pool, reflecting C that was fixed during several preceding growing seasons, thus confirming our hypothesis. The TTs of three to five years for C in the NSC pool represent averages, and reserve pools likely integrate C from a range of years. The sugars in maple sap thus likely comprise a substantial amount of C fixed in the most recent growing season mixed with C even older than the average TT of the mixture; that is, > 3–5 years. These findings suggest that the maple trees' ability to mobilize C during sap ascent comes from a buffered pool that depends on the tree's C balance of many growing seasons, rather than being dependent on the very last few growing seasons only.

Having said that, we also have to point out the possibility that, following an exceptionally unfavorable growing season (e.g. affected by strong drought), trees may be forced to use proportionally more C from older reserves during springtime mobilization than they usually would. According to weather data (http://climate.weather.gc.ca) from the nearby meteorology stations Notre Dame de la Paix, QC (*c.* 20 km away from S1) or Montebello Sedbergh (*c.* 11 km from S2), however, the year 2013 showed higher than average mean temperatures during late spring and a short heat wave during June, but there is no record of a strong drought. It should further be noted that sugar maple is a masting tree, and strong masting events have been reported to negatively affect the yield of maple sap most likely due to a depletion of the available reserve pools (Rapp & Crone, 2015). We are not aware of strong masting occurring on our sites in 2013, and available data from the North American Maple Project in Vermont suggest that the proportion of trees with heavy seed production was relatively small in 2013 compared to strong masting years (Rapp & Crone, 2015). Also, the maple sap sugar concentrations observed during our sampling were well within the expected range of 1 to 6% that is typical for sugar maple (Larochelle *et al.*, 1998). Overall, we conclude that our sampling represents a typical situation and that the mobilization of C from several year-old reserve pools is a regular phenomenon.

The average $\Delta^{14}C$ and $\delta^{13}C$ values in the sugar samples showed surprisingly little temporal variation, which is indicative of a relatively well-mixed reserve pool contributing to the ascending sap

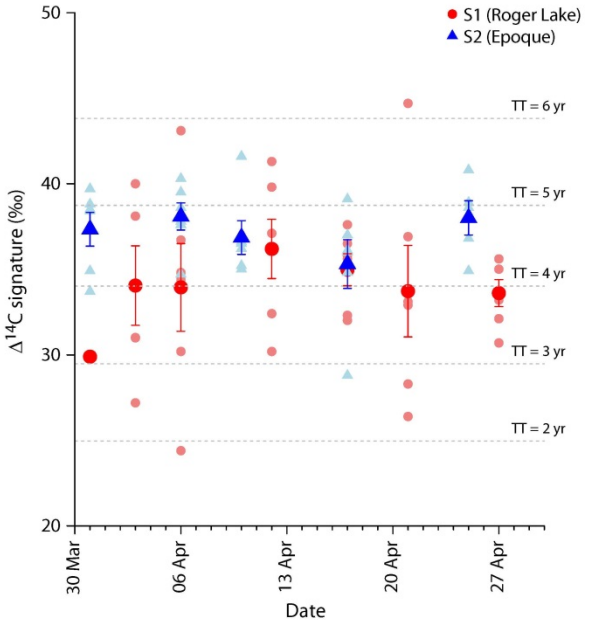

Fig. 4 $\Delta^{14}C$ signatures of xylem sap sugar samples of sugar maple (*Acer saccharum*) on two sites (Roger Lake, S1 in reddish colors; Epoque, S2 in blueish colors) throughout the sampling period. Light-colored small symbols represent individual samples, the bigger symbols in saturated color represent mean values with respective standard errors. Dashed lines indicate the $\Delta^{14}C$ as estimated by the one-pool, steady state model by Gaudinski *et al.* (2001). The model estimates the turnover time (TT) of a pool, that is, the time to empty the entire carbon pool if inputs were to cease. TT estimates expected for the year 2014 are indicated at the right-hand side.

C. The reserve pool being well-mixed implies regular interconversion of newly fixed sugars and already existent starch reserves, something that was also postulated by Richardson *et al.* (2013) after reporting almost identical ages (7–14 years, depending on site) for starch and sugar pools in stems of red maple. This also supports the idea of sugar being regularly mobilized from this pool during sap ascent.

We did, however, observe differences between the two sites. Individual trees at S1 had more pronounced variation in sugar concentrations and isotopes than trees at S2. While $\delta^{13}C$ values showed little temporal variation within a given site, the overall mean $\delta^{13}C$ differed by almost 2‰ between the two sites. Similar differences have been reported elsewhere for maple sap sugar samples from different sites (Peck & Tubman, 2010) and are assumed to reflect differences in environmental conditions. A higher overall $\delta^{13}C$, as found here at S1, could for example, indicate drier conditions at S1 compared to S2. In fact, average summer monthly precipitation (June–August) was lower in site S1 (90 mm, based on climate data from Notre Dame de la Paix) than on site S2 (111 mm, Montebello Sedbergh) during the years preceding our sampling (2009–2013) and also over longer temporal horizons (97 vs 115 mm, 1998–2013, respectively). Drier conditions result in trees keeping stomatal conductance lower in order to reduce water losses via transpiration, thereby also reducing fractionation against the heavier isotope. Drier conditions at S1 could be enhanced by the coarser soil texture (and presumably lower water holding capacity) observed at S1 (C. Messier, pers. comm.). At the same time, conditions forcing trees to reduce stomatal conductance could generally indicate a less favorable site. While this statement remains speculative, it would match the observation of a slightly higher temporal variability in the radiocarbon signature of S1, potentially due to more variability growing season C balance.

Recent findings from other studies corroborate our results by showing that the average age of C used to fuel regular metabolism like stem and root respiration usually ranges from recently assimilated (i.e. 0 years) to a few years (Schuur & Trumbore, 2006; Carbone *et al.*, 2013; Muhr *et al.*, 2013; Trumbore *et al.*, 2015). Together, these recent findings suggest that trees in general rely on a mixture of recently assimilated and stored C to fuel growth and metabolism on a more regular basis than usually assumed in many of the current models of tree growth (Le Roux *et al.*, 2001). Further evidence indicates that trees mobilize even older NSC reserves to recover from major disturbances. For instance, stump sprouts in red maple emerging after tree removal were found to be formed from NSC up to 17 years old (Carbone *et al.*, 2013), while up to 10 years old NSC was used for new root growth in a tropical forest after hurricane damage (Vargas *et al.*, 2009).

Continuous isotope labeling studies have indicated that reserves up to several years old are used to grow leaves (Keel *et al.*, 2006) and early wood (Kagawa *et al.*, 2006). A 32-year-long radiocarbon record of mature leaves of red maple from the Ottawa region (McNeely, 1994) indicates that leaf biomass in maple is partly formed from reserve C. Our data allow not only detecting the use of storage C for building leaf biomass but also allowing estimations of the proportion of stored vs newly-assimilated C in leaf tissues. The $\Delta^{14}C$ of the maple leaves was always higher than the atmospheric $\Delta^{14}C$ of the year of growth, being closer to the atmospheric $\Delta^{14}C$ of 1–2 growing seasons before (TT of *c.* two years estimated by the steady state model). Our findings offer a possible explanation for this offset by assuming that the old sugars that were mobilized at high concentrations in the xylem during late winter/early spring remain available until the onset of leaf growth later in spring. Assuming that the reserve C allocated to leaf growth originated from a pool with a similar TT as found in our investigation (i.e. 3–5 years), and further assuming that the rest of the leaf biomass was formed from newly assimilated C (i.e. of zero age) in spring, a simple two-pool mass balance approach then would suggest that 20–60% of the leaves' final biomass originated from reserve C, and the other 40–80% from new assimilates. Assuming the size of a tree's total reserve pool to be equivalent to one- to four-times the amount of C contained in foliage (Hoch *et al.*, 2003; Würth *et al.*, 2005) the storage pools may allow buffering many years of defoliation – if these pools can be accessed.

Forest stands in Canada periodically are affected by outbreaks of forest tent caterpillar, resulting in large-scale defoliation that can persist over several growing seasons (Cooke & Lorenzetti, 2006) and forcing trees to use reserve C during recovery (Hartmann *et al.*, 2008). However, while single defoliations did not cause declines in sugar content of maple sap in the following spring (Gregory & Wargo, 1986), repeated defoliations over several growing seasons

could possibly result in progressive C storage depletion, tree decline and death (Hartmann & Messier, 2008). Also, current climate models project that eastern Canada will be among the regions most likely to be affected by decreasing soil moisture, both in the near-term and the longer-term (Collins *et al.*, 2013; Kirtman *et al.*, 2013), and predict increased occurrence of extreme drought stress in the future. Drought reduces C assimilation via stomatal closure and may force trees to use stored C for survival (McDowell *et al.*, 2008). While our results suggest that sugar maple trees are well protected against such stresses experimental evidence is still lacking.

Further investigations on this reserve pools may provide substantial insights on tree storage regulation, mobilization and use of NSC. Manipulations of the tree carbon balance, for example with starvation via girdling, may allow unveiling allocation priorities under declining C availability via changes in ^{14}C signals in different pools. Although sweet sap production is inherently linked to the density of wood ray parenchyma cells in stem xylem (Gregory, 1982), it still needs to be shown whether and how long sugar maple trees can maintain high NSC mobilization rates following heavy stresses such as extreme drought or heavy insect defoliation. Combining sweet sap and wood parenchyma analysis may then allow assessing whether and to what degree stem NSC is metabolically relevant or merely sequestered (Sala *et al.*, 2012).

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Author contributions

All authors contributed to the design of the research. Fieldwork was carried out by C.M. and S.D, laboratory analysis of sugars by H.H. and radiocarbon analysis by J.M. Data analysis was done by J.M., H.H., X.X. and S.T., data interpretation by all authors. The manuscript was written by J.M. with major contributions by all co-authors.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Tree and sap data for sites Roger Lake (S1) and Epoque (S2)

Please note: Wiley Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.

9.8. Chapter 8: Old fresh sugar maple syrup

Muhr J., Messier C., Delagrange S., Trumbore S., Xu X. & Hartmann H. (2016) How fresh is maple syrup? Sugar maple trees mobilize carbon stored several years previously during early springtime sap-ascent. *New Phytologist*, **209**, 1410-1416.

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OPINION PAPER

Carbon starvation during drought-induced tree mortality – are we chasing a myth?

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Abstract

Drought-induced tree mortality has received much attention in the recent past. McDowell et al.'s (2008) hydraulic framework links tree hydraulics with carbon dynamics and proposes two non-exclusive mortality mechanisms: carbon starvation (CS) and hydraulic failure (HF). CS is often referred to as the (partial) depletion of non-structural carbohydrates (NSC) in response to stomatal closure, reduced C assimilation and sustained C storage dependency during longer droughts. HF describes a lethal level of xylem dysfunction from runaway embolism during severe droughts. While HF can be readily inferred from the percentage loss of conductivity in vascular tissues at the time of death, CS is much more difficult to assess.

Starvation is usually defined as a lack of food leading to suffering or death. In plants photosynthetic sugars play many functional roles, not only as a source of catabolic energy. For example, sugars are important for osmotic regulation of cell pressure and recent studies suggest a potential link between xylem parenchyma sugars and embolism repair following drought. Hence, carbon limitation could have a direct impact on tree hydraulics and HF; however, empirical evidence for such a mechanism is still inconclusive.

Although HF appears to be predominant during drought mortality, our limited understanding of the roles of NSC in hydraulic function precludes any premature refutation of CS as a mechanism in drought-induced tree mortality.

Plant carbon dynamics during drought – no water, no carbon

Recent observations of increased drought-induced tree and forest mortality across the globe (e.g.,Allen *et al.*, 2010; Settele *et al.*, 2014) have incited a wave of investigations on the underlying causal mechanisms (Hartmann *et al.*, 2015). Even under non drought conditions, plants have to spend hundreds of molecules of water for each molecule of carbon dioxide they assimilate (Taiz & Zeiger, 2002). In vascular plants stomata play a critical role in regulating this exchange. When soil water availability is reduced, root signaling via phytohormones cause stomatal closure as a means to decrease water loss via transpiration (Brodribb & McAdam, 2011). However, stomatal closure also reduces CO_2 diffusion into the leaf and hence carbon assimilation. It is therefore no surprise that the plant carbon balance is tightly coupled with its water balance.

McDowell et al.'s (2008) hydraulic framework employed this relationship to explain how drought severity and drought duration, in interaction with a species' stomatal behavior and its capacity to maintain a minimum water

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potential, may lead to either hydraulic failure (irreversible xylem dysfunction, HF) or carbon starvation (CS), i.e. insufficient carbon supply to meet a plant's demand for both osmotic and metabolic functioning (McDowell, 2011). An anisohydric (aniso = unequal, hydric = relating to moisture) species, i.e. a species that can maintain very low water potentials during drought, would also experience very low xylem water tension with the threat of cavitation and run-away embolism during a severe drought – and hence may die from HF. On the other hand, more conservative (isohydric) species that cannot maintain very low water potentials during drought would face sustained reductions in carbon assimilation during longer droughts and may then have to rely on stored carbohydrates (or other stored carbon compounds) until these are depleted – and would die from CS.

Carbon starvation – what exactly does that mean?

Merriam-Webster® online dictionary defines starvation as *"suffering or death caused by having nothing to eat or not enough to eat: the condition of someone who is starving"*. Plants produce their own food during photosynthesis in form of sugars like glucose. While drought reduces stomatal conductivity and hence glucose production, plants usually store large amounts of carbon compounds and hence do not suffer immediately after the onset of drought. On the other hand, glucose and other non-structural carbon compounds (NSC) derived from glucose play many roles in plant functioning, not only as sources of catabolic energy. Besides their role as substrates for plant defense compounds, carbohydrates are the building blocks for structural biomass and serve also as signaling substances for the genetic regulation of metabolic processes like photosynthesis or respiration (Koch, 1996). Also, sugars are important for controlling osmotic cell pressure to prevent tissue desiccation and to maintain physiological functioning under decreasing water availability (Morgan, 1984). Hence, severe reductions in carbohydrates not only influence plant catabolism but plant functioning in general and, in particular, the hydraulic functioning.

There are many potential interactions relating severe reductions in NSC (i.e. the lack of 'free' carbon compounds) to the plant water balance, for example xylem and root growth, osmotic regulation or xylem embolism repair. Observations of xylem recovery from large loss of hydraulic conductivity after drought have initially been explained by basal area growth, i.e. replacement of embolized vessels with new functional ones (Brodribb *et al.*, 2010). Also, plants may overcome water deficits by allocating carbon to root growth to maintain a functional equilibrium of the root:shoot ratio under drought conditions (Poorter & Nagel, 2000) or to mycorrhizal hyphae allowing exploring greater soil volumes (Lehto & Zwiazek, 2011). Moreover, recent theoretical advances show that embolism may be repaired with water inflow from surrounding functional vessels and this inflow would be driven by an osmotic gradient established via importing low molecular-weight sugars into embolized conduits (Brodersen *et al.*, 2010; Nardini *et al.*, 2011). However, methodological artefacts during conductivity measurements may yield spurious evidence for embolism repair (Sperry, 2013; Wheeler *et al.*, 2013) and the prevalence of xylem refilling in trees may be greatly overestimated (Cochard & Delzon, 2013; Delzon & Cochard, 2014). On the other hand, Thorsten Knipfer presented during the 2015 Xylem International Meeting in Bordeaux experimental results showing that embolism repair in grapevines, observed via X-ray microtomography, was related to the presence of carbohydrates in xylem parenchyma cells. While such results have not yet been shown for tree species (Knipfer, T, personal communication), evidence from a variety of drought manipulations in potted trees suggests a linkage between xylem vulnerability and xylem carbohydrate content (Adams et al., in preparation) and is hence supportive of a similar mechanism acting in trees. The fact that trees store substantial amounts of NSC in parenchyma cell in the vicinity of xylem vessels (Plavcová & Jansen, 2015) also suggests a potential functional role for the maintenance of water transport. However, given the lack of empirical support for a functional linkage between xylem carbohydrates and hydraulic function such suggestions are still rather speculative (Fig. 1).

Carbon starvation – does it exist beyond theory?

Carbon dynamics during starvation has so far been investigated only in cell cultures, excised tissues or shaded herbaceous plants (e.g., Journet *et al.*, 1986; Brouquisse *et al.*, 1991; Dieuaide-Noubhani *et al.*, 1997; Devaux *et al.*, 2003; Tcherkez *et al.*, 2003; Morkunas *et al.*, 2012). Experimental studies on drought-induced tree mortality are rather inconclusive with respect to carbon storage changes. Carbohydrate concentrations sometimes decline (e.g., Galiano *et al.*, 2011; Galvez *et al.*, 2013; Poyatos *et al.*, 2013) and sometimes increase during lethal drought (e.g., Galvez *et al.*, 2011; Anderegg *et al.*, 2012; Hartmann *et al.*, 2013b) while HF (measured as substantial loss of branch conductivity) has been observed in many other studies (e.g., Anderegg & Anderegg, 2013; Duan *et al.*, 2013; Mitchell *et al.*, 2013). Moreover, very few investigations have been carried out that were able to partition drought effects on carbon assimilation from drought effects on a tree's capacity to translocate stored carbon from sources to sinks (McDowell & Sevanto, 2010). Declining plant hydration during drought negatively affects phloem transport either via phloem

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viscosity buildup or reduced phloem cell turgor (Sevanto, 2014; Mencuccini *et al.*, 2015) and impeded carbon translocation may be yet another causal mechanism of drought-induced tree mortality (Sala *et al.*, 2010). Only two studies have addressed this mechanism explicitly and showed that phloem transport failure may indeed be responsible for drought mortality (Hartmann *et al.*, 2013a; Sevanto *et al.*, 2014). Impeded carbon transport across plant tissues may be causing different mechanisms to operate in different plant tissues and may cause HF to occur in above-ground tissues while belowground tissues die from CS (Hartmann *et al.*, 2013a; Hartmann *et al.*, 2013b). How such processes interact with other changes in environmental conditions, such as elevated temperature, has so far only been investigated under controlled experimental conditions. Such studies show that elevated temperatures speed up plant carbon expenditure by increasing respiration rates (Adams *et al.*, 2009) and push plants faster into a negative carbon balance (Zhao *et al.*, 2013) and could – likely – cause or hasten CS. However, elevated temperature also increases water vapor pressure deficit and hence decreases xylem water potential thereby yielding a greater risk of HF. Such interactions may explain why experimental drought manipulations of potted plants indicated a prevalence of HF as mortality mechanism while reduced carbohydrate concentrations were common but not universal with mortality (Adams, in preparation).

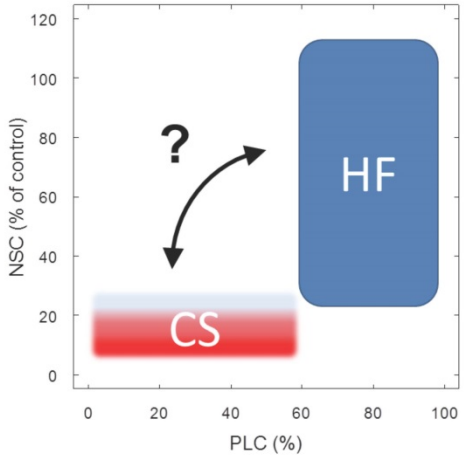

Figure 1: Working definitions for carbon starvation and hydraulic failure. Carbon starvation is defined as 'depletion' (compared to control trees) of carbohydrate pools. Minimum levels of carbohydrates may vary across plant types and even across plant tissues but are currently unknown (indicated by fuzzy shape). In contrast, thresholds for hydraulic failure (loss of conductivity) also depend on plant type and other factors but the range of thresholds is comparably well defined (indicated by clear shape). Interactions between minimum carbohydrate levels and hydraulic function, like carbohydrate-mediated embolism repair (arrow), are still not well understood and require further investigations.

Carbon starvation – are we chasing a myth?

There are still many unresolved questions with respect to tree carbon metabolism during drought and simple measurements of NSC concentrations may not be very insightful with respect to the tree carbon balance (Ryan, 2011). Carbon starvation can clearly not be defined as a complete depletion of NSC pools, as starvation in mammals occurs before blood sugar levels are down to zero (Hoch, 2015). Also, some NSC may be sequestered in woody tissues and are likely not be available for plant metabolism (Sala *et al.*, 2012). While thresholds for HF are well defined and have been reported for both conifers (e.g., Brodribb & Cochard, 2009) and angiosperms (e.g., Barigah *et al.*, 2013b; Urli *et al.*, 2013) minimum thresholds of NSC concentrations required for survival are currently unknown and this prevents defining useful working definitions for CS (Fig. 1). Methodological issues in measuring carbohydrates in plant tissues still prevent us from accurately determining absolute carbohydrate concentrations (Quentin *et al.*, 2015) and trees may shift carbon catabolism away from carbohydrates towards lipids during carbon limitation (Fischer *et al.*, 2015; Hanf *et al.*, 2015). Hence carbohydrate concentration, often assessed in studies on tree responses to drought, may be elusive and misleading.

Future research should focus on the role of NSC, including lipids, in key plant tissues like meristems that are essential for regrowth after disturbance and for plant survival (Barigah *et al.*, 2013a). As long as the functional roles of NSC in such key tissues are not clearly identified and addressed in focused studies, any attempt to refute the existence of CS during drought-induced tree mortality will be premature and scientifically unsound.

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9.9. Chapter 9: New avenues for research on carbohydrates

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Review

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Understanding the roles of nonstructural carbohydrates in forest trees – from what we can measure to what we want to know

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Contents

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Summary

Carbohydrates provide the building blocks for plant structures as well as versatile resources for metabolic processes. The nonstructural carbohydrates (NSC), mainly sugars and starch, fulfil distinct functional roles, including transport, energy metabolism and osmoregulation, and provide substrates for the synthesis of defence compounds or exchange with symbionts involved in nutrient acquisition or defence. At the whole-plant level, NSC storage buffers the asynchrony of supply and demand on diel, seasonal or decadal temporal scales and across plant organs. Despite its central role in plant function and in stand-level carbon cycling, our understanding of storage dynamics, its controls and response to environmental stresses is very limited, even after a century of research. This reflects the fact that often storage is defined by what we can measure, that is, NSC concentrations, and the interpretation of these as a proxy for a single function, storage, rather than the outcome of a range of NSC source and sink functions. New isotopic tools allow direct quantification of timescales involved in NSC dynamics, and show that NSC-C fixed years to decades previously is used to support tree functions. Here we review recent advances, with emphasis on the context of the interactions between NSC, drought and tree mortality.

I. Introduction

Living systems require a minimum of *c.* 20 elements to synthesize complex molecules and biological constructs, such as membranes, nuclei, filaments or ribosomes (Williams, 1991). Carbon (C) – the backbone of life – plays a central role because it can bond with many other elements, such as hydrogen and oxygen, to form large macromolecules composed of many repeated subunits. One family of such macromolecules, carbohydrates, are the plant metabolic currency because they provide both the building blocks for biomass *and* the energy required for biological chemical reactions and for the synthesis of other organic compounds. Structural carbohydrates are long-chained molecules used to build and solidify biomass components and structures, including cell walls, stalks and

stems, whereas nonstructural carbohydrates (NSC) are the major substrates for both primary and secondary plant metabolism.

The study of NSC in trees has a long tradition and dates back > 100 yr. Hartig (1878), in his impressive early work on tree physiology, distinguished between nonorganized (i.e. soluble) and organized (starch, which he called endosperms) reserve compounds, whereas Fischer (1891) had already recognized different storage compound strategies in trees, identifying 'starch' and 'fat' (i.e. lipid storing) tree species. Seasonal dynamics in reserve compounds of forest tree species were investigated early in the last century (Fabricius, 1905) and have remained a focus in tree physiology research (e.g. Sinnott, 1918; Hoch *et al.*, 2003). The response of NSC concentrations to environmental cues has been studied in apple trees for > 80 yr (e.g. De Long *et al.*, 1930; Oliveira & Priestley, 1988), whereas recent observations of increased tree and forest mortality due to drought and heat have sparked new interest in the role of NSC in tree survival under changing environmental conditions (e.g. Galiano *et al.*, 2011; Adams *et al.*, 2013; Anderegg & Anderegg, 2013; Hartmann *et al.*, 2013a,b; Obrien *et al.*, 2014).

NSC dynamics in tissues of trees and woody plants have been the focus of literature reviews (Oliveira & Priestley, 1988; Loescher *et al.*, 1990; Kozlowski, 1992; Magel *et al.*, 2000; Dietze *et al.*, 2014) and research (Lacointe *et al.*, 1994; Hoch *et al.*, 2003; Richardson *et al.*, 2013) during recent decades. Most of these have relied on the quantification of temporal variations in NSC concentrations or content (concentration × tissue biomass) to infer plant C dynamics (e.g. Galiano *et al.*, 2011; Piper, 2011; Hartmann *et al.*, 2013b; Mitchell *et al.*, 2013). However, recent findings have emphasized the difficulty of measuring NSC accurately in plant tissues (Quentin *et al.*, 2015), which in turn limits how changes in NSC can be quantitatively compared to other tree C fluxes. Only a few studies have investigated C fluxes by concomitantly quantifying several NSC sinks including growth, respiration and storage (Hartmann *et al.*, 2015b; Klein & Hoch, 2015). Although these approaches may be much more insightful for tree C relationships than concentration or pool size measurements (Ryan, 2011), they still infer fluxes from changes in NSC concentration over time and may require the assumption of steady state on longer timescales.

One major theme we emphasize throughout this review is that measured concentrations of NSC in tree organs cannot be interpreted explicitly in terms of 'storage' functions, but that storage is the result of asynchronies in the supply and demand for C currency compounds that occur across a range of timescales and, in part, reflect distances between source and sink tissues. Thus, NSC concentrations not only have been used as an easily measurable metric of C storage, but also have been interpreted as a plant function (storage) *per se* (Fig. 1). Such circular reasoning (Dietze *et al.*, 2014) is also reflected in the prevailing assumption embedded in models that C supply from photosynthesis – an easily measured parameter, at least at the leaf level and over short time periods – controls plant growth, as opposed to stoichiometric, genetic or other factors that are more difficult to assess (Körner, 2015). Research spanning molecular biology to ecophysiology and ecology will be needed to break out of this cycle and to achieve predictive understanding of how plant NSC interact with environmental change.

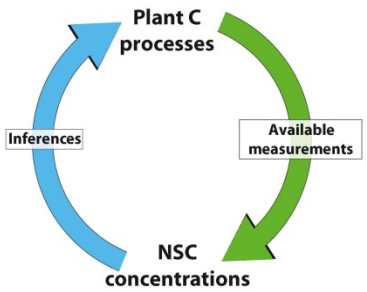

Fig. 1 An illustration of how research on the roles of nonstructural carbohydrates (NSC) in plant functioning can suffer from circular logic. Processes in plant carbon (C) relationships are often difficult to measure directly (e.g. use of different substrates in respiration) and are investigated using available measurements, like changes in NSC concentrations. In turn, these changes are frequently interpreted as a process (e.g. reserve formation/accumulation) although the regulatory mechanisms driving this process have not been addressed directly. Such circular reasoning leads to investigations on 'what we can measure' instead of 'what we want to know'.

Especially as the combinations of stresses experienced by trees are altered in the context of climate change and enhanced atmospheric CO_2 (Niinemets, 2010; Trumbore *et al.*, 2015a), questions about the central role of NSC are critical for predicting resilience of trees that differ in life stage (ontogeny) and life strategies (phylogeny). In this paper, we give particular emphasis to the regulation of NSC storage in trees. This is because trees are long-lived organisms likely to encounter many stressful periods throughout their lifetimes (Petit & Hampe, 2006; Niinemets, 2010) and trees may have to allocate NSC to long-term storage at the expense of other sinks to ensure survival (Wiley & Helliker, 2012). In forests, NSC pools are large enough for their changes to be important in annual stand-level C balance (Richardson *et al.*, 2013) and given the central role of NSC in plant functioning, it is not surprising that many vegetation models are based on the analogy of mobile C as currency or cash flow (McDowell *et al.*, 2011; Klein & Hoch, 2015). Hence, a thorough understanding of the process-oriented functioning of NSC in general and its interaction with C storage is key to realistic predictions of forest dynamics under changing environmental conditions (Dietze *et al.*, 2014), as well as to understanding feedbacks between forests and the global C cycle. Questions that need to be addressed to increase our understanding of the role of NSC in plant functioning include: How important is NSC storage in tree C allocation strategies? How are NSC used to mediate storage with demand from other plant functions (growth, defence, reproduction, osmoregulation, symbiosis)? Is there a critical threshold below which NSC are no longer available to support these functions, and what defines it? Can we predict how NSC influence tree response to multiple stress factors, and how that might vary with tree growth stage or life strategy?

Our goal in this review is to provide an overview of recent progress in answering these questions, geared to those who may be entering the field. We begin with an overview (section II) of the

different forms and functions of NSC in trees, giving examples of how one type of stress, drought, can act to alter those functions. In section III, we review tools, including new isotopic approaches, used to measure and infer dynamics of NSC. Section IV summarizes how application of these tools has resulted in new ideas of storage in trees that includes mixing and transport within tree stems, and section V proposes manipulative experiments designed to further elucidate the role of NSC in trees subjected to environmental stress.

Throughout the paper, and especially in section II, we use drought as an example of environmental stress that impacts NSC in trees. Drought-induced tree mortality has been recognized as a major survival threat for global forest ecosystems (Allen *et al.*, 2010) and there is substantial evidence that the global vulnerability to climate change-induced forest die-off is highly underestimated (Allen *et al.*, 2015). Recent studies focusing on drought-induced tree mortality, whether based on field observations or manipulative experiments, have failed to provide a clear line of evidence of the physiological processes during drought-induced mortality (Hartmann, 2015; Hartmann *et al.*, 2015a) and how NSC is affected by drought (Hoch, 2015). As a result, the interest in what factors control allocation of C to NSC storage pools and the overall role of storage in plant resilience to environmental stress has increased recently (Wiley & Helliker, 2012; Dietze *et al.*, 2014). Although our understanding of physiological responses to environmental stress such as drought is quite well developed for particular processes including photosynthesis (Flexas *et al.*, 2005; Atkin & Macherel, 2009; Körner, 2015), this is not the case for many whole-plant processes such as regulation of NSC partitioning across different sinks, especially during stress.

II. NSC in plant function: synthesis, classes, roles and responses to drought

In the Calvin cycle during photosynthesis, CO_2 is bound to a 5-carbon molecule but the resulting unstable 6-carbon molecule ultimately splits into two 3-carbon triose phosphate molecules. These subsequently form the primary product of photosynthesis, glucose, which in turn is used for the synthesis of most other plant compounds and structures. Primary and secondary cell walls are made of structural carbohydrates including cellulose, the most common organic polymer with an estimated annual production of 1.5×10^{12} tons globally (Klemm *et al.*, 2005), and hemicellulose. Plants lack enzymes for cellulose degradation (Pallardy, 2008), so C in these compounds is not available to the plant for future use (but see Murneek, 1929, and Hoch, 2007, for a potential role of hemicelluloses as mobile reserve compounds). By contrast, NSC can undergo frequent transformations including the conversion of sucrose to starch in leaves during daytime and conversion back into sucrose during night, or the transformation of glucose and fructose into sucrose for transport (Gibon *et al.*, 2009).

Carbohydrates comprise a variety of different compounds that serve a range of functions. Here we concentrate on those that make up the majority of NSC (i.e. form the biggest C stores). Monosaccharides are mainly simple sugars containing more than three C atoms, but the most common monosaccharides have five (*pentoses* like arabinose, xylose or ribose) or six (*hexoses* like glucose, fructose or galactose) C atoms. These molecules are usually used as osmolytes and as substrates for respiration and for the synthesis of other molecules (Table 1). In contrast to monosaccharides, disaccharides such as sucrose (table sugar) can be hydrolysed to smaller carbohydrates and sucrose is the most common transport sugar in plants. Oligosaccharides such as stachyose or raffinose usually contain between three and nine simple sugars and can be important compounds in phloem transport in some tree species. Polysaccharides are long chains of monosaccharides that can have a linear form, such as cellulose and amylose, or a branched structure, such as amylopectin. Amylose and amylopectin are the constituents of the most common storage carbohydrate, starch, and have the advantage over other carbohydrates in being osmotically inactive, allowing plants to accumulate them in large quantities. Besides NSC, lipids are important storage compounds and are used by plants as substrates for respiration but also for plant defence and communication (Table 1), although we cannot thoroughly review recent and very exciting developments, especially those in volatile organic compounds, here.

Thus, NSC are directly or indirectly involved in all plant functional processes of primary and secondary metabolism (Fig. 2) and some of these functions, such as growth, comprise a whole suite of individual processes which are under complex regulatory control in response to environmental cues (Taiz & Zeiger, 2002). We will briefly highlight some processes that are key to understanding NSC dynamics, in particular storage. These processes are tightly linked to plant metabolism (e.g. photosynthesis and respiration), defence, osmoprotection and osmoregulation, transport of C and water, export and exchanges with symbionts. Hence, NSC storage is an integrative function relating all of these processes (Fig. 2). As examples of the complexity of tree response to environmental stressors, we have added in each case how drought can change NSC. For conciseness, however, many other processes, such as the biosynthesis of secondary metabolites for attraction of pollinators or as antioxidants, are not covered here because they do not represent, at least under normal conditions, a large proportion of the plant C balance (Kesselmeier *et al.*, 2002). They are, nonetheless, quite important for plant functioning (Swain, 1977).

Table 1 Classes, examples and functional roles of common carbohydrates (and lipids) in plants

Class	Examples	Functional roles
Monosaccharide		
5-C sugars	Arabinose, xylose, ribose	Metabolism & osmolytes
6-C sugars	Glucose, fructose, galactose	
Di- and Oligosaccharides		
	Sucrose, raffinose, stachyose	Transport sugars
Polysaccharides		
Straight chain	Hemicellulose, cellulose	Structural biomass
Branched	Amylose, amylopectin	Storage
Lipids		
	Triglicerides, fatty acids	Metabolism Energy storage
	Terpenoids and derivatives	Defence Communication Antioxidants

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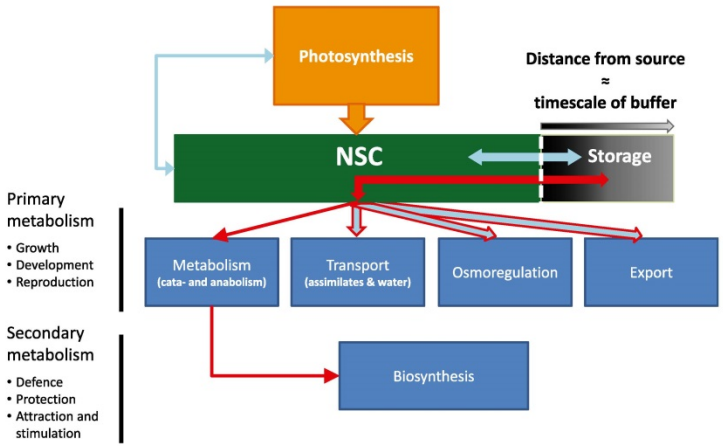

Fig. 2 The primary products of photosynthesis, that is, nonstructural carbohydrates (NSC) play a central role in plant functioning as the substrate for all plant metabolic activity. Hence, all catabolic and anabolic processes, including primary or secondary metabolites, are tightly coupled with the availability of NSC. Storage (indicated by a separate NSC pool) shares this central importance with the NSC pool. As pictured, storage NSC can be exchangeable with more recently fixed NSC. One major area of current debate is whether allocation of new photosynthetic products to storage is an actively regulated process (in which case storage would compete with the other processes pictured) or is merely an overflow buffer that balances inequalities in supply and demand. Actively regulated C allocation processes are indicated by red arrows, overflow processes by turquoise arrows. Note that allocation can be mediated by both processes although overflow likely may not be very important for metabolism and biosynthesis. The timescale of buffering provided by NSC storage depends on its distance from sources like photosynthetic or conductive tissues (see also Figs 3 and 4).

1. Photosynthesis and metabolism

During photosynthesis plants transpire hundreds of molecules of water for each molecule of CO_2 they assimilate (Taiz & Zeiger, 2002). Most of this water is transpired and only a small fraction is used to provide electrons for the light-dependent reactions of photosynthesis. Vascular plants control water loss from transpiration via regulation of stomatal aperture when soil water availability is reduced or when water vapour deficit is high. Regulation in response to declining soil water availability is triggered by root signalling via phytohormones causing stomata to close (Brodribb & McAdam, 2011); however, stomatal closure also reduces CO_2 diffusion into the leaf. According to a current framework for plant drought responses (McDowell *et al.*, 2008), stomatal closure during drought reduces C uptake and forces trees into NSC storage dependency, thereby linking a tree's capacity to mobilize and use stored NSC as a source for metabolic activity, defence against pathogens and for osmoregulation directly to tree survival (McDowell, 2011). However, drought also decreases plant water potential and cell turgor required for cell expansion following mitosis and this leads to a concomitant reduction in C demand for growth. Because growth declines earlier during drought than photosynthesis, NSC pools may actually increase in early phases of drought (Muller *et al.*, 2011) but metabolic needs for respiration and osmotic adjustment may reduce NSC pools during longer droughts until the plant dies (McDowell, 2011).

Observations of NSC concentration changes in trees during drought are rather inconclusive, though; concentrations may increase or decrease or not change at all (e.g. Galiano *et al.*, 2011; Galvez *et al.*, 2011, 2013; Anderegg & Anderegg, 2013; Mitchell *et al.*, 2013; Poyatos *et al.*, 2013) and this response can even vary across plant organs of individual trees (Hartmann *et al.*, 2013b). Our current understanding of NSC dynamics during drought is very limited because research has focused on NSC concentrations, and aboveground compartments, rather than on all of the functions and organs that are affected by drought. The interplay of sink activities (e.g. growth vs NSC) at the whole-tree level has so far been assessed only in natural settings using a top-down (ecosystems to tree) approach (e.g. Klein & Hoch, 2015) or under strictly controlled experimental conditions on small trees (e.g. Hartmann *et al.*, 2015b).

Plant metabolism involves biochemical pathways that break down larger molecules into smaller units that are either oxidized to release energy (catabolism) or processed in other reactions to produce different molecules (anabolism). The energy and the C skeletons required for biosynthesis and cellular maintenance are produced by carbohydrate catabolism (respiration), generally releasing 30–40% (Poorter *et al.*, 1990) and theoretically up to *c.* 80% of previously assimilated C (Amthor, 2000). Respiration in plants can be achieved mainly via the glycolytic and the tricarboxylic acid (TCA) pathways. Carbohydrates are converted into pyruvate or malate via glycolysis or the oxidative pentose phosphate pathway (OPP), respectively. Glycolysis generates reducing power in the form of a net gain of both ATP and NADH, OPP produces pentoses as well as reducing equivalents in the form of NADPH. Hence, OPP as a major source of reducing power and metabolic intermediates for biosynthetic processes plays a rather anabolic role (Kruger & von Schaewen, 2003). Yet another type of respiratory pathway, originally identified in thermogenic inflorescences of the Araceae, the 'voodoo lily' (Meeuse, 1975), diverts

electrons from the cytochrome pathway into an alternative pathway, often referred to as 'cyanide-resistant respiration' (McIntosh, 1994). This pathway occurs under conditions of imbalance between upstream respiratory C metabolism and downstream electron transport and helps to prevent the generation of harmful active oxygen species (Vanlerberghe & McIntosh, 1997). Plants can employ the alternative oxidase pathway to adjust energy production vs substrate oxidation under stressful conditions like drought (Atkin & Macherel, 2009).

Drought affects tree metabolism through combined effects associated with higher temperatures (in 'hot' droughts) and transport limitation, as stomatal closure slows or stops water transport between roots and canopy. Respiration (production of CO_2) is normally used as the measure of metabolism in trees and generally increases with temperature (Ryan, 1991), at least up to a certain temperature optimum, but respiration can acclimate to elevated temperature within a few days (Bryla *et al.*, 1997) and this acclimation may be due to reduced substrate availability (Atkin & Tjoelker, 2003). By contrast, drought alone reduces whole-plant respiration (Hartmann *et al.*, 2013a) and root respiration (Bryla *et al.*, 1997; Burton *et al.*, 1998; Hartmann *et al.*, 2013b). Also, drought-induced declines in whole-plant respiration can occur even if NSC increases in the canopy due to reduced transport of substrates from sources to sinks (Hartmann *et al.*, 2013a,b). In combination with elevated temperature, however, drought leads to higher cumulative respiratory fluxes in leaves compared to drought only, causing a greater whole-plant C loss and potentially starvation (Adams *et al.*, 2009). Moreover, drought seems to hamper not only NSC mobilization, but also other catabolic processes, resulting in reduced use of alternative respiration substrates like lipids or proteins (Fischer *et al.*, 2015).

2. Defence

Plants produce numerous organic compounds that are involved in growth processes (primary metabolites) or play a role in a wide variety of functions ranging from defence and attraction to physical protection (secondary metabolites). Given the common metabolic origin of these compounds, the photosynthetic carbohydrates, it becomes self-evident that investigations of NSC dynamics should also consider the potential role of secondary metabolites in response to stress.

Although it is commonly believed that plants under stress are more vulnerable to additional disturbances (Manion, 1991), stresses such as drought usually result in increased concentrations of secondary metabolites (Gershenzon, 1984; Mattson & Haack, 1987) which likely reflects the accumulation of NSC from decreased growth sink activity (Herms & Mattson, 1992). However, accumulation of NSC may only occur during early phases of drought, and declining NSC availability during longer droughts may cause a reduction of allocation to defence compounds (Steele *et al.*, 1995). Patterns of NSC concentration change may also differ for canopy/distal branches, stems and roots, depending on how drought has affected transport of NSC within the plant. Additionally, the catabolism of fatty acids fuels the synthesis of volatile organic compounds (VOC), including jasmonic acid and methyl jasmonate, that provide protection against cellular and tissue damage from reactive oxygen species (Holopainen, 2004). Such a protection is particularly important during drought stress (Apel & Hirt, 2004; Cruz de Carvalho, 2008). How NSC availability and the production of VOCs from fatty acid catabolism interact at the whole-plant level, particularly under drought conditions, is not known.

3. Osmoregulation and osmoprotection

Plants can actively control the osmotic cell pressure to avoid tissue water deficit and to maintain physiological functioning under decreasing water availability by increasing the concentration of compatible (i.e. not interfering with other metabolic processes) substances (Morgan, 1984). Plants employ a variety of different compounds for osmoregulation: mainly sugars, sugar alcohols and amino acids, but also hydrophilic proteins (Chaves *et al.*, 2003). These are synthesized in the cytoplasm from imported carbohydrates and cause a decrease in the cell osmotic potential that triggers diffusion of water into the cytoplasm from the vacuole and/or from neighbouring tissues (Morgan, 1984). Compatible organic solutes protect plants during desiccation and against freezing damage by stabilizing proteins complexes and membranes (Bohnert *et al.*, 1995; Ögren *et al.*, 1997; Oliver *et al.*, 2011). Declines in NSC during extended droughts will also affect osmoregulation and osmoprotection directly via availability of sugars and indirectly via reduced synthesis of compatible solutes. However, the minimum thresholds of NSC required to maintain osmoregulation and osmoprotection are currently unknown (Adams *et al.*, 2013).

4. Transport

NSC allows translocation of energy and C skeletons for biomass production across plant organs; that is, from sources to sinks. In source organs (e.g. photosynthetic tissue, storage organ during remobilization) sugars are either actively loaded into the phloem against a sucrose concentration gradient between mesophyll and phloem companion cells (CC) or along such a gradient ('passive loading'; Rennie & Turgeon, 2009; De Schepper *et al.*, 2013). Active phloem loading occurs in the apoplast via sucrose uptake transporters and requires energy (Ayre, 2011), whereas symplastic phloem loading traps larger molecules in CC that are synthesized from sucrose diffusing through plasmodesmata in the cell membranes of bundle sheath cells (BSC). This passive loading process is driven by a sucrose gradient between BSC and CC that is maintained by the conversion of sucrose to oligosaccharides, like stachyose or raffinose in CC (Turgeon, 1996) or by maintaining a high concentration gradient of sucrose or sugar alcohols between mesophyll cells and CC without polymer trapping (Turgeon, 2010). Although most woody species exhibit phloem cell characteristics indicative of symplastic loading, several tree species including ash (*Fraxinus*), apparently use a redundant loading strategy involving both apoplastic and symplastic transport (Gamalei, 1989; Oner-Sieben & Lohaus, 2014).

Phloem transport is thought to be driven by a pressure build-up in source tissues (Münch, 1930) which is created when water moves

into the highly concentrated phloem solution via osmosis. The resulting hydrostatic pressure in the phloem cells then pushes the sap to adjacent cells and all the way to sink tissues (meristems, storage tissues) where the pressure is released by actively withdrawing or leaking out of sugars from the phloem (Ryan & Asao, 2014). Hence, phloem transport is dependent on a plant's capacity to build up hydrostatic pressure via water movement into the phloem from adjacent xylem (Hölttä *et al.*, 2009). During drought, however, declining plant hydration causes a reduction in xylem water potential and in combination with reduced NSC availability can negatively affect phloem transport. Lower water availability in the xylem and less water movement into the phloem increases phloem viscosity and reduces phloem cell turgor, hence impairing sap movement through the vascular network (Sevanto, 2014; Mencuccini *et al.*, 2015). Although elevated temperatures during drought may reduce phloem sap viscosity to some degree, reduced NSC and water availability during drought have a much greater negative effect on phloem transport (Allen *et al.*, 2015).

Recently, the potential role of NSC in maintenance of xylem transport has been highlighted. Due to the great size of trees, long-distance transport of water faces a high pathway resistance and gravitational forces that cause strong xylem tensions and increase the risk for xylem cavitation (Sperry & Tyree, 1988). The hydraulic system of trees is operating close to the threshold of cavitation (Choat *et al.*, 2012) but hydraulic failure – that is, complete interruption of water transport due to runaway xylem embolism – may not occur as frequently as previously thought, at least not under nondrought conditions (Cochard & Delzon, 2013). Recent theoretical advances show that embolism may be repaired via water inflow from surrounding nonembolized vessels and driven by an osmotic gradient established via importing low molecular-weight sugars into embolized conduits (Brodersen *et al.*, 2010; Nardini *et al.*, 2011). This mechanism is currently hotly debated because methodological artefacts associated with conductivity measurements may yield spurious evidence for general occurrence of embolism repair (Sperry, 2013; Wheeler *et al.*, 2013). Although some suggest that the frequency of xylem refilling in trees may be greatly overestimated (Cochard & Delzon, 2013; Delzon & Cochard, 2014), others proclaim xylem embolism repair to be a common phenomenon in trees (e.g. Trifilò *et al.*, 2015). Although there is no agreement on the importance of embolism repair, trees maintain sometimes large fractions of living ray and axial parenchyma cells in their stems (Morris *et al.*, 2016) and these cells contain substantial amounts of NSC that could play important roles not only for embolism repair, but also for osmoprotection against freezing or as C reserve for pathogen defence responses in conducting xylem tissue (Plavcová & Jansen, 2015).

5. Export: symbiotic interactions and root exudation

Complex biological and ecological processes occur in the rhizosphere with potentially synergistic and positive effects for plant growth and development (Bais *et al.*, 2006). Belowground interactions with rhizosphere bacteria (Yang *et al.*, 2009), mycorrhizal fungi (Ruiz-Lozano *et al.*, 1995) or nitrogen-fixing bacteria (Sprent, 2009) can assist plants in tolerating abiotic stress while being competing sinks in a plant's NSC partitioning. Over two-thirds of terrestrial plants interact with arbuscular mycorrhizal fungi which provide them with resources, such as water and minerals, in exchange for carbohydrates (Smith & Smith, 2011), and up to 20% of the plant photosynthetic C yield can be allocated to fungi (Wang *et al.*, 1989; Smith & Smith, 2011). Carbohydrate transporters in plant cell membranes regulate this exchange at the plant–fungus interface (Doidy *et al.*, 2012), providing glucose as the main exchange carbohydrate to the fungus (Schüßler *et al.*, 2006; Nehls *et al.*, 2010), but it remains uncertain whether the plant or the fungi control this exchange (Fitter *et al.*, 2011).

In addition to contributing to soil processes through symbionts, roots exude a wide range of small molecular weight compounds directly into the rhizosphere, including inorganic ions, oxygen and water, enzymes, and a diverse array of C-rich primary and secondary metabolites (Uren, 2007). Root exudation has been recognized as an important mediator for interactions between roots and both pathogenic and beneficial soil microbes, soil invertebrates and roots of competing plants (Walker *et al.*, 2003; Weir *et al.*, 2004). Root exudation represents a substantial C cost to the plant, yet the proportion of soluble sugars (as opposed to mucilage or litter) may be small at < 1% of total photosynthetic yield (Uren, 2007). Although difficult to measure, studies demonstrate that exudation increases with drought and other stresses (Henry *et al.*, 2007), including during winter freezing of soil in alpine forest (Scott-Denton *et al.*, 2006).

6. Storage

Storage in plants has been defined as 'resources that build up in the plant and can be mobilized in the future to support biosynthesis' (Chapin *et al.*, 1990, p. 424) so as to buffer any asynchrony of supply and demand which may occur on diel, seasonal or decadal (or longer) temporal scales and across plant organs. As such, storage plays a critical role in most if not all plant functional processes and is of a central importance for understanding plant functioning at the whole-tree level. Basically all models of woody vegetation, spanning scales from individual trees to forests and ecosystems, include some NSC storage component; however, the limited understanding of resource redistribution within plants in general and, in particular to and from storage, is mirrored by the lack of realism in the implementation of allocation to storage in models (Dietze *et al.*, 2014; Fatichi *et al.*, 2014).

In their early work on plant storage economy, Chapin *et al.* (1990) differentiated three distinct processes: (1) accumulation (build-up of resources when supply exceeds demand); (2) reserve formation (metabolically regulated synthesis of storage compounds, competing with other sinks like growth and defence); and (3) recycling (reutilization of compounds involved in growth or defence during later metabolization). This definition includes, therefore, both an overflow process (accumulation) and an actively regulated component of storage (reserve formation). Interestingly, even some 30 yr after this seminal work, there is still discussion about whether C storage may be either 'passive' (*sensu* accumulation) or 'active' (*sensu* reserve formation) or both (Sala *et al.*, 2012; Wiley & Helliker, 2012).

Trees are long-lived organisms displaying delayed maturity and therefore have to secure long-term survival to maintain population fitness (Petit & Hampe, 2006). Reserve formation may be an evolutionary response to C limitation allowing survival during unfavourable conditions, such as extended shading or recurrent severe herbivory (Wiley & Helliker, 2012). However, storage pool concentrations may increase even under unfavourable conditions, as observed in evergreen conifers with increasing altitude (Hoch & Körner, 2012). but such increases may be due to cold temperatures reducing growth more than photosynthesis. This growth limitation hypothesis (Körner, 1998) explains accumulation of carbohydrates from photosynthesis by limitations of sink activity (slow growth), and has further led to the idea that trees are not C-limited under current atmospheric conditions (Körner, 2003). Moreover, NSC concentrations in tree tissues have been shown to increase even when other sink activities such as latex production are increased (Silpi *et al.*, 2007) or when source activity was decreased, for example through defoliation (Wiley *et al.*, 2013). Such results have been taken as supportive evidence for upregulation of storage formation as to increase the safety margin in the face of environmental stochasticity (Sala *et al.*, 2012). In fact, NSC storage has been linked to seedling survivorship under unfavourable conditions such as sub-canopy shade (Kobe, 1997) and drought (Obrien *et al.*, 2014), but data on the role of NSC in survival of mature trees are sparse, inconclusive or missing.

III. Tools and approaches for quantifying NSC dynamics

Mature trees store enough NSC to refoliate the canopy at least once (Würth *et al.*, 2005) or up to four times (Hoch *et al.*, 2003). Both concentrations and pool sizes can show strong seasonal and interannual variation. When these are summed to the stand level these differences are large enough to be a significant component of net ecosystem exchange (Tschaplinski & Hanson, 2003) and provide an explanation for mismatches between eddy covariance-based net ecosystem exchange measurements and growth-based net primary production (Richardson *et al.*, 2013). This section explores the methods used to measure NSC in plant tissues and to infer its dynamics using new isotopic tools.

Although Chapin *et al.* (1990) stated that concentration and pool size (concentration × tissue mass) are both useful for describing the production and use of NSC in plants, several problems limit inference of NSC dynamics and their role in tree- and stand-level C budgets from concentration measurements alone. First and foremost, absolute concentrations of sugars and starches are hard to quantify accurately. A recent laboratory inter-comparison by Quentin *et al.* (2015) demonstrated large unexplained biases in NSC concentration measurements with only some uncertainty related to different extraction and quantification procedures. In addition, trees may meet metabolic needs by shifting from carbohydrates to other reserve compounds such as lipids, or by recycling existing metabolites like proteins (Fischer *et al.*, 2015) and both are rarely measured in tree ecophysiological studies. Such problems severely limit the usefulness of any metric derived from concentrations measurements for mass-balance approaches for whole-plant C balance estimation, for example, by comparison with measures of net photosynthesis and respiration fluxes (Hartmann *et al.*, 2015b). Direct assessments of whole-plant C balance are already challenging for smaller individuals (Zhao *et al.*, 2013) but are exceedingly difficult in mature trees (Ryan, 2011) and hence new approaches are needed.

Measurements of NSC concentrations are useful for estimating changes in the net C balance of trees (Hoch, 2015), but we advocate a shift from measuring only NSC concentrations and pool sizes to a more process-oriented approach that emphasizes NSC fluxes and especially functions. One basis for this shift is the fact that storage has a temporal dimension: that is, imbalances between C sources and sinks that cause changes in NSC concentrations can take place on day–night, seasonal or even longer timescales, depending on the location, chemical nature and function of substrate materials. This temporal dimension can be addressed using a suite of isotopic tracers (see Box 1), whether via stable isotope labelling (Epron *et al.*, 2012), application of a multi-year label associated with FACE experiments (reviewed in Niinemets, 2010) or using the decades-old radiocarbon labelling associated with atmospheric nuclear bomb testing (Trumbore, 2006). Recent developments in laser-based analytical devices now allow online measurements of the isotopic composition of plant gas exchanges for real-time tracing of ^{13}C label through individual sugars and starch in different organs to respiration as CO_2 (Bahn *et al.*, 2013; Blessing *et al.*, 2015). Newly introduced approaches from other disciplines, such as cavity-enhanced Raman spectroscopy, also allow *in situ* investigations of respiratory quotients that have not been possible before (Keiner *et al.*, 2013; Hanf *et al.*, 2015) and site-specific labelling of metabolic substrates may allow insights into biochemical pathways of C metabolism (Tcherkez *et al.*, 2012). The following sections will address how the application of these new tools in investigations of plant C relationships allow us to go from just measuring 'how much NSC and where they are located' to 'under which conditions and to what extent are NSC used' to support plant function.

IV. What is the spatial and temporal distribution of NSC in trees?

The most commonly used measure of NSC dynamics has been tracking temporal variation in NSC concentrations in plant tissues. These measures indicate a range of temporal dynamics according to tissue type (Fig. 3). Sugars produced in the leaf are converted to starch and stored in the chloroplast during daytime (i.e. when supply > demand) to be remobilized and used for growth during the night (Geiger *et al.*, 2000). This diel dynamic is under tight genetic orchestration and allows plants to avoid C starvation (i.e. depletion of starch before new supply by photosynthesis) even during night-time periods of variable length (Gibon *et al.*, 2004).

Stems and coarse roots comprise the main woody volume of trees. Thus, even though their NSC concentrations are relatively low, they account for most of a tree's NSC-stock. Despite the fact that the central cylinder of the stem is often composed of vascularly dysfunctional secondary xylem (heartwood), in a number of species living parenchyma cells in wood rays span from the outer trunk inwards and into the sapwood–heartwood transition zone (Frey-

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Box 1 Tools for assessing nonstructural carbohydrate (NSC) dynamics on timescales ranging from hours to centuries

Assessments of NSC concentrations have a long tradition, dating back more than a century (Hartig, 1878). However, the diversity of extraction protocols and of measuring methods (e.g. high-performance liquid chromatography, photometric analysis, gas chromatography) along with a varying efficiency of extraction protocols to specific tissue matrixes makes NSC concentration measurements inaccurate and hence do not allow comparisons between studies (Quentin *et al.*, 2015). Given that such measurements are nonetheless precise (high repeatability within a given lab) they can be used for comparisons between treatments and changes in NSC over time within an experiment/observation, as long as no changes in tissue matrix chemistry occur (e.g. heartwood formation). For such studies, the use of natural ^{13}C abundances allows inferences on physiological parameters like water-use efficiency (Farquhar & Richards, 1984) or storage remobilization (Helle & Schleser, 2004). Some compounds such as lipids or amino acids may not be regularly measured but still represent important reserves.

Pulse labelling is useful to investigate processes that involve several pools, like source–sink relationships, and for pools with turnover times from days to months, in exceptions a couple of years (Kagawa *et al.*, 2006; Keel *et al.*, 2007). Pulse labelling allows studying how fast and where the labelled carbon (C; e.g. photosynthetic sugars) is allocated among competing pools. Allocation priorities can be estimated by comparing isotopic signals across the ensemble of pools (Hartmann *et al.*, 2013b) and the decay over time allows estimating turnover times of the pools. Pulse labelling normally involves the use of ^{13}C (where the label can reach levels up to 50–100 times background levels (1%), or 14C (where levels up to 10^3 times background can be achieved depending on radiation regulations, and even higher if radiation protocols are followed (Carbone & Trumbore, 2007; Epron *et al.*, 2012)).

Continuous labelling can be applied to investigate processes that unfold over several months to many years. Due to the greater running cost the strength of the applied isotopic tracer is usually much lower than for a pulse label (e.g. 10^1–10^2 per mil vs 10^2–10^3 per mil VPDB). Continuous labelling allows estimating patterns of long-term allocation to pools such as stem or root woody biomass, as in FACE experiments (Körner *et al.*, 2005) but usually requires increasing $[CO_2]$ beyond current levels and hence does not allow inferences on 'normal' plant functioning (Epron *et al.*, 2012). This can be avoided by first reducing $[CO_2]$ below ambient before adding the tracer, as has been done in open-top chamber experiments (Hartmann *et al.*, 2013a), but this usually implies the use of seedlings or saplings instead of mature trees.

The bomb ^{14}C approach builds upon the doubling of the atmospheric radiocarbon concentration during thermonuclear weapons during the early 1960s and its subsequent exponential decrease from oceanic and terrestrial CO_2 uptake as well as emissions of ^{14}C-free CO_2 derived from fossil fuel combustion (Trumbore, 2006; Muhr *et al.*, 2013). By comparing the ^{14}C signal of plant biomass samples with the atmospheric radiocarbon curve (Levin & Hesshaimer, 2000), one can derive the average time since assimilation of the CO_2 in a given biomass pools, that is, often referred to as 'C age' (Richardson *et al.*, 2013, 2015). The ^{14}C signal hence allows estimating the degree of reserve use (i.e. the contribution of stored C) in processes that draw upon such pools, including respiration.

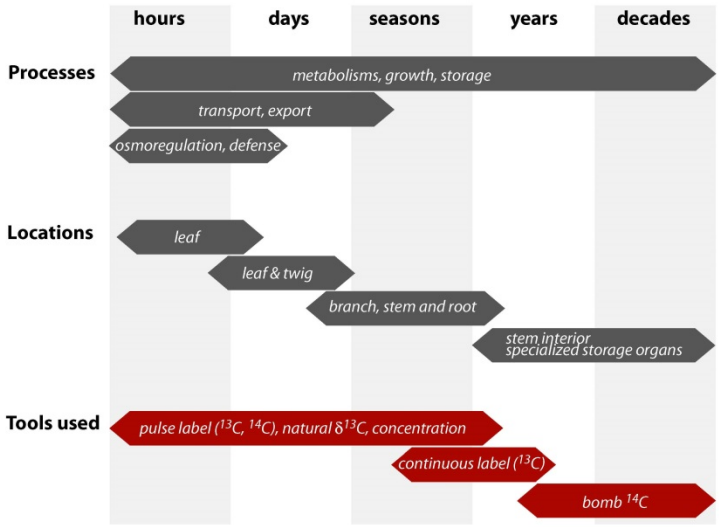

Figure Box 1. Temporal scales of the processes related to plant NSC storage, locations where these processes occur and the tools used for investigations. Note that for longer investigations spanning from seasons to decades isotopic tracers are required.

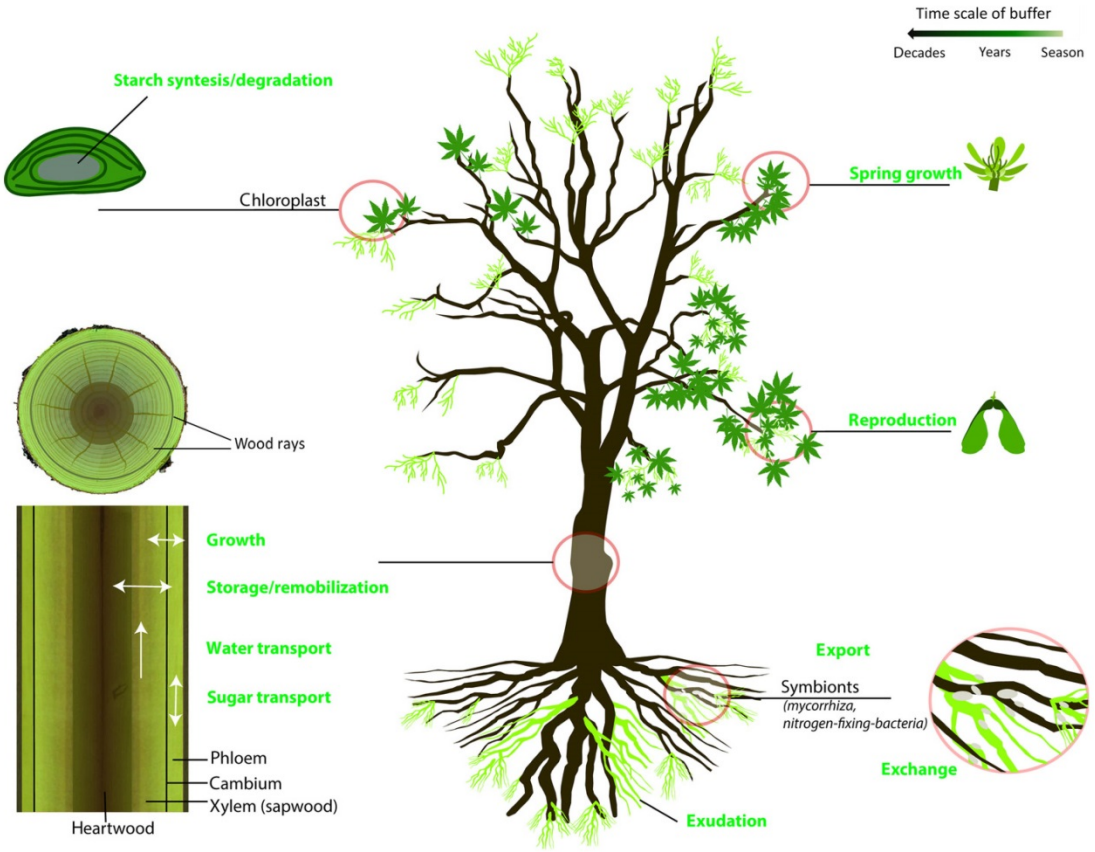

Fig. 3 Storage sites within the tree body, functional roles of nonstructural carbohydrates (NSC) and their temporal scales. Chloroplasts in leaves store starch during diel cycles to buffer diurnal over-supply of sugars and night-time demand for growth. Reproductive organs are generally made from current season photosynthetic carbon (C). Lighter green parts of the tree body (fine branches, fine roots, sapwood and phloem) indicate locations for seasonal storage pools, whereas darker green and brown parts (trunk, large branches, coarse roots) contain storage pools that may be mobilized only in small proportions during normal functioning but can make up most of the C source during catastrophic events that occur on decadal/centennial timescales, like severe and repeated herbivory, wind and ice-storm damage (branch breakage) or fire. Different pools are interconnected via assimilate translocation within the phloem (vertical transport) or via wood rays spanning radially from phloem in the trunk sapwood and heartwood. NSC are used at these different timescales to support metabolism (respiration), to provide C skeletons for structural and reproductive growth, to allow C translocation across organs, to maintain hydraulic integrity (osmoregulation and –protection, embolism repair) and for export (root exudation, exchanges with symbionts).

Wyssling & Bosshard, 1959; Ziegler, 1964; see Fig. 3). These rays connect living parenchyma cells in the sapwood (vascularly functional xylem) with the phloem and are thus interconnected with the assimilate translocation system, allowing both introduction and removal of carbohydrates (Ziegler, 1964). NSC concentrations usually decrease from the outer towards the inner sapwood zone in stems but remain constant from the sapwood–heartwood transition into the heartwood (Hoch *et al.*, 2003).

Seasonal and interannual variations in NSC concentration in woody branch and stem tissues have been reported in many temperate, tropical and subtropical tree species and other woody plants (Kozlowski & Keller, 1966). These patterns of accumulation and loss of NSC (especially starch) may or may not be correlated with cambial activity, depending on the species/life strategy studied (Cameron, 1923; Cockerham, 1930; Wight, 1933). For example, observations of increased NSC concentrations in branches and stems at bud break in several deciduous species led to the conclusion that initial growth in springtime is independent of storage compounds (Hoch *et al.*, 2003), whereas investigations using isotopic tracers (Kagawa *et al.*, 2006) or natural abundance ^{13}C (Helle & Schleser, 2004) suggested that growth of branch sections (longitudinal growth) and stems (tree-ring early wood), was fuelled by C from storage pools in branches and in sapwood that was fixed in previous years. Another possible reason for NSC increase at the end of the dormant season is photosynthesis in the bark of fine branches (Offermann *et al.*, 2011; Bloemen *et al.*, 2013).

Bomb radiocarbon measurements permit estimation of the mean time elapsed since C in NSC (NSC-C) was originally fixed from the atmosphere (see Box 1). The few measurements in trees made to date demonstrate an overall pattern of increasing mean age of NSC-C with greater distance from its sources in the canopy and phloem (Figs 3, 4). For example, in two temperate trees, the C extracted from branches had NSC with ^{14}C signatures indicating that it was fixed in the current year, whereas in fine roots it was several years old (Richardson *et al.*, 2015). Radiocarbon signatures of 'mobile' (i.e. water- or methanol-extracted) NSC reflect that its C is derived from recent photosynthetic products in the outermost growth rings of stems and coarse roots (Richardson *et al.*, 2015). With increasing distance from the phloem, mean NSC-C ages increase, to decades or even older (Trumbore *et al.*, 2015b). This 'mobile' C is consistently younger than the cellulose from which it was extracted (Richardson *et al.*, 2013, 2015; Trumbore *et al.*, 2015b), indicating that mixing of younger NSC-C inward into the stem exceeds fluxes of older NSC-C outward (Fig. 4). Data from a range of temperate tree species, ages and life traits (evergreen, deciduous) indicate that this net inward mixing of NSC is a common pattern that seems to scale across different tree species (Fig. 5). Starch extracted from stem wood tissue generally has the same ^{14}C content as low molecular weight carbohydrates, indicating either rapid exchange between these pools, or that the methods of extracting mobile NSC (which includes soluble compounds other than sugars) and starches are not actually separating them effectively by mean age (Trumbore *et al.*, 2015b).

Both radiocarbon analyses (Carbone *et al.*, 2013) and long-term ^{13}C label studies have demonstrated that stem wood (especially early wood in deciduous trees; Mildner *et al.*, 2014), leaf buds, new leaves and new fine roots (Keel *et al.*, 2007; Gaudinski *et al.*, 2009; Muhr *et al.*, 2016) are built from NSC-C derived from a mixture of new and older source C. Further, the observation that CO_2 emitted from tree stems (Carbone *et al.*, 2013; Muhr *et al.*, 2013) and respired by roots (see review by Hopkins *et al.*, 2013) is derived at least in part from C fixed years to decades previously, supports the idea that more distal, older, NSC stores in stem sapwood and living roots form part of a mixed pool available for supporting plant functions (Carbone *et al.*, 2013; Fig. 6).

Together, the combination of observational constraints – seasonal variations in NSC concentration and isotopic studies demonstrating contributions of multi-year C pools to growth and metabolism – has led to the formulation of new conceptual models of NSC dynamics. Models that explain both decades-old C in NSC in interior stems with younger C emitted from tree stems require a two-pool model with a 'fast' pool that varies in size seasonally and a 'slow' pool that acts as a buffer on multi-annual timescales (Richardson *et al.*, 2013). An alternative model (Trumbore *et al.*, 2015b) does not define pools but rather the timescales for lateral mixing and metabolism of NSC in the stem. In this model, it is the decline of mixing and metabolic rates within the stem that define the timescales on which old C persists. A mixture of C derived from metabolism of fast and slow pools is emitted from tree stems, with greater contributions from the 'slow' (or more distal) pool fuelling metabolism in the dormant season in temperate deciduous trees (Fig. 5).

Application of these models to various tree species and ontogenies highlights differences in NSC dynamics depending on the 'vigour' of the tree, with fast-growing 'vigorous' trees a larger 'fast' (and young) NSC pool which fuels current metabolism (Carbone *et al.*, 2013) and also mixes in toward the centre of the tree stem and to more distal and older pools (Trumbore *et al.*,

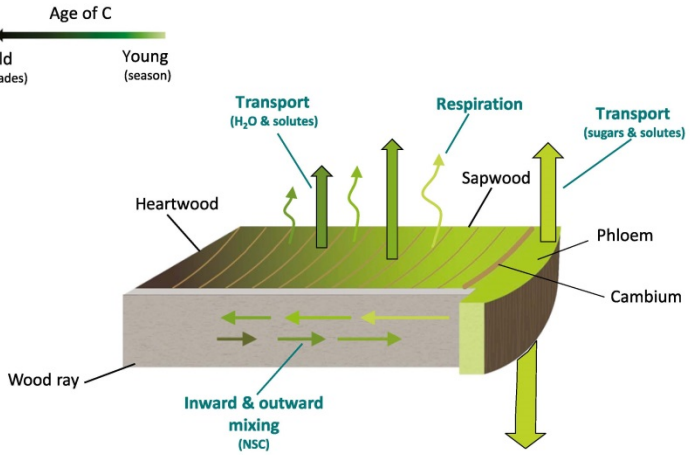

Fig. 4 Age pattern of translocated, stored and mobilized/metabolized nonstructural carbohydrates (NSC) in a schematic representation of a stem section. 'Young' carbon (C) is translocated in the phloem (light green) and deposited within parenchyma cells within the sapwood by transport via wood rays (grey). This inward transport creates a net inward mixing effect, that is, NSC located within a given tree ring is younger (see colour offset) than the structural biomass of that ring. CO_2 evolved from local metabolism carries the same ^{14}C signature (~age) as the locally stored NSC. During periods of storage dependency (e.g. drought, girdling), the outward translocation of stored NSC from inner stem parts causes an increase in the ^{14}C signature of the NSC in more peripheral tissues, such as in the phloem. This 'older' C may then be transported to other plant organs (e.g. roots).

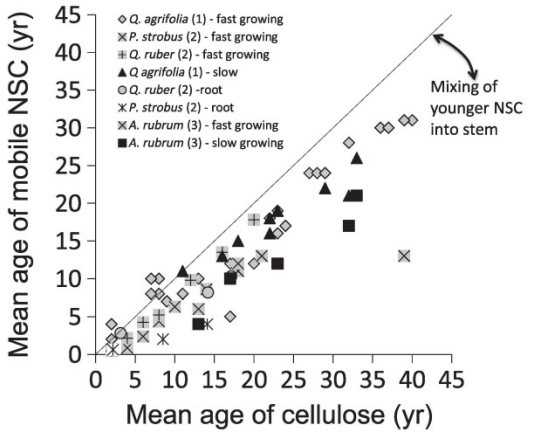

Fig. 5 Mean age of carbon (C; i.e. mean time elapsed since C was fixed from the atmosphere) for cellulose from tree rings (ref. 2; Richardson *et al.*, 2015) or in a defined section of stem core (ref. 1;, Trumbore *et al.*, 2015b) and (ref. 3; Carbone *et al.*, 2013) compared to the mean age of C in 'mobile' nonstructural carbohydrates (NSC) extracted from the same wood sample. The line indicates the 1 : 1 relationship. Points falling below the line indicate that C in mobile phases is consistently 'younger' than the structures where it is found. This indicates that even if NSC is mixed both outward and inward, the net movement is of younger C inward (see also Fig. 3). Abbreviations in the key: *Q*, *Quercus*; *P*, *Pinus*; *A*, *Acer*.

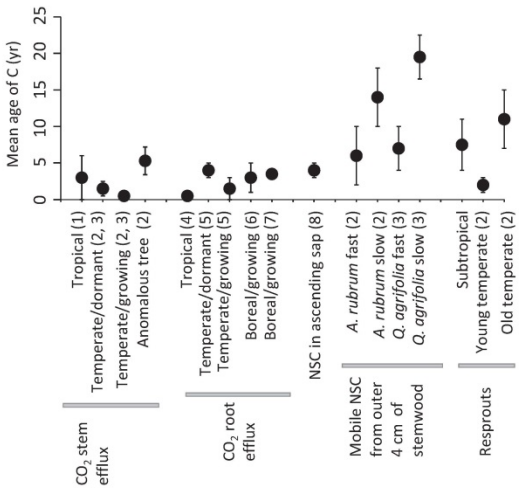

Fig. 6 Radiocarbon age (years ± 1 SD) of nonstructural carbohydrates (NSC) and its products (stem and root CO_2 efflux, biomass) for studies carried out in different biomes. Data are taken from (1) Muhr *et al.* (2013), (2) Carbone *et al.* (2013), (3) Richardson *et al.* (2015), (4) Trumbore (2006), (5) Hopkins *et al.* (2013), (6) Czimczik *et al.* (2006), (7) Schuur & Trumbore (2006), (8) Trumbore *et al.* (2015b); data on subtropical resprouts are from Vargas *et al.* (2009), young and old temperate from (2).).
Figure abbreviations: *Q*, *Quercus*; *A*, *Acer*.

2015b). By contrast, slow-growing, less vigorous trees produce a smaller 'fast' NSC pool which cannot always satisfy current metabolic demand, making the use of 'slow' (distal and old) pools necessary when fresh C supplies are low (Carbone *et al.*, 2013). Such conceptual models can be used to formulate hypotheses that are testable in mature trees, and suggest reasons why some trees may be more resilient to, for example, damage or extreme climate stress than others (Niinemets, 2010).

Results of isotope studies over a range of timescales highlight why NSC concentrations and pool sizes are not by themselves sufficient to quantify storage pool use. Fluxes into or out of NSC pools can only be detected when the pool sizes substantially change, and even then, they reflect net not gross fluxes. For example, Hartmann *et al.* (2015b), by assessing whole-tree C balance, showed that allocation of newly fixed C to NSC pools in stems can be maintained even when the net change in pool size is zero or even negative. In this study only the use of an isotopic marker (^{13}C) allowed tracing the flux of newly-assimilated C into the storage pool. The absence of response of NSC pools during periods of strong sink activity (Hoch *et al.*, 2003) can similarly be interpreted as indicating rapid turnover, if there are also C sources to offset C losses. Without information on both pool sizes and fluxes into and out of the pool such inferences cannot be made but fluxes are difficult to assess especially at the whole-tree level (Ryan, 2011). In the following section, we will highlight a selection of fundamental questions related to the role of NSC in plant functioning and show how some of the tools described above have been successfully employed to gain new and important insights.

V. Studies on the use of NSC in plant functioning – progress towards answering longstanding questions

1. To what extent are older or more distal NSC stores available for metabolism?

The use of storage reserves to support catabolism cannot be easily inferred from measurements of NSC concentration changes if transport processes, such as C translocation across organs, are not also addressed (Adams *et al.*, 2013). NSC dynamics needs to be tracked in all organs, especially in roots, where sustained negative C balance can contribute to tree mortality (Landhäusser & Lieffers, 2012). For some species living in colder climates, the respiratory demand of several months can be covered by a single day's C gain (Körner, 1998), making changes in NSC concentrations difficult to detect. However, as noted above, C isotopes in respired CO_2 can provide a sensitive detector for shifts in the balance of older and younger C sources for metabolism. Although this may be complicated by the fact that CO_2 efflux (e.g. from stems) can reflect a combination of respired (*in situ*) vs transported (remotely respired) CO_2 (Teskey & McGuire, 2002; Bloemen *et al.*, 2013), incubations of stem increment cores or roots can also be used to determine the isotopic signature of respiration sources (J. Muhr, unpublished data).

Several outstanding questions surrounding the role of NSC in tree resilience and mortality can now be addressed by combining available tools with manipulative studies. For example, one question related to the models of NSC in tree stems is whether

stores available to the plant are well-mixed or follow a first-in, first-out rule for accessibility. If accessible NSC are a well-mixed reservoir, we would expect the radiocarbon signature of metabolized C following treatments like girdling or shading that remove sources of newly fixed C to increase, thereby reflecting the use of NSC-storage sources, and then plateau. Moreover, if successively older and older (more and more distal) pools are accessed, we expect the mean age of respired CO_2 to continue increasing over time. Similarly, tracking how the age of both respired C and NSC change in various organs during tree death can shed light on whether residual NSC might be inaccessible for metabolism or simply not used in processes when trees approach mortality.

2. Do plants under stress shift sources of C substrates used in metabolism?

When under stress, plants may shift from NSC to burn fats or lipids, which are not normally quantified. Such shifts in substrate can be detected by measurement of the respiratory quotient (RQ = CO_2 evolved over O_2 consumed during respiration, as more O_2 is consumed to oxidize lipids than carbohydrates). New optical methods, including cavity-enhanced Raman spectroscopy, allow continuous *in situ* monitoring of changes in respiratory quotient associated with a shift from carbohydrate to lipid substrates for metabolism (Keiner *et al.*, 2013; Hanf *et al.*, 2015). Further, CO_2 evolved from lipid metabolism is more strongly depleted in ^{13}C than CO_2 evolved from carbohydrates (Gleixner *et al.*, 1993; Bowling *et al.*, 2008) and the natural abundance of ^{13}C can therefore be used as indictor for substrate changes and storage use. Fischer *et al.* (2015) used such measures of RQ and ^{13}C in respired CO_2 to show that trees under C limitation (from shading) switched from progressively declining carbohydrates to stored lipids to fuel respiration but this was not observed during drought. The observed decreasing NSC content in shaded trees during the experiment would likely have been interpreted as a preferential use of NSC in respiration, if alternative respiration substrate content, RQ and $\delta^{13}C$ of respired CO_2 had not also been assessed along with NSC content. Ideally, measurements of RQ can be combined with other isotopic measures including substrate age as part of field manipulation experiments, although this may rely on development of field-robust O_2 sensors of suitable accuracy. Such manipulations would allow investigations on long-term dynamics of substrate use, either during phenological or ontogenetic development or during periods of prolonged stress.

3. Is allocation to storage maintained under C limitation?

Limiting plant C availability via defoliation or reductions of atmospheric [CO_2] may indicate allocation priorities and several recent studies have tracked how such limitations affect C reserves. For example, maintenance of NSC concentrations (used as measure of storage) during concomitant reductions of growth occurred even when the supply of new C had been constrained via defoliation (Wiley *et al.*, 2013; Puri *et al.*, 2015) or in trees strongly affected by drought (Klein, 2015). These studies are clever attempts to tackle C storage regulatory dynamics. However, they cannot resolve whether this maintenance of NSC under constrained C supply was in fact new reserve formation or mere accumulation in response to growth declines from either reduced turgor (and hence reduced demand for NSC; Muller *et al.*, 2011) or from defoliation-induced hormonal changes affecting cambial activity (Puri *et al.*, 2015). By contrast, a direct manipulation of C availability via low atmospheric CO_2 used ^{13}C labelling of new photosynthetic products to show that allocation to storage was maintained even though the NSC pool size decreased (Hartmann *et al.*, 2015b).

Corroborative evidence from radiocarbon analysis of springtime ascending xylem sap in sugar maple (*Acer saccharum*) indicates that the sugars mobilized to fuel leaf-out have been assimilated during several preceding growing seasons, likely by regular allocation of NSC to a well-mixed 3–5 yr 'deep' functional storage pool (Muhr *et al.*, 2016). Such studies also show that results from lab experiments may be meaningful for general plant functioning, as observed in the field. Moreover, NSC in the maple tree sap represent the mobilized storage pool that can be used, at least to some degree, to grow the leaf canopy in spring and hence investigations on NSC dynamics in this pool may avoid potentially misleading definitions of operationally defined 'available' (i.e. extractable but maybe not metabolically important) NSC stored in tree stems.

Whether 'regular' allocation to storage under field conditions is also maintained under C limitation can be tested by using stem girdling to control C availability to tree tissues below the phloem cut, and then look at how that affects metabolically available C in the tree. A study is underway to follow how girdling (i.e. cutting off of new assimilates) affects the ^{14}C content of the storage pool mobilized during sugar maple springtime xylem sap ascent (Muhr *et al.*, 2016). This study will assess functional processes (such as respiration or synthesis of biomass and defence compounds) that rely on storage after girdling in trees of different ages, species and life strategy (e.g. shade-tolerant maple vs pioneer beech) and hence with likely different storage use strategies.

4. What controls storage?

Reserve formation can be achieved either via direct upregulation of storage processes (e.g. starch or lipid synthesis) or by downregulating allocation to competing sinks such as growth ('quasi-active storage'; Dietze *et al.*, 2014). Regulatory processes at the molecular and biochemical levels using gene expression and enzyme activity have so far been investigated only in annual herbs and over diel timescales (Smith & Stitt, 2007). but findings from such studies may not be relevant for long-lived organisms like trees. In studies that actually addressed regulatory processes in plant C relationships in citrus trees the modification of source–sink relationships caused a genetic downregulation of photosynthetic activity as to avoid excessive carbohydrate accumulation in branches (Nebauer *et al.*, 2011); however, further implications on whole-tree storage have not been addressed in this study. Importantly, a better understanding of molecular storage regulation in tree species is key to improve modelling efforts of vegetation dynamics (Dietze *et al.*, 2014).

Directly addressing storage regulatory mechanisms in mature forest trees will remain a major – and likely impossible – challenge.

However, one can learn much about tree functioning from studies on cloned seedlings and saplings, because even small trees are nonetheless perennial plants with particular life strategies for long-term survival (Petit & Hampe, 2006). By reducing atmospheric [CO_2], such small trees can be forced into gradually developing storage dependency of different degree (e.g. C compensation, C starvation) and their allocation strategies investigated by assessing changes in sink activities (via changes in biomass and in tracer signals) along with the underlying molecular (i.e. genetic) or biochemical (i.e. hormonal) regulatory patterns, similar to what has been done in Arabidopsis (Smith & Stitt, 2007; Hummel *et al.*, 2010). Only such approaches will have a hope of resolving recent debates on the control of storage components. Reserve formation should be independent of abundant C supply (surplus) and should be maintained even under decreasing C limitation, likely down to the C compensation point (Fig. 7). The storage pool size may decrease but sustained allocation can be traced with isotopic markers added to the atmosphere (Hartmann *et al.*, 2015b) and should be accompanied by distinct transcript patterns of genes encoding for storage processes, like starch or lipid synthesis (Koch, 1996) and associated enzymatic activities. Although accumulation may be controlled by a similar genetic orchestration in response to sugar signalling when concentrations are high (Smeekens, 2000; Rolland *et al.*, 2006) reserve formation as an evolutionary trait of 'being a tree' (Petit & Hampe, 2006) should be independent of C availability until short-term survival is at stake and reserves must be remobilized (transition from C compensation to C starvation, see Fig. 7).

5. Is there a link between use of internal-stem NSC and tree water transport?

A topic of current hot debate is the potential ability of trees to repair embolism during routine functioning and during drought via importing low molecular-weight sugars into embolized conduits (Brodersen *et al.*, 2010; Nardini *et al.*, 2011). High NSC concentrations at death during drought-induced mortality were found in several gymnosperm species and associated with lower xylem vulnerability but the existence of a causal relationship still needs to be formally tested (Henry Adams, personal communication). A potential approach to test this relationship could build on local labelling of stem parenchyma NSC via bark photosynthesis (Vandegehuchte *et al.*, 2015) and tracing of this signal within the xylem stream following a post-labelling drought phase (to induce embolism). Girdling above and below the labelling surface in trees that lack axial parenchyma cells, such as conifers, can prevent vertical transport of assimilates and would force NSC to be deposited into sapwood parenchyma. Such studies may take advantage of the high traceability of ^{14}C markers (Carbone & Trumbore, 2007; Carbone *et al.*, 2007) because only small amounts of stem-assimilated $^{14}CO_2$ would likely be stored within stem parenchyma cells and further diluted following embolism adsorption and xylem upward transport. Although leaching of NSC into xylem water may occur also in the absence of embolism repair in control trees, a greater tracer recovery in trees with embolism would nonetheless indicate a causal link between NSC and embolism repair, given that the latter is also detected in those trees.

6. What is the role of symbionts in plant C allocation strategies?

The exchange of NSC between plants and symbionts, including insects including ants as well as nitrogen-fixing bacteria or mycorrhiza, are important NSC sinks affecting the whole-plant C balance (Pringle, 2016). Radiocarbon measurements of mycorrhizal fruiting bodies and parasitic plants indicate that C being transferred is mostly fixed within the last year (Gaudinski *et al.*, 2009), which would seem to indicate that this is a high priority, although few systematic measurements exist. Questions about the role of NSC supply can be answered with manipulative experiments (Udvardi & Poole, 2013) and some studies have cleverly manipulated C supply via shading or low CO_2 and used isotope labelling to quantify tradeoffs between plant C availability and nitrogen

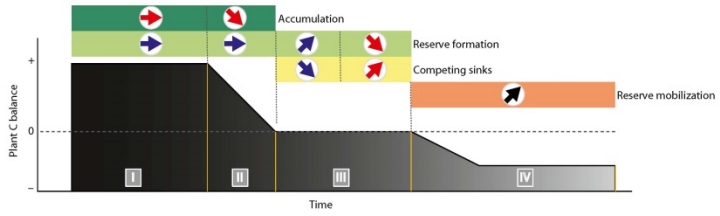

Fig. 7 Nonstructural carbohydrates (NSC) storage formation hypothesized to be an actively regulated (blue arrows) or overflow (red arrows) process. At positive carbon (C) balance (phase I), allocation to storage may result from either accumulation (dark green box) or reserve formation (light green box) or both. If NSC is present in excess of demand for growth or other sinks (accumulation), genetic upregulation of reserve formation may be triggered by sugar signalling-induced expression of genes encoding for storage processes (e.g. starch and lipid formation). However, as C availability decreases (phase II) both accumulation and storage upregulation via sugar sensing should decrease whereas reserve formation may still be actively upregulated. When plants reach the C compensation point (CCP; phase III; carbon balance = daily assimilation − daily respiration = 0) accumulation ceases and allocation to storage (reserve formation) must compete with other sinks including growth (yellow box). At CCP, storage upregulation is unlikely to be triggered by sugar signalling because NSC accumulation decreases. Sustained allocation to storage (relative to other sinks) would then be indicative for active storage upregulation, independent of NSC excess, whereas decreased allocation to storage would provide evidence against reserve formation having a high allocation priority. Under C starvation (phase IV) storage may be remobilized to fuel life-maintaining sinks (e.g. respiration, maintenance) but this mobilization is independent of storage allocation regulation (orange box).

(^{15}N) and phosphorus (^{33}P) uptake (Fellbaum *et al.*, 2014; Zhang *et al.*, 2015). Interestingly, nutrient uptake did not decrease even though shaded or low-CO_2 plants decreased the absolute amount of C transferred to mycorrhiza. Instead, plants optimized internal resource distribution by allocating proportionally more C and N to aboveground tissues to maximize the potential for CO_2 assimilation (Zhang *et al.*, 2015). A similar resource limitation experiment applied atmospheric N_2 removal to force rhizobia to 'cheat' on their hosts, thereby addressing plant sanctions for nonrewarding symbionts (Kiers *et al.*, 2003). Such clever experimental designs coupled with the use of isotopic tracers allow investigations on resource exchanges between plants and symbionts and may help in elucidating whether plants or their partners determine the rate of sink activity.

7. How do NSC allocation strategies vary with plant traits and are these linked to resilience to stress?

A better understanding of the how NSC are allocated across different functional groups of trees (fast- vs slow-growing, angiosperm vs gymnosperm, deciduous vs evergreen) may help us to predict vulnerability/response to different combinations of environmental stresses (Niinemets, 2010). For example, one might expect mature rainforest tropical trees that have reached the canopy to be dominated by 'fast' NSC pools, because there is not really a dormant season. However, Muhr *et al.* (2013) demonstrated that CO_2 emitted from tropical tree stems was from C that on average was as old (or older) than that from temperate trees (Carbone *et al.*, 2013; Fig. 6). Linking wood anatomy and life traits to the overall dynamics of NSC will require measurements across all forest biomes, and may also help answer questions about the potential role of NSC in xylem cavitation repair during drought, whereas investigation of the age of C fuelling resprouting after massive tissue dieback may be linked to the recurrence frequency of 'emergencies' such as large-scale damage or fire.

8. What is the role of elevated CO_2 in NSC dynamics and what are the consequences for trees?

We have focused here on the current debates surrounding drought and tree mortality, but obviously a change in CO_2 supply is an ongoing (and even, since the end of the last glaciation, long-term) factor to be considered. Whole-Tree Chamber (WTC) experiments demonstrated that elevated [CO_2] can exacerbate seasonal acclimation of leaf respiration to elevated temperature and to moderate drought (Crous *et al.*, 2011), whereas Free-Air CO_2 Enrichment experiments (FACE) showed that elevated [CO_2] may substantially enhance leaf net photosynthetic rates (Ellsworth *et al.*, 2012) although both net leaf C assimilation and carboxylation capacity depend on leaf N content (Ellsworth *et al.*, 2004). At the whole-tree and ecosystem levels, elevated [CO_2] had no effect on tree radial growth (Bader *et al.*, 2013), while observed increased forest net primary productivity was closely linked to N availability and may diminish over time (Norby & Zak, 2011). Such observations and chamber experiments demonstrate that elevated [CO_2], even under concurrent elevated temperature, leads to increased plant NSC content (Wullschleger *et al.*, 1992; Vu *et al.*, 2002). However, NSC dynamics are rarely investigated in FACE experiments (but see Körner *et al.*, 2005) even though their role in plant resilience to stress may become a determinant factor vegetation responses to climate change (Niinemets, 2010). How changes in NSC from elevated [CO_2] translate into altered tree function (e.g. changes in metabolism, nutrient acquisition, secondary metabolite production, resilience to damage, growth, etc.) has yet to be investigated fully and needs to be a research priority in ongoing studies. The current evidence, however, does not indicate any alleviatory effects of elevated CO_2 during drought-induced tree mortality (Allen *et al.*, 2015).

VI. Summary and conclusion

Application of isotopic tools that can distinguish recently fixed from stored carbon in nonstructural carbohydrates (NSC) have led to a more quantitative understanding of NSC dynamics in trees. They show that multi-annual storage reserves contribute normally to tree function, and demonstrate definitively that measurements of NSC concentrations alone do not provide sufficient information to understand tree functioning and how it is affected by stress. Combining these new tools and experiments offer promise to make progress on longstanding and fundamental questions about the role of NSC storage, particularly in drought stress and mortality in trees.

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9.10. Chapter 10: You just can't get enough but you still have to share

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REGULAR ARTICLE

Plant carbon limitation does not reduce nitrogen transfer from arbuscular mycorrhizal fungi to *Plantago lanceolata*

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Abstract
Aims The stress-gradient-hypothesis predicts that interactions among organisms shift from competition to facilitation as environmental stress increases. Whether the strength of mutualism will increase among symbiotically associated organisms when partners are forced into resource limitation remains unknown. Plants exchange photosynthetic carbohydrates (plant C) for nutrients in mycorrhizal symbiosis but how this exchange varies with plant C limitation is not fully understood.
Methods We investigated the influence of plant C availability and of arbuscular mycorrhizal fungi (AMF) on plant nitrogen (N) uptake and resource allocation using ^{13}C and ^{15}N labeling. We grew *Plantago lanceolata* with and without AMF *Rhizophagus irregularis* under ambient (400 ppm, AC) and low (100 ppm, LC) atmospheric [CO_2] and physically restricted plant root but not mycorrhizal access to soil N.
Results We found that plants grown under LC used AMF to obtain the same amount of N as those grown under AC, but the amount of newly fixed C correlated with the acquisition of N only under LC. The LC plants allocated more of their C to aboveground tissues.
Conclusions Overall our results suggest a more beneficial role of symbiosis under C limitation. The tight reciprocal control on N transfer and C allocation under C limited conditions supports the stress-gradient hypothesis of mutualistic symbiotic functioning.

Keywords Symbiosis · Resource allocation · Plant carbon limitation · Stable isotopes ^{13}C and ^{15}N · Stress-gradient hypothesis

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Introduction

Arbuscular mycorrhizal fungi (AMF) can form parternerships with over two-thirds of terrestrial plants and provide numerous resources (e.g., water, nutrients) in exchange for photosynthetic carbohydrates (plant C) (Smith and Smith 2011). AMF are generally thought to be responsible for taking up immobile nutrients (such as phosphorus (P), copper or zinc) but recent work also highlights their role in nitrogen (N) uptake (Leigh et al. 2009). N and P, heterogenously distributed in soils, limit plant growth in most ecosystems (Vitousek and Howarth 1991). Via their extraradical mycelium, AMF increase plant root arsorbing surface area with positive consequences for plant survival and growth (Smith and Read 2008). The fact that AMF transfer N to their hosts

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has been shown in both laboratory (Tanaka and Yano 2005) and field experiments (Blanke et al. 2011; Cavagnaro et al. 2012). However, the underlying mechanisms regulating AMF-mediated N transfer remain unclear (Fitter et al. 2011; Govindarajulu et al. 2005; Jin et al. 2005).

Growth and survivial of AMF rely on the provision of photosynthates from their host plants (Heinemeyer et al. 2006; Johnson et al. 2002) and recent studies using root organ cultures showed that plant C availability influenced nutrient transfer in plant-mycorrhizal symbiosis (Fellbaum et al. 2012; Hammer et al. 2011; Kiers et al. 2011). The concentration and types of plant carbohydrates determined nutrient uptake and transfer via AMF (*Glomus intraradices*) in axenic cultures of transformed carrot (*Daucus carota*) roots (Bücking and Shachar-Hill 2005). However, these root culture studies supplied plant tissues with extraneous carbohydrates and hence do not allow conclusions on whole-plant C-N exchange mechanisms. AMF nutrient transfer to host plants (*Plantago lanceolata*), using whole-plant system, did not change when plants were grown at 440 ppm as opposed to 1500 ppm [CO_2]. In contrast, AMF permitted more plant nutrition gain per unit of C invested into fungi for plants grown under low CO_2 (Field et al. 2012). In another study using whole-plant system, differences in sucrose investment did not affect nutrient (N, P, Zn) uptake by plants via arbuscular mycorrhizal fungi (Gabriel-Neumann et al. 2011). Thus, plant C supply may not be the only factor controlling AMF nutrient transfer.

According to the stress-gradient hypothesis (Bertness and Callaway 1994; Brooker et al. 2008), interactions among symbiotically associated organisms shift from competition to facilitation as environmental stress increases for one or both symbiotic partners. However, whether the functional complementarity between plant and AMF, in terms of C supply and N transfer, are reinforced under extreme environmental conditions has not been fully investigated. Cost and benefit in symbiotic exchanges depend on the relative resource availability and their balance between the symbiotic partners (Grman 2012; Grman and Robinson 2013) but how this mutualistic relationship changes when both symbiotic partners are forced into resource limitation remains unknown.

To address these issues, we devised an experiment where plant-AMF systems were forced into C limitation by strongly reducing ambient [CO_2]. Previous studies that relied on shading to reduce C supply showed that plants allocated more C to above- than below-ground organs and regulated C transfer to AMF by adjusting C allocation (Bethlenfalvay and Pacovsky 1983; Son and Smith 1988; Fellbaum et al. 2014). However, shading can cause shifts in C allocation to enhance shoot elongation for light capture independent of AMF (Pierik et al. 2009). In contrast, direct manipulation of atmospheric [CO_2] can force plants into C limitation without inducing such artifacts (Hartmann et al. 2013). Under such conditions, changes in C allocation among plant organs and/or between plant and AMF could extend results from shading experiments and provide deeper insights into the mechanisms that controling plant-AMF interactions.

Within the plant-mycorrhiza-soil system, we investigated N transfer dynamics under ambient and under induced reductions in plant C availability. We used C and N stable isotope labelling to quantify net C gain of plants and N fluxes from mycorrhiza to plants under experimental conditions. The main goals were to investigate: (1) whether sudden changes in plant (*P. lanceolata*) C supply influenced AMF (*Rhizophagus irregularis*) mediated N transfer and the cost-benefit ratio of the symbiotic exchange; and (2) whether internal plant C and N allocation changed under C limited conditions. Because plant carbohydrate supply to AMF determined nutrient uptake and transfer in vitro root cultures (Bücking and Shachar-Hill 2005; Kiers et al. 2011), we hypothesized that: (1) less N would be transferred from AMF to host plants under plant C limitation; (2) plants would receive more N per unit C invested under C limitation thereby decreasing the cost-benefit ratio, (3) internal plant resource allocation would shift to above-ground tissues in order to compensate for C limitation, and (4) AMF-mediated N transfer plays a more important role in plant growth and survival when plants are forced into C limitation.

Materials and methods

Microcosm design

Microcosms were made of PVC material (14 cm height × 17 cm length × 12 cm width) and separated into two compartments (Fig. 1, modified from (Hodge et al. 2001): HOST (11 cm length) and LABEL (6 cm length) separated by a 3-mm thick plate also made of PVC). The

plates were perforated evenly with 144 holes (6 mm radius) with the lowest row 3 cm higher than the base to prevent transfer of water and dissolved nutrients between chambers. To allow hyphae but prevent roots to penetrate, we glued a 20-μm nylon mesh on the HOST side and a 65-μm nylon mesh on the LABEL side of the plates (Walder et al. 2012). An air gap formed between the mesh membranes on either side of the 3-mm thick PVC plates thereby preventing both mass flow and nutrient diffusion across plates (tested in a pre-experiment). A visual water level indicator (float, Technoplant GmbH, Germany) was fixed in the corner of each compartment and ~2 cm of gravel was added to the bottom of each compartment to allow soil drainage. HOST compartments were filled with 1000 g, LABEL compartments with 500 g of sterile growth substrate (treated at 170 °C for 2 h) consisting of a carbon-free 1:1 vermiculate: sand mixture (Hartmann et al. 2013).

Biological material and growth conditions

Seeds of *P. lanceolata L.* were surface sterilized with a 2.5 % HCl solution for 10 min, then rinsed with distilled H_2O and germinated on moist filter paper in a Petri dish (Olsson and Johnson 2005). Three 1-week-old seedlings were planted in each HOST compartment and thinned 60 days after planting, leaving two seedlings per microcosm. Thinning minimized biomass variation across microcosms. Each microcosm was treated with 2 g of inoculum containing expanded clay and *R. irregularis* spores (110 infective units per gram) or with 2 g autoclaved inoculum in the nonmycorrhizal controls (Walder et al. 2012). In addition, soil microbial organisms, excluding AMF, were added to HOST compartments to homogenize microbial communities (Koide and Li 1989). To do so, we first washed 100 g field soil (collected from native *P. lanceolata* habitat) with deionized water and sieved the supernatant liquid through a 32-μm sieve to obtain 1 L of microbial wash. We added 10 mL of this microbial wash to each pot. The microcosms were then randomly arranged and evenly spaced on a glasshouse table (90 days after planting). Plants were left to grow under controlled environmental conditions on a 16–8 h day–night cycle. Day and night temperatures were set at 24 and 20 °C, respectively. Photosynthetically active radiation (PAR) measured daily at the leaf surface ranged from 450 to 550 μmol photons m^{-2} s^{-1}. Microcosms were irrigated twice a week with 50 mL of a nutrient solution (1/10 of N and P content as in Thornton and Bausenwein 2000, i.e. 0.15 mmol/L NH_4NO_3, 2.1 mmol/L $CaCl_2$, 0.75 mmol/L $MgSO_4$, 0.5 mmol/L K_2SO_4, 0.0307 mmol/L NaH_2PO_4, 0.0026 mmol/L Na_2HPO_4, 50 μmol/L H_3BO_3, 10 μmol/L $FeC_6H_5O_7$, 8.6 μmol/L $MnSO_4$, 2 μmol/L $ZnSO_4$ and 1 μmol/L $CuSO_4$).

Experimental design

Eight glass growth chambers (80 cm height×75 cm length×45 cm width, c. 250 L volume) were placed on a glasshouse table to provide controllable headspace for manipulation of atmospheric [CO_2]. The table was covered with closed-cell rubber foam mats of ethylene propylene diene monomer creating an airtight seal (details see Hartmann et al. 2013). The chambers were equipped with an automated watering system (drippers) that could be activated without opening the chambers. On August 15 (i.e. 90 days after planting), we transferred four microcosms into each chamber (two treated with active AMF inoculum and two with autoclaved inoculum, Fig. 1) and randomly arranged them on a plastic tray within the growth chambers. Plants were allowed to acclimate for 1 week. We applied a ^{15}N-labeled solution (2 g NH_4NO_3 with $\delta^{15}N$= 171.3‰, in 10 ml distilled water) to the LABEL compartment 97 days after planting. During the experimental period each HOST compartment was watered twice a week until water level indicator readings indicated soil saturation. As the volume of LABEL compartment was only 1/4 of the size of the HOST compartment, each LABEL compartment received 1/4 of the amount of water applied to the HOST compartment. Microcosms were also irrigated every second week with 50 mL of the nutrient solution only at HOST side.

Chambers were flushed with mixtures of CO_2-free air (Gamnitzer et al. 2009; Hartmann et al. 2013) and fixed amounts of ^{13}C-depleted ($\delta^{13}C$=−36.41±0.005‰) from a gas bottle with pure CO_2. Air inlet and outlet pipes went through the greenhouse table inside the chambers (Fig. 1), and the airflow was fixed at 10 L min^{-1} independent of CO_2 concentration. We started by flushing all eight chambers with ambient [CO_2] (i.e. 400 ppm, hereafter AC) for 1 week after plants were installed (90 days after planting). Subsequently, we progressively decreased [CO_2] in half of the chambers down to 100 ppm. This concentration was identified as the whole-plant C compensation point where no measurable difference between the daily sum of assimilated C and

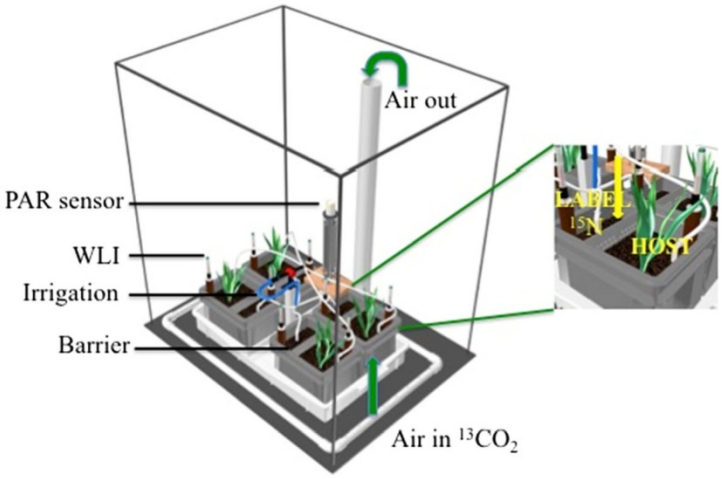

Fig. 1 Schematic view of four microcosms within one chamber showing the ventilation and irrigation systems. Two pots for mycorrhizal plants were treated as one replicate for each chamber, while the other two pots for non-mycorrhzal plants was treated as control. PAR, photosynthetically active radiation; WLI, water level indicator; Barriers were attached with nylon mesh screens (25 μm for HOST and 65 μm for LABEL compartment side, respectively)

the daily sum of respired C could be observed over a 2-week period. Thereafter, we continued to use 100 ppm [CO_2] as the level of the C limitation treatment (hereafter LC treatment) and maintained this concentration until the end of the experiment. The ambient C treatment (AC) maintained a level of 400 ppm CO_2 throughout the experiment.

Sample collecting and analysis

Three mycorrhizal (M) and three non-mycorrhizal (NM) microcosms were destructively harvested 90 days after planting – i.e. before the start of the experiment and as a measure for pre-treatment condition. Two M and two NM microcosms within each of the four chambers per treatment (representing one replicate) were also harvested 146 days after planting (i.e. after a 7 days acclimation period to chamber conditions and an additional 49 days of treatment). Plants were harvested quickly (within 3 min) from experimental chambers to reduce the influence of outside atmospheric CO_2 on the remaining microcosms.

Shoots were clipped at the soil surface and roots were carefully rinsed with water to remove adhering sand particles, and the total fresh weight of each recorded. Root subsamples (~2 g fresh roots) were randomly picked and cut into 2 cm lengths for the assessment of mycorrhizal colonization. The remaining shoots and roots were weighed and then dried at 70 °C for 48 h and weighed again to determine the fresh to dry biomass ratios.

Root subsamples were cleaned with 10 % KOH, acidified with 1 % HCl and stained with lactoglycerol mixture (1:1:1 lactic acid, glycerol and water), as described in Phillips and Hayman (1970). The proportion of root length colonized by arbuscular, vesicles, and hyphae was estimated using the magnified intersections method (Brundrett et al. 1996). At least 100 root intersections for each sample were inspected under a compound microscope at ×200 magnification. To quantify hyphal length density in the LABEL compartment, we used the modified membrane filter method (Jakobsen et al. 1992) and at least 50 views were counted at ×125 magnification using a compound microscope (Carl Zeiss, Inc., Axiolab, Jena, Germany). Hyphal length (in m g^{-1} soil) was estimated following the modified Newman formula (Tennant 1975).

Dried plant material was milled to a fine powder. For both shoots and roots, C and N concentrations and isotopic ratios were determined with an isotope-ratio mass spectrometer (IRMS; Deltaplus XP and Delta C prototype Finnigan MAT, respectively, Finnigan MAT, Bremen, Germany; 0.1‰ precision).

Mycorrhizal responses were calculated as MR= 100×(M−NM)/NM, where M are values for mycorrhizal plants, and NM are paired values for nonmycorrhizal plants grown within the same chamber. Mycorrhizal growth response was calculated with M and NM based on whole plant dry weight while mycorrhizal N response was determined with M and NM derived from whole plant N content.

Calculation of newly fixed-C and transferred-N

The CO_2 supplied to the chamber system had a very distinct isotopic signature ($\delta\ ^{13}C_{chamber}$=−36.41±0.005 ‰, compared to $\delta\ ^{13}C_{atmosphere}$~−8.5 ‰). Before we transplanted the pots into the chambers, we measured $\delta^{13}C$ and $\delta^{15}N$ on sacrificed plant tissues, and these are $\delta^{13}C_{t0\_plant}$ and $\delta^{15}N_{t0\_plant}$. The $\delta^{15}N$ of the nutrient solution added to the LABEL compartment ($\delta^{15}N_{LABEL}$) at the beginning of the experiment was 171.3‰.

We assumed that fractionation from CO_2 fixation was constant during the experiment and calculated the proportion of newly fixed-C in the plant C pool as:

$$\text{Newly fixed}-\text{C} = \frac{\left(\delta^{13}C_{t1_{plant}} - \delta^{13}C_{t0_{plant}}\right)}{\left[\left(\delta^{13}C_{chamber} - \delta^{13}C_{fractionation}\right) - \delta^{13}C_{t0_{plant}}\right]} \quad (1)$$

where $\delta\ ^{13}C_{chamber}$ is the isotopic signature of air leaving the chamber, $\delta^{13}C_{t0\_plant}$ is the isotopic signature of the plant tissue before we moved them into the chambers, $\delta\ ^{13}C_{t1\_plant}$ is the isotopic signature of plant tissues at each sampling time, and $\delta\ ^{13}C_{fractionation}$ was calculated as $\delta\ ^{13}C_{atmosphere}$ - $\delta^{13}C_{t0\_plant}$.

A similar mixing model equation was used for calculating the proportion of transferred-N:

$$\text{Transferred}-\text{N} = \frac{\left(\delta^{15}N_{t1_{plant}} - \delta^{15}N_{t0_{plant}}\right)}{\left(\delta^{15}N_{LABEL} - \delta^{15}N_{t0_{plant}}\right)} \quad (2)$$

where $\delta^{15}N_{LABEL}$ was 171.3‰, $\delta^{15}N_{t0\_plant}$ is the isotopic ^{15}N signature for the plants before being moved into the chambers, and $\delta\ ^{15}N_{t1\_plant}$ is the isotopic signature for the plants at each sampling time.

Statistical analysis

We analyzed our data with linear mixed models (LMMs) as implemented in package lme4 (Bates et al. 2013), using CO_2 treatment, AMF, and their interaction as fixed factors, and individual chamber as random factor. We set each chamber as our biological replicate units and thus had four replicates per treatment. To meet assumptions of normality of errors (Shapiro-wilk test) and homogeneity of variances (Levene's test), we transformed data using arcsine [square-root] (mycorrhizal root colonization, arbusculars, vesicles) or log10 (newly-fixed C, plant N, transferred N) when necessary. We used linear regression on the mycorrhizal N response (MNR) with the mycorrhizal growth response (MGR) and also on the newly fixed-C with transferred-N to test for correlations. All statistical analyses were conducted in R (v2.15.3, The R Foundation for Statistical Computing).

Results

Plants with AMF showed larger increases in below- but not above-ground biomass under AC (Fig. 2). In contrast, the presence of AMF had no significant effect on shoot and root biomass of plants under LC (Fig. 2, Table 2). The mycorrhizal colonization rate was 44.23 (± 42.18)% and 33.28 (± 11.30)% of root length for M plants grown under AC and LC, respectively, with no significant differences. Mycorrhiza-infected plants grown under AC and LC had no significant differences in hyphal length density (HLD), root length colonization, vesicles or arbuscules (Table 1).

AMF, but not [CO_2], had a significant effect on total plant N and also on the total amount of N transferred by AMF (Fig. 3, Table 2). Patterns seen in total biomass were also seen in total C (Fig. 4a, b, Table 2); i.e. AFM-infected plants grwon under AC ended the experiment with more C in below- but not above-ground tissues, while M and NM plants under LC did not differ in the final mass of shoot or root C. M plants grown under LC had significantly less total root C than those grown under AC, though shoots contained the same total amount of C (Fig. 4a, b, Table 2).

Under AC, plants with AMF fixed more total C in shoots and roots during the experiment (as determined by the amount of ^{13}C label incorporated) (Fig. 4). Under LC, however, plants with AMF contained more C only in shoots not in roots (Fig. 4c, Table 2).

AMF significantly increased newly-fixed C allocation ratios (shoot/root) under LC, but had no effect under AC (Fig. 5a). For NM plants under LC and all plants under AC, newly-fixed C allocation ratios were not significant different from one (Fig. 5a). Transferred N allocation ratios (shoot/root) were significantly greater under LC than that under AC for M plants (Fig. 5b).

LC significantly reduced plant nitrogen use efficiency (the amount of biomass (g) produced per unit of total N (mg), Fig. 6). Mycorrhizal growth responses had no relationship with mycorrhizal nitrogen response under AC, but showed a positive correlation under LC (although not significant, R^2=0.64, P=0.13; Fig. 7a).

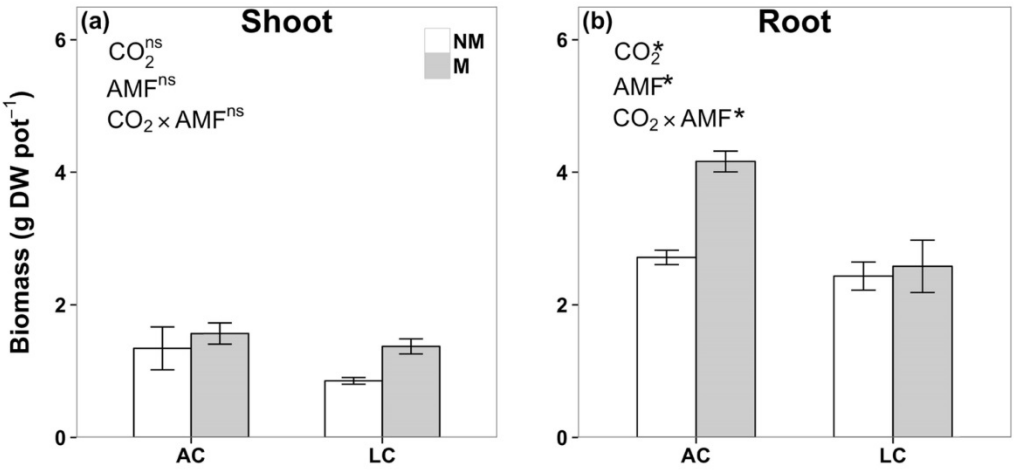

Fig. 2 Final shoot (**a**) and root (**b**) biomass (DW pot^{-1}) for non-mycorrhizal (NM, *white boxes*) and mycorrhizal (M, *grey boxes*) *P. lanceolata* grown for 49 days under ambient (AC, 400 ppm) and low CO_2 (LC, 100 ppm), respectively. Values are means (n=4)±1 SE. Statistically significant effects of single factors (CO_2 and AMF) and interactions (CO_2 × AMF) are shown with treatment letters (*** if P<0.0001, ** if P<0.001, * if P<0.05, ns if not significant). DW, dry weight

Furthermore, transferred-N was positively correlated with newly fixed-C content (R^2=0.92, P<0.05) for M plants grown under LC but not AC (Fig. 7b).

Discussion

Our results showed that plant C limitation did not reduce net N transfer from fungi to plants. However, C-limited host plants with AMF symbionts optimized the use of resources by increasing both C and N allocation to shoots to alliviate C limitation. The positive relationship between transferred-N and newly fixed-C under LC suggests that AMF-mediated N transfer plays an essential role in plant net C gain under C limitation.

N and C transfer under plant C limitation

Contrary to our initial hypothesis, the amount of new-N transferred to plants by AMF was independent of plant C availability (Fig. 3). This is in accord with a study by Hodge and Fitter (2010), in which shading had no effect on AMF-mediated N transfer to *P. lanceolata*. In another study, shaded *Medicago truncatula* maintained high AMF colonization rates and did not reduce gene expression of a putative ammonium transporter in the plant host (Fellbaum et al. 2014), also indicating that AMF maintained the rate of N transfer to C limited plants. In all of these studies, including ours, N and P concentrations of added fertilizer (except the additional labled-N) were in much lower concentrations than under normal field conditions and forced plants to rely on the AMF

Table 1 Arbuscular mycorrhizal fungal colonization parameters: extraradical hyphal length density (HLD, m g^{-1}) in the LABEL compartment; root length colonization (RLC, %), arbuscular (%) and vesicle (%) in the HOST compartment under ambient (AC, 400 ppm) and low (LC, 100 ppm) CO_2 for mycorrhizal (M) and non-mycorrhizal (NM) plants. Values are means±SD (n=4)

	AC		LC	
	NM	M	NM	M
HLD (m g^{-1})	0.00 (± 0.00)^a	6.85 (± 5.63)^b	0.00 (± 0.00)^a	2.20 (± 2.27)^b
RLC (%)	0.00 (± 0.00)^a	44.23 (± 42.18)^b	0.10 (± 0.79)^a	33.28 (± 11.30)^b
Arbuscular (%)	0.00 (± 0.00)^a	6.93 (± 5.80)^b	0.00 (± 0.00)^a	4.78 (± 1.61)^b
Vesical (%)	0.00 (± 0.00)^a	12.32 (± 9.75)^b	0.14 (± 0.28)^a	6.92 (± 7.32)^b

Different superscripts indicate significant differences between treatment levels

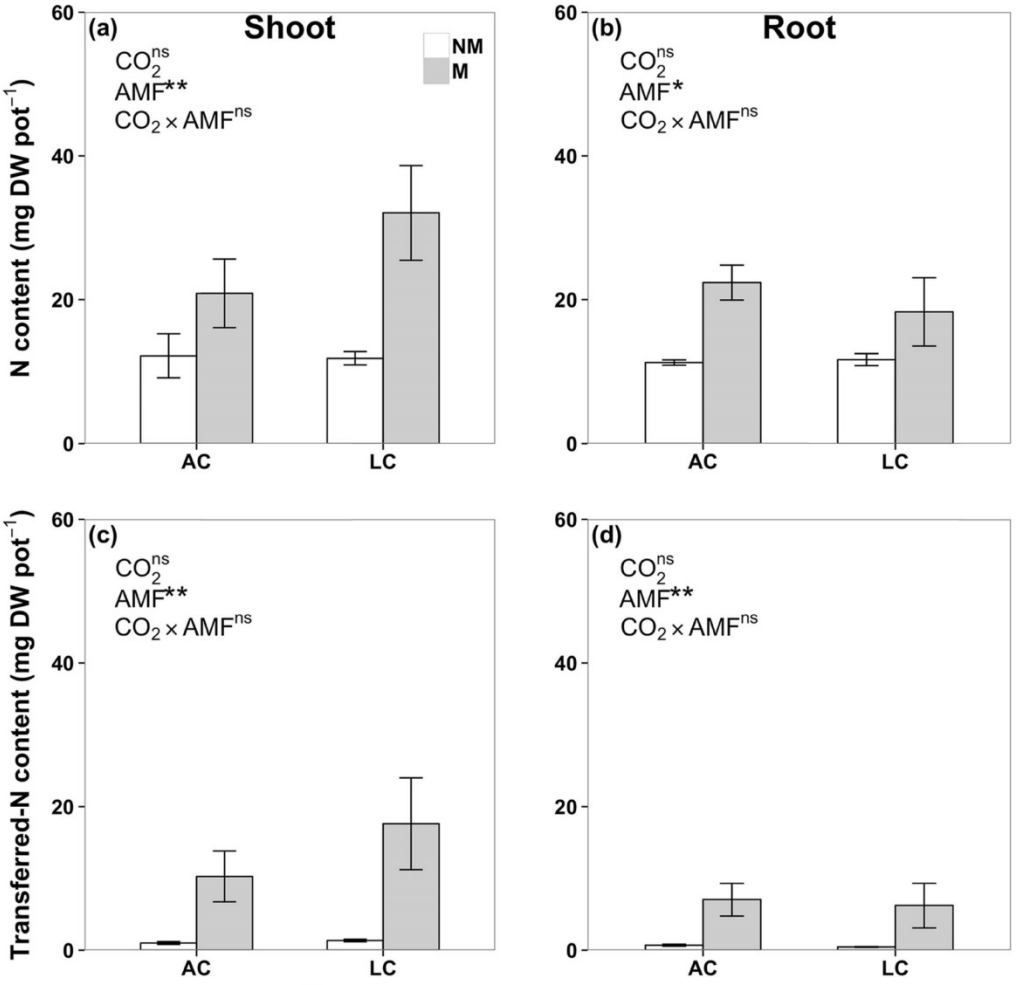

Fig. 3 Total nitrogen and transferred-N (i.e. ^{15}N-labeled, mg DW pot^{-1}) for shoots (**a**, **c**) and roots (**b**, **d**) of non-mycorrhizal (NM) and mycorrhizal (M) *P. lanceolata* under ambient (AC, 400 ppm) and low CO_2 (LC, 100 ppm), respectively. Values are means (n= 4)±1 SE. Asterisks indicate significant differences between NM and M plants. Statistically significant effects of single factors (CO_2 and AMF) and interactions (CO_2 × AMF) are shown with treatment letters (*** if P<0.0001, ** if P<0.001, * if P<0.05, ns if not significant). DW, dry weight

Table 2 ANOVA F ratios for the fixed effects of CO_2 concentration, AMF association and their interaction on plant biomass (dry weight), carbon (C) content, newly fixed-C (NewC), nitrogen (N) content, and transferred-N (NewN) for *Plantago lanceolata*

Source of variation		Biomass $F_{1,6}$	C content $F_{1,6}$	NewC $F_{1,6}$	N content $F_{1,6}$	NewN $F_{1,6}$
Shoot	CO_2	3.22	3.88^	30.39**	1.50	1.22
	AMF	3.79^	3.01	12.34*	14.43**	63.81***
	Interaction	0.60	0.56	0.09	0.95	0.05
Root	CO_2	9.66*	9.73*	95.86***	0.90	1.29
	AMF	21.38**	23.32**	0.29	13.04*	48.95***
	Interaction	14.20**	16.17**	6.58*	1.36	0.01

^P< 0.1; *P< 0.05; **P< 0.01; ***P< 0.001

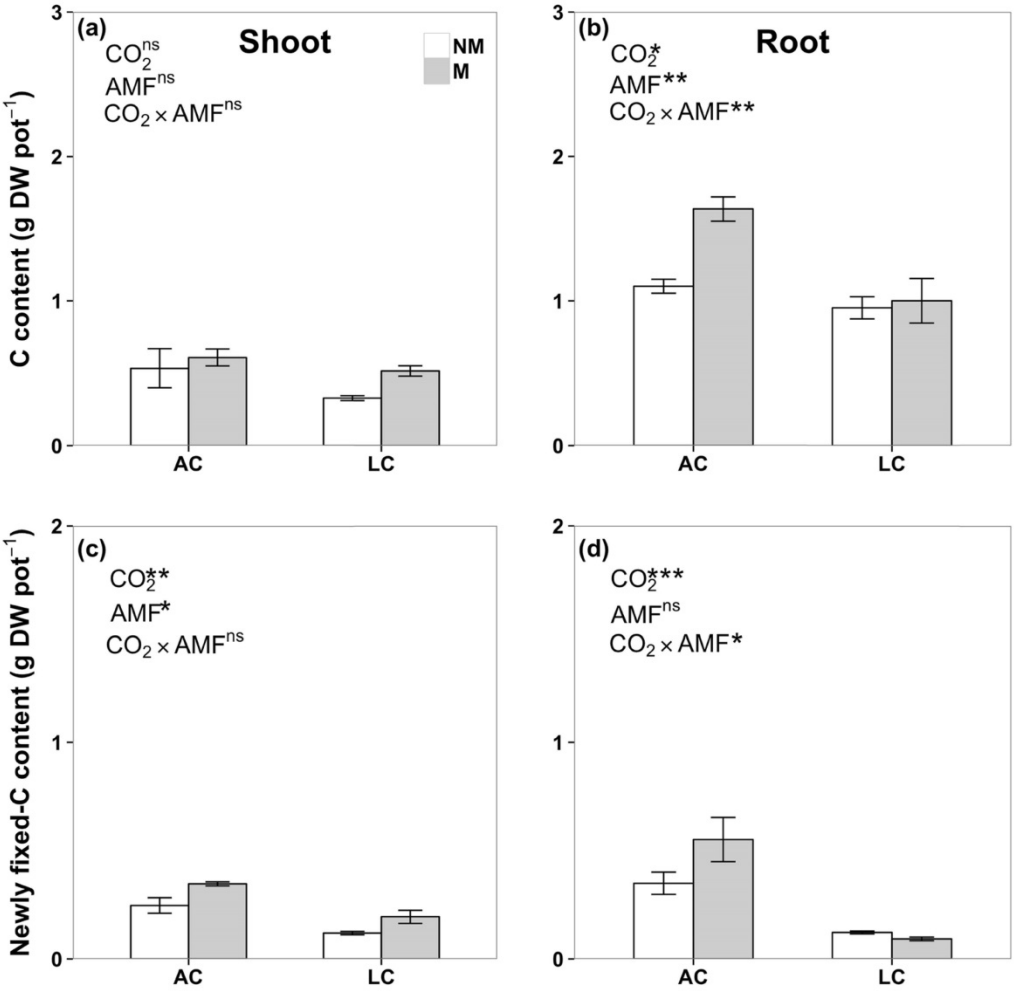

Fig. 4 Total plant C (**a**, **b**) and newly fixed-C (**c**, **d**, g pot^{-1}) for non-mycorrhizal (NM, *white boxes*) and mycorrhizal (M, *grey boxes*) *P. lanceolata* under ambient (AC, 400 ppm) and low CO_2 (LC, 100 ppm), respectively. Values are means (n=4)±1 SE. Statistically significant effects of single factors (CO_2 and AMF) and interactions (CO_2 × AMF) are shown with treatment letters (*** if P<0.0001, ** if P<0.001, * if P<0.05, ns if not significant). DW, dry weight

symbiosis for nutrient supply. Therefore, N transfer was maintained when plants had the chance to interact with AMF, even when C supply decreased.

Low [CO_2] did not reduce mycorrhizal root colonization in our study. Growth of mycelium under low carbohydrate levels and low soil nutrient availability was also observed by Olsson et al. (2014) and in plants where C availability was reduced by shading (Knegt et al. 2014). AMF are obligate biotrophs and colonizing and transferring N, even to low-quality hosts, secures a sustained C suply to fungi. Hence, under both C and N limitation, maintaining plant-AMF exchange may be a high priority for both plant hosts and symbionts because it provides a means for C-limited plants to get nutrients for survival and growth and represents a "strategic investment to retain future bargaining power" for AMF (Fellbaum et al. 2014).

In contrast, results from in vitro root organ cultures suggested that C availability controlled nutrient transfer in AM symbioses (Bücking and Shachar-Hill 2005; Fellbaum et al. 2012). It is important to consider, however, that these studies did not consider shoot-mediated effects on nutrient sink strength and uptake (Smith and Smith 2011). Shoots also play a key role in determining C transfer to AMF because plant shoots are the actual C source for both the plant and AMF (Gabriel-Neumann

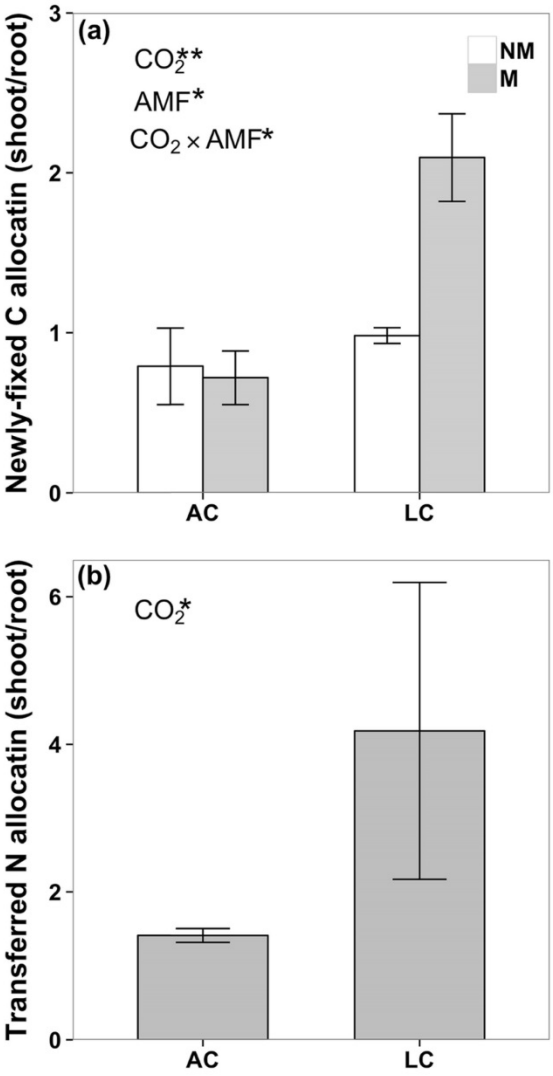

Fig. 5 Newly fixed-C (**a**) and transferred-N (**b**) allocation for shoots and roots for non-mycorrhizal (NM, *white boxes*) and mycorrhizal (M, *grey boxes*) *P. lanceolata* under ambient (AC, 400 ppm) and low (LC, 100 ppm) CO_2, respectively. Values are means (n=4)±1 SE. Statistically significant effects of single factors (CO_2 and AMF) and interactions (CO_2 × AMF) are shown with treatment letters (*** if P<0.0001, ** if P<0.001, * if P<0.05, ns if not significant)

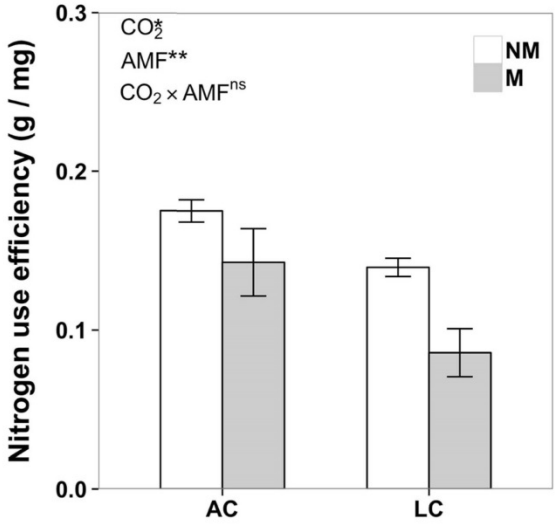

Fig. 6 Whole-plant nitrogen use efficiency (g / mg, productivity per unit N) for non-mycorrhizal (NM, *white boxes*) and mycorrhizal (M, *grey boxes*) *P. lanceolata* under ambient (AC) and low (LC) CO_2, respectively. Values are means (n=4)±1 SE. Statistically significant effects of single factors (CO_2 and AMF) and interactions (CO_2 × AMF) are shown with treatment letters (*** if P<0.0001, ** if P<0.001, * if P<0.05, ns if not significant)

et al. 2011). The contrasting results from shading (Hodge and Fitter 2010) and axenic culture experiments (Fellbaum et al. 2012) may be due to the fact that C availibility was extremly low in the latter study (80–90 % of original root C had been exhausted before adding sucrose). Once the amount of added sucrose to the root compartment went beyond a critical level (i.e., 5 mM), N transfer by the extraradical mycelium did not increase anymore and gene expression only showed moderate changes (Fellbaum et al. 2012). This suggests that C availibility may not control N transfer unless the plant and mycorrhizal symbiosis are extremely C limited. The fact that both low CO_2 in our experiment and shading in Hodge and Fitter (2010) did not reduce plant nutrient uptake in a whole-plant system highlights the influence of shoots as strong nutrient sinks (Stonor et al. 2014). In addition, increasing carbohydate availibility under elevated CO_2 reduced N acquisition in *Pinus halepensis* via its ectomycorrhizal fungi, *paxillus involutus* (Kytöviita et al. 2001), but increased AMF-mediated plant ^{15}N uptake for three arbuscular mycorrhizal fungi, *Gigaspora margarita*, *Glomus clarum* and *Acaulospora morrowiae* (Cheng et al. 2012). These results suggest that C availibity does not always control AMF-mediated N transfer and that the overall effect may depend on mycorrhizal types.

Plant resource allocation strategy

Plants preferentially allocate C to tissues responsible for acquiring the most limiting resources (Johnson et al. 2013). Consequently, plants grown under C limitation

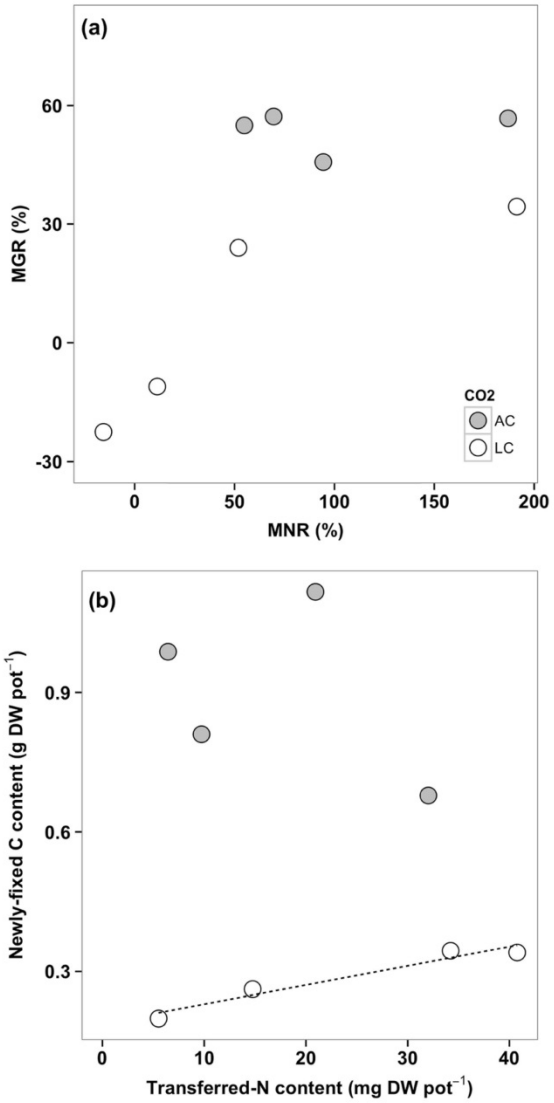

Fig. 7 Mycorrhizal nitrogen response and mycorrhizal growth response for (**a**), newly fixed plant-C content (g pot^{-1}) and (**b**) AMF-transferred-N (mg pot^{-1}) for *P. lanceolata* grown under ambient (AC, *grey*) and low CO_2 (LC, *white*) conditions. Only under LC, newly fixed-C showed positive relationship with AMF-transferred-N (*slashed line*, R^2=0.92, P<0.05)

are expected to allocate more biomass to aboveground tissues to enhance C assimilation (Sage and Coleman 2001). As shading itself can shift C allocation to enhance shoot elongation (Pierik et al. 2009), inferring regulatory mechanisms of plant C transfer to AMF from C allocation data in shading experiments may be problematic (Bethlenfalvay and Pacovsky 1983; Son and Smith 1988). We found that under LC only M plants but not NM plants allocated more resources to shoots (Fig. 5). In contrast, we found no preferential allocation of newly fixed-C between shoots and roots for NM plants under either CO_2 level. Thus, the observed shift in C allocation towards shoots in M plants under LC apparently resulted from the interaction with AMF rather than from resource allocation optimization alone.

Mechanisms that control the transfer of C between plant and AMF are not well understood. The total amount of C allocated to shoots was the same under both CO_2 levels, while allocation to roots was less under LC than that under AC, even though levels of AMF root colonization were not different. If shoots control C supply to AMF, we suggest as a possible mechanism that plants shifted C allocation to shoots in order to maintain C supply to AMF under plant C and N limitation. However, a proof for such a mechanism would require investigating metabolomics (i.e. genes encoding for carbohydrate transporters) and how they respond to AMF colonization under varying C availability.

Plant-AMF interaction under nutrient limitation

Our results raise the intriguing question why the bilateral exchange of plant-supplied C for microbially-acquired N was sustained even under low C availability. We found overall lower N use efficiency (new C fixed per unit transferred N) in plants under LC than under AC (Fig. 6). The plant growth response mediated by AMF (mycorrhizal growth response, MGR) can be positive or negative and depends on the mycorrhizal nutritional response (MNR). We found that the mycorrhizal growth response showed a positive trend with mycorrhizal nutritional response (although not significant, R^2=0.64, P=0.13) only under LC. In contrast, plants under AC did not grow more biomass even when they received more N via AMF (likely due to limitations from otherelements, such as P, Fig. 7a) and the amount of transferred-N mediated by AMF was positively correlated with newly-fixed C at LC but not AC (Fig. 7b). These results suggest a more synergistic relationship between plants and mycorrizae when environmental constraints intensify.

Consistent with the stress gradient hypothesis (Bertness and Callaway 1994; Brooker et al. 2008), our results indicated that the strength of mutualism between symbiotically associated organisms increased when habitat stress increased. Indeed, plants invested more C in AMF and received more benefits in return

under stressful (C limited) than optimal (high CO_2) conditions (Aghili et al. 2014). Shading had a significant effect on AMF-mediated N transfer in roots and shoots of plants associated with *Glomus aggregatum* but not with *R. irregularis* (Fellbaum et al. 2014), indicating that decreasing C availibility does not reduce benefits for plants associated with *R. irregularis* as in our study. When not given the choice of their plant host, even *G. aggregatum*, transferred more nutrients per unit C to C-limited (shaded) hosts, causing a shift in the cost-to-benefit ratio in favor of the shaded plant (Fellbaum et al. 2014). Similarly, *P. lanceolata* grown at a gradient representing the Palaeozonic CO_2 decline (from 1500 to 440 ppm) received proportionally more nutrients from AMF under lower [CO_2], i.e. the symbiotic relationship became more beneficial under declining C availability (Field et al. 2012). These results suggest that the mycorrhizal symbiosis may function as an insurance strategy that allows the plant to maintain productivity even when C becomes limiting. In turn, the N investment by AMF could also be an importrant stragegy to maintain or increase plant C assimilation and consequently to enhance the probability of a future investment return.

Conclusion

Terrestrial plants with mycorrhizal symbionts have co-evolved for 400 million years. However, details of how and why these interactions change with atmospheric CO_2 levels remains an important question. Here we showed that forcing plants to their photosynthetic compensation point did not reduce AMF-mediated N transfer. Plants under C limitation with AMF increased their C allocation to shoots, presumably to increase future C supply. Whether plants can control C supply to AMF by changing C allocation among different organs requires further investigation. However, the mutualistic relationship between symbiotically associated organisms became stronger, rather than weaker, under C stress, suggesting mycorrhizal symbiosis must have a high priority for stressed plants.

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9.11. Chapter 11: To defend or not defend, that is the question

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Original Article

Release of resource constraints allows greater carbon allocation to secondary metabolites and storage in winter wheat

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ABSTRACT

The atmospheric CO_2 concentration ($[CO_2]$) is rapidly increasing, and this may have substantial impact on how plants allocate metabolic resources. A thorough understanding of allocation priorities can be achieved by modifying $[CO_2]$ over a large gradient, including low $[CO_2]$, thereby altering plant carbon (C) availability. Such information is of critical importance for understanding plant responses to global environmental change. We quantified the percentage of daytime whole-plant net assimilation (A) allocated to night-time respiration (R), structural growth (SG), nonstructural carbohydrates (NSC) and secondary metabolites (SMs) during 8 weeks of vegetative growth in winter wheat (*Triticum aestivum*) growing at low, ambient and elevated $[CO_2]$ (170, 390 and 680 ppm). R/A remained relatively constant over a large gradient of $[CO_2]$. However, with increasing C availability, the fraction of assimilation allocated to biomass (SG + NSC + SMs), in particular NSC and SMs, increased. At low $[CO_2]$, biomass and NSC increased in leaves but decreased in stems and roots, which may help plants achieve a functional equilibrium, that is, overcome the most severe resource limitation. These results reveal that increasing C availability from rising $[CO_2]$ releases allocation constraints, thereby allowing greater investment into long-term survival in the form of NSC and SMs.

Key-words: carbon allocation; CO_2; growth; respiration; storage carbohydrates.

INTRODUCTION

For more than 40 years, the control mechanisms of whole-plant carbon (C) allocation have been of central interest to plant scientists (Mooney, 1972). Plants fix CO_2 from the atmosphere and partition the resulting photosynthetic products (carbohydrates) among several uses, including growth of structural biomass, synthesis of secondary metabolites and metabolic processes like respiration and osmoregulation. The atmospheric CO_2 concentration ($[CO_2]$) has increased from 170 ppm during glacial periods of the past million years to the current ~400 ppm and is expected to increase up to 430 ppm to 1000 ppm by the year 2100 (Cubasch *et al.*, 2013). However, little is known about how increased $[CO_2]$ alters partitioning of fixed C among different sinks or about the processes regulating allocation (Dietze *et al.*, 2014).

Historically, the regulation of plant C partitioning was thought to be driven by C supply (i.e. source activity), but recent evidence suggests that sink activity may be the main determinant (Fatichi *et al.*, 2014; Korner, 2015). Experiments manipulating source activity via $[CO_2]$ availability can elucidate the contributions of sources and sinks for whole-plant C allocation (Fatichi *et al.*, 2014). However, such studies are still sparse, not only because of the expense of manipulating $[CO_2]$ (Gerhart & Ward, 2010), but also because of challenges in assessing both whole-plant C balance (assimilation minus respiration; A – R) and how non-respired C is distributed among sink components, that is, structural growth (SG), nonstructural carbohydrates (NSC), secondary metabolites (SMs) and export (e.g. to rhizosphere symbionts or as volatile organic compounds (VOCs)) (Fig. 1).

In many plant species, short-term stimulation of leaf-level photosynthesis by elevated $[CO_2]$ (Franks *et al.*, 2013; Gerhart & Ward, 2010) is often associated with down-regulation of photosynthesis in the long term (Lee *et al.*, 2011; Long *et al.*, 2004). However, leaf-level net photosynthetic rates cannot be directly scaled up to whole-plant assimilation for a number of reasons. For example, leaf-level assimilation measurements are usually done under ideal light and temperature conditions (Kirschbaum, 2011) and therefore may overestimate actual assimilation rates. Scaling up from leaf to whole-plant gas exchange also requires parameterization of leaf phenology and light distribution. In addition, photosynthesis in non-leaf tissues (bark, stems) may not scale in the same way as in leaves. Therefore, direct whole-plant assessment of gas exchange is a more suitable method for determining whole-plant responses to different $[CO_2]$ (Atkin *et al.*, 2007; Boote *et al.*, 2013).

Respiration (R) is an essential plant functional process providing energy and metabolic intermediates needed for photosynthesis, which in turn provides substrates for R. Some studies have reported that whole-plant R may account for up to 30–50% of the gross photosynthetic CO_2 uptake in herbaceous plants, and due to the interdependency of these processes this proportion remains relatively constant under a gradient of

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approach for reducing plant C availability, it may also trigger phytochrome-induced growth stimulation (Casal, 2013) and defence suppression (Ballare, 2014), independent of C availability. Direct manipulations of atmospheric [CO_2] suffer less from such side effects and allow plants to adjust to C (rather than light) availability.

Because allocation is a continuous process, the whole-plant fluxes (A – R derived from gas exchange) and pools were monitored over the vegetative growth phase using a unique chamber design implemented in a greenhouse. Supplemental nutrients were applied to the substrate in order to minimize nutrient limitations on plants. Allocation to reproduction (seed production) was purposely excluded in this study because pollination has been shown to be problematic and incomplete in these growth chambers, likely due to light limitation. Changes in primary (NSC, including soluble sugars, starch and fructans) and SMs (including flavonoid glycosides and benzoxazinoids) of plant tissues (leaves, stems and roots) were used to partition biomass into SG (SG = Biomass – NSC – SMs) and nonstructural components (NSC and SMs). Specifically, we hypothesized that, with increasing [CO_2] (170, 390 and 680 ppm): (1) the proportion of C allocated to R remains constant but decreases in biomass (SG + NSC + SMs) (relative to A); (2) NSC and SMs increase while SG decreases; and (3) biomass (SG + NSC + SMs) increases in stems and roots but decreases in leaves, in accordance with the functional equilibrium hypothesis.

MATERIALS AND METHODS

Plant material

A cultivar of winter wheat from a local seed plantation (Bioland Hof Jeebel Biogartenversand, Salzwedel, Germany) adapted to Central Europe was used in our study. On 7 January 2015, seeds were germinated in plates filled with sand and were watered every day. On 13 and 14 January 2015, seedlings of similar height were transplanted into pots (diameter 11 cm, height 24 cm) filled with C-free quartz sand. Four pots (12 seedlings per pot) and two pots (10 seedlings per pot) were randomly placed in each plant chamber immediately after transplanting (Fig. 2). We provided the plants with supplemental nutrients by watering pots with a continuous through-flow of a modified Hoagland nutrient solution (Hoagland & Arnon, 1950) to avoid nutrient limitation (other than C).

Growth chambers

Twelve aquarium-style glass chambers (80 cm high × 75 cm long × 45 cm wide) were used to isolate the atmosphere around the plants and allowed controlling [CO_2] (Fig. 2). Chambers were placed on a greenhouse table covered with closed-cell rubber foam mats made of ethylene propylene diene monomer for airtight sealing (as in Hartmann *et al.* (2013)). Air in the chambers was flushed continuously at a flow rate of 14 l min^{-1} through inlet tubing located at the bottom and outlet tubing at the top of the chamber. The [CO_2] of incoming air was controlled by first scrubbing all CO_2 from atmospheric air using a molecular sieve and then adding pure CO_2 from a gas tank (Schnyder, 1992). The CO_2-free air was separated into three air circuits (four chambers per circuit); in each circuit, [CO_2] was measured sequentially with a Vaisala® (*GMP 343*) at intervals of 10 min, using a custom-built switching valve controlled by a micro-logger (Campbell® CR1000). Measured [CO_2] were compared against pre-set values (170, 390 and 680 ppm, respectively), and, if required, CO_2 supply to the airstream was adjusted via mass flow controllers to maintain target [CO_2] levels. To minimize differences in light levels, treatments were randomly assigned on the table, and three of four chambers per treatment were used to grow plants while the other one was used to monitor [CO_2] directly in the chamber.

In each chamber, air temperature and photosynthetic photon flux density were monitored continuously (see Hartmann *et al.*, 2013). During the day, average temperatures increased

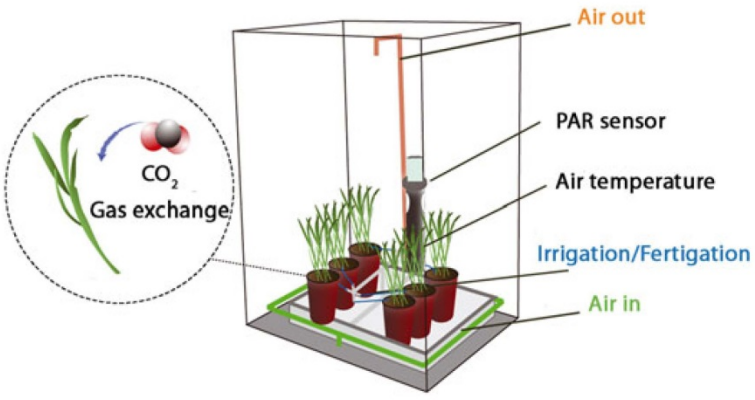

Figure 2. Schematic view of one of the 12 chambers showing the ventilation system and the photosynthetically active radiation (PAR) sensor. Air was flushed continuously through inlet tubing located at the bottom and outlet tubing at the top of the chamber. Four pots (12 seedlings per pot) and two pots (10 seedlings per pot) were randomly placed in each plant chamber. Note that only pots containing 12 seedlings were harvested. Supplemental nutrients were provided by watering the pots with a continuous through-flow of a modified Hoagland nutrient solution. [Colour figure can be viewed at wileyonlinelibrary.com]

from ca. 12 °C at 6:30 (local time) to ca. 20–24 °C in the afternoon, and then decreased to ca. 16.5 °C at 22:00. During the night, air temperature declined from ca. 16 to 11 °C. All plants were grown in a 16/8 h light/dark regime using supplemental greenhouse lamps (Son-T Agro® 430 W HPS bulbs, primary light range = 520–610 nm, Philips® Lighting Co., Somerset, NJ, USA). The average daily total photosynthetic photon flux density inside the chambers from 23 January to 20 March was 7.79 ± 0.92 mol m^{-2} d^{-1}.

Destructive harvesting and biomass processing

Allocation priorities likely differ between growth stages of winter wheat (seedling, tillering, stem elongation, flowering and grain filling), and we concentrated on the vegetative growth period (seedling and tillering stage) to rule out the potential confounding effects of developmental stage on plant allocation. According to the growth scale of Zadoks *et al.* (1974), seedling growth of winter wheat is achieved before the occurrence of nine unfolded leaves on the main shoot. Plants were therefore harvested when three, six and eight leaf sheaths were completely developed, denoted as 3 L, 6 L and 8 L periods, respectively. For 390 and 680 ppm treatments, this occurred 3, 7 and 9 weeks after transplanting, respectively, while development in the low $[CO_2]$ treatment was slower, and plants were sampled 3.5, 8 and 10.5 weeks after transplanting. Sampling was always conducted between 16:00 and 21:00. At each sampling, three pots (12 seedlings per pot) from three chambers of each $[CO_2]$ treatment were destructively harvested. Fresh biomass of whole-plant leaves, stems and roots of each pot were determined, and then all tissues were shock-frozen in liquid nitrogen and stored at −80 °C, freeze-dried and weighted for dry biomass. Dry tissues were homogenized and ground to fine powder using a ball mill (Retsch® MM400, Haan, Germany) and stored at −20 °C until analysis.

Whole-plant gas exchange

Gas exchange data were measured with a Picarro® 2101-i (precision 0.01–0.4%, Picarro Inc. Santa Clara, CA, USA) for the first 7 weeks (extending beyond the 6 L period) under 680, 390 and 170 ppm $[CO_2]$. However, because the Picarro failed after week 7, gas exchange for week 8, 9, 10 and 11 were measured with a Spectronus® FTIR (Fourier transform infrared spectroscopy, Ecotech Pty Ltd., Knoxfield, VIC, Australia) using otherwise the same hardware and the same sequence protocol as mentioned above. Reference gases were used to verify and ensure comparability across devices.

Within 2 h, the $[CO_2]$ of air leaving the 12 chambers and 6 reference air samples (taken from the air circuit) were measured sequentially at intervals of 6 min 40 s, and the sequence was controlled by a micro-logger (Campbell® CR1000) and a custom-built switching valve unit. Transition periods after valve switching were excluded from analysis, and a core period of 3 min 20 s was used to estimate instantaneous whole-plant gas exchange, defined as:

$$\Delta[CO_2] = \frac{[CO_2]_{\text{non-plant}} - [CO_2]_{\text{plant}}}{M} \quad (1)$$

where $[CO_2]_{\text{non-plant}}$ is the $[CO_2]$ of outgoing air from the chamber without plants, and $[CO_2]_{\text{plant}}$ is the $[CO_2]$ of outgoing air from chambers with plants. M is the number of plants grown in the chamber.

The whole-plant gas exchange rate was assumed to be constant within the 2 h cycle and whole-plant C flux at hour j was calculated as:

$$C_j\ (\text{g h}^{-1}) = \frac{\Delta[CO_2]\ (\mu\text{mol mol}^{-1}) * \text{VFR}\ (\text{l min}^{-1}) * 60\ \text{min} * 12\ (\text{g mol}^{-1})}{22.4\ (\text{l mol}^{-1}) * 1000000} \quad (2)$$

where VFR is the volumetric flow rates of air going through the chambers, that is, 14 l min^{-1}, and 22.4 l mol^{-1} is the molar volume of gas at normal conditions. The cumulative daily whole-plant net C assimilation (A_{cum_n}) and night-time respiration (R_{cum_n}) over the duration of the experiment (n = number of days d, j = hour of day) were then computed using the following equation:

$$A_{cum\_n} = \sum_{i=1}^{n} \sum_{j=7}^{22} C_j \quad (3)$$

$$R_{cum\_n} = \sum_{i=1}^{n} \sum_{j=23}^{24} C_j + \sum_{i=1}^{n} \sum_{j=1}^{06} C_j. \quad (4)$$

A_{cum_n} and R_{cum_n} between 3 L and 6 L were used to calculate the fraction of C allocated to R. Whole-plant A and R collected over the last two or three days prior to biomass sampling were averaged for calculations of mass-based (i.e. specific) rates of daily net assimilation and respiration.

While C-free quartz sand was used to avoid any C input into the system other than from plant photosynthesis, it was technically impossible to sterilize the all components of such a greenhouse facility (air, water, plant material) to a degree that would completely avoid microbial infestation. Hence, microbial respiration of root exudates or litter may be included in the gas exchange measurements. However, given that root exudation (Grayston *et al.*, 1997; Phillips *et al.*, 2009) represents a much smaller C flux than respiration (<10% versus 30–50%) (Atkin *et al.*, 2007; Gifford, 1995; Pons & Poorter, 2014), gas exchange data represent mainly plant assimilation and respiration (Hartmann *et al.*, 2015; Hartmann *et al.*, 2013).

NSC analysis

Concentrations of soluble sugars and starch in plant tissues were determined using methods described in Raessler *et al.* (2010). For soluble sugars, usually 1 ml (or 0.5 ml for small samples) of sterilized water was added to 50 mg (or 10 mg for small samples) dry biomass. The mixture was vortexed, incubated at 65 °C for 10 min and then centrifuged at 12 000 *g* for 10 min. The supernatant was collected and stored on ice, and the remaining pellet was re-extracted twice using the same procedure. The supernatants were pooled and then diluted at a ratio of 1:20 (or 1:8 for small samples) and stored at −20 °C. For starch, 50 mg (or 10 mg for small samples) dry biomass

were extracted with 0.35 ml (or 0.175 ml for small samples) of water and 0.5 ml (or 0.25 ml for small samples) of perchloric acid (52%). The mixture was vortexed, incubated at 65 °C for 10 min and then centrifuged at 12 000 *g* for 10 min. The supernatant was collected, and the remaining pellet was re-extracted. The supernatants were pooled and then diluted at a ratio of 1:55, stored at −20 °C.

Sucrose, glucose and fructose of both soluble sugars and starch extractions were determined by High-Performance Liquid Chromatography coupled with Pulsed Amperometric Detection (HPLC-PAD), using a Dionex® ICS 3000 ion chromatography system equipped with an autosampler (Thermo Fisher GmbH, Idstein, Germany). Starch concentrations were calculated by subtracting glucose and half of the sucrose concentrations in water-soluble extract from glucose concentrations in the hydrolyzed extract then multiplying a conversion factor of 0.9 (Sullivan, 1935). Fructan concentrations were calculated using the same method but with fructose instead of glucose concentrations, given that fructan is one of the most important C storage compounds in wheat (Pollock & Cairns, 1991). Total NSC concentrations reported are the sum of soluble sugars, starch and fructans.

SM analysis

Samples from 680 ppm at 8 L were used for the identification of major SMs in leaf, stem and root tissues; 800 μl methanol and two glass beads were added to 30 mg of dry biomass. The mixture was bead-beaten for 40 s at 6.0 m s^{-1} with a FastPrep Instrument (MP Biomedicals, Santa Ana, USA), then vortexed and centrifuged at 13 000 *g* for 5 min. The supernatant was collected and then analysed by HPLC coupled to a mass spectrometer. Compounds were separated on a Nucleodur Sphinx RP18ec column with dimensions of 250 × 4.6 mm and a particle size of 5 μm (Macherey Nagel, Dueren, Germany) using an Agilent 1100 series HPLC with a flow rate of 1.0 ml min^{-1}. The column temperature was maintained at 20 °C. Phenolic compounds were separated using 0.2% (v/v) formic acid and acetonitrile as mobile phases A and B, respectively, with the following elution profile: 0–28 min, 5–61% B in A; 28–30 min 100% B; and 30–35 min 5% B.

Compound detection and quantification were accomplished with an Esquire 6000 ESI ion-trap mass spectrometer (Bruker Daltronics, Bremen, Germany). Flow coming from the column was diverted in a ratio of 4:1 before entering the mass spectrometer electrospray chamber. ESI-MS was operated in negative mode scanning $m\ z^{-1}$ between 50 and 1600 with an optimal target mass of 400 $m\ z^{-1}$. The mass spectrometer was operated using the following specifications: skimmer voltage, 60 V; capillary voltage, 4200 V; nebulizer pressure, 35 psi; drying gas, 11 l min^{-1}; gas temperature, 330 °C. Capillary exit potential was kept at −121 V. Compounds of leaves and stems were identified by comparing the fragmentation patterns with previously reported wheat phenolic profiles (Moheb *et al.*, 2011; Wojakowska *et al.*, 2013). Root compounds were identified based on profiles of benzoxazinoids in grasses (Wouters *et al.*, 2014).

For quantification, 500 (300) μl 95% methanol and two glass beads were added into 50 (30) mg samples, the mixture extracted using the same procedure described above but the remaining pellet was re-extracted again. Supernatants were pooled and then analysed by HPLC-UV using the same chromatographic conditions as above. The UV wavelengths 240, 260, 280 and 330 nm were monitored. All compounds were quantified using external standard curves. Feruoylputrescine (HPC Standards GmbH, Cunnersdorf, Germany) was used to quantify ferulic acid-based compounds in leaves and stems, and also putrescine-containing compounds in roots. Luteolin 6-C-glucoside and apigenin 6-C-glucoside (Sigma-Aldrich) were used to quantify luteolin-based and apigenin-based compounds in leaves, respectively. Chrysoeriol (LGC standards, Middlesex, UK) was used to quantify chrysoeriol-based and tricin-based compounds in leaves. DIMBOA-Glc and HDMBOA-Glc in roots were quantified by standards DIMBOA-Glc and HDMBOA-Glc purified from maize roots (Wouters et al., 2014). Leaf and stem compounds were quantified at a UV absorption spectrum of 330 nm, and root compounds at 280 nm.

Elemental analysis

C and N concentrations of tissues were determined by an elemental analyser (varioEL II). To do so, 15 mg dry samples were weighed into tin foil, combusted and separated by specific columns, and then assessed with a thermal conductivity detector.

C balance

Tissue-specific C, NSC and SM content was calculated by multiplying tissue-specific C (Supporting Information Table S1), NSC and SM concentrations by tissue dry mass. C content in tissue-specific NSC and SM was then calculated by multiplying tissue-specific NSC and SM content by their mass proportion of C (0.4 for NSC and specific fraction to each compound for the different SMs (Supporting Information Table S2)). C allocation to biomass, NSC and SMs was defined as the difference in C content of whole-plant biomass, NSC and SMs between 6 L and 3 L divided by the difference in A_{cum_n} between 6 L and 3 L. Note that quantifying allocation to biomass requires relatively large increase in biomass; therefore, C allocation between 8 L and 6 L is not reported, given that average biomass increment of low [CO_2] plants was smaller than its standard deviation within and across chambers.

When net C gain estimated from gas exchange exceeded the C that accumulated in biomass, the difference was attributed to an 'unaccounted carbon pool' (UCP). UCP was calculated by mass balance using the following equation:

$$UCP = A_{cum\_n} - R_{cum\_n} - SG - NSC - SMs. \quad (5)$$

Data analysis

All of the data were analysed using Levene tests and Tukey's HSD tests that treated each growth chamber as a biological

replicate ($n = 3$), to check homogeneity of variances and detect significant differences between treatments, respectively. Data were log-transformed when variance was not homoscedastic. All statistical analysis was conducted in R, version 3.23 (R Development Core Team, 2014).

RESULTS

Whole-plant gas exchange and tissue biomass

Whole-plant daily assimilation, respiration and net carbon gain increased with increasing [CO_2], but the increase was much stronger at 170 ppm (compared to 390 ppm) than at 390 ppm (compared to 680 ppm) (Fig. 3a–c). However, after 9 weeks, plants grown at 390 and 680 ppm exhibited no significant difference in day-time assimilation (A), night-time respiration (R) and net carbon gain (A – R; Fig. 3a–c). Initially, R/A in plants grown at 170 and 390 ppm [CO_2] were higher than that in plants grown at 680 ppm [CO_2], but the difference was not statistically significant and disappeared over time (Fig. 3d). At 3 L, mass-based A increased with increasing [CO_2] (Fig. 4a). Mass-based R was higher at 390 and 680 ppm [CO_2] that at 170 ppm [CO_2], but there was no significant difference between the two higher [CO_2] treatments (Fig. 4b). Both mass-based A and R declined strongly over time at 390 and 680 ppm [CO_2] but much less at 170 ppm [CO_2] (Fig. 4a,b).

Biomass varied with [CO_2] in a way similar to whole-plant assimilation and respiration. At 3 L and 6 L, the dry mass of all tissues increased with increasing [CO_2], and the increase was much greater comparing plants grown between 170 and 390 ppm than between 390 and 680 ppm (Fig. 5a–d). However, at 8 L, there was no significant difference in all tissues between 390 and 680 ppm [CO_2] treatments (Fig. 5a–d).

NSC and SMs

The response of soluble sugars and total NSC (soluble sugars + starch + fructans) to [CO_2] differed between tissues and developmental stages. At 3 L, plant leaves grown at 170 ppm [CO_2] had slightly higher concentrations of soluble sugars and total NSC than plant leaves grown at 390 ppm [CO_2], but the opposite was observed in stems and roots (Fig. 6a–f). However, while the concentrations of soluble sugars and total NSC (soluble sugars + starch + fructans) remained relatively constant at 170 ppm [CO_2] between 3 L and 6 L, they doubled at 390 ppm [CO_2] and increased by more than three times at 680 ppm [CO_2] over the same period (Fig. 6a–f). Whole-plant NSC content increased in proportions that were much higher than increases in total plant biomass (Fig. 7). At 6 L, concentrations of soluble sugars and total NSC (soluble sugars + starch + fructans) were significantly higher at 680 ppm than at 390 ppm [CO_2] while the difference between 390 and 170 ppm [CO_2] was much smaller. Between 6 L and 8 L, the concentrations of soluble sugars and total NSC (soluble sugars+ starch + fructans) increased at 390 ppm [CO_2] but showed a declining trend at 680 ppm [CO_2] (Fig. 6a–f).

SM concentrations were much higher in leaves and roots than that in stems (Fig. 6g–i). Leaf SM concentrations significantly increased with increasing [CO_2] over all developmental stages (Fig. 6g). At 3 L, stem SM concentrations significantly

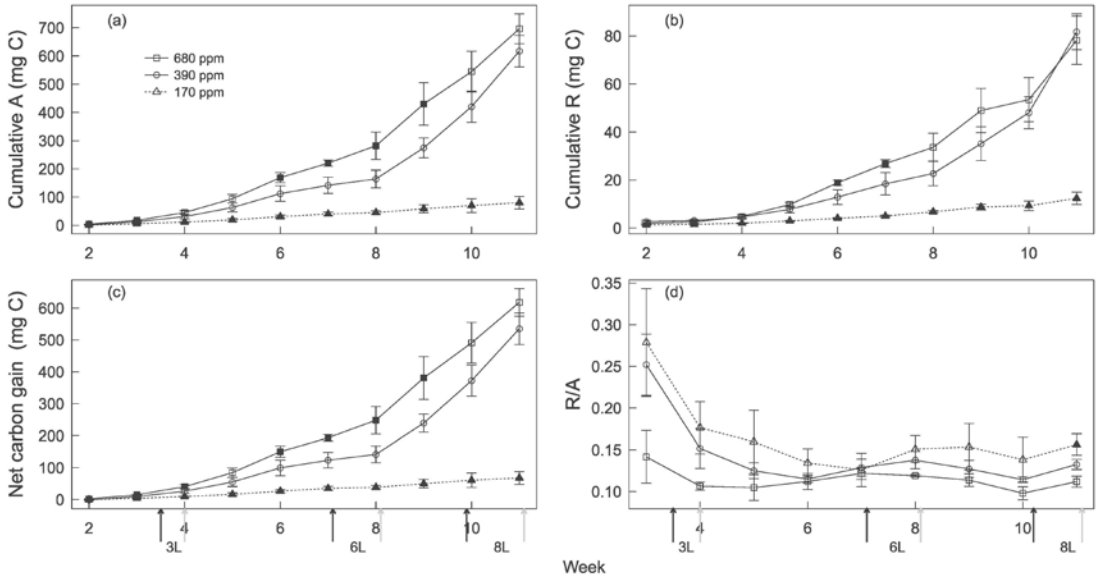

Figure 3. Weekly cumulative whole-plant net carbon assimilation A (a), respiration R (b), net carbon gain (c) and R/A (d) of winter wheat (*Triticum aestivum* cv. Genius) for the three [CO_2] treatments: 680 ppm [CO_2] (squares, black line); 390 ppm [CO_2] (circles, black line); 170 ppm [CO_2] (triangles, dashed line). Values are the means (mg C) of three individual chambers; error bars represent ±1 SD. Black arrows indicate sampling dates for 680 and 390 ppm [CO_2] treatments; grey arrows indicate sampling dates for 170 ppm [CO_2] treatment. Significant differences between 680 and 170 ppm [CO_2] treatments compared to ambient [CO_2] (390 ppm) are indicated by filling of symbols ($P < 0.05$, Tukey's HSD). Harvests were carried out after emergence of three, six and eight leaf sheaths; denoted as 3 L, 6 L and 8 L periods, respectively.

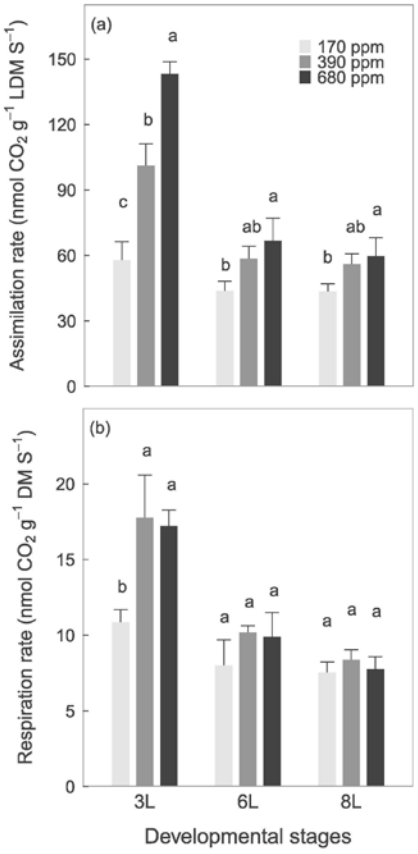

Figure 4. Leaf mass-based net assimilation rate (a) and whole-plant mass-based night-time respiration rate (b) of winter wheat (*Triticum aestivum* cv. Genius) for the three [CO_2] treatments: 680 ppm [CO_2] (black bars); 390 ppm [CO_2] (dark grey bars); 170 ppm [CO_2] (light grey bars). Values are the means (nmol CO_2 g^{-1} s^{-1}) of three individual chambers; error bars represent ±1 SD. Different letters indicate significant differences between [CO_2] treatments ($P < 0.05$, Tukey's HSD). Harvests were carried out after emergence of three, six and eight leaf sheaths; denoted as 3 L, 6 L and 8 L periods, respectively.

increased with increasing [CO_2], but at 6 L and 8 L there was no significant difference across [CO_2] treatments (Fig. 6h). At 6 L and 8 L, root SM concentrations were significantly lower at 170 ppm than at 390 ppm [CO_2]. By contrast, root SM concentrations were slightly higher at 390 ppm than at 680 ppm [CO_2], although this difference was statistically not significant (Fig. 6i). Note that most leaf SMs are C-based compounds whereas most root SMs contain N (Supporting Information Figs S1 & S2). Within leaves, low [CO_2] tended to decrease the concentrations of Luteolin-based, chrysoeriol-based, tricin-based and apigenin-based SMs, rather than ferulic acid-based SMs (Supporting Information Fig. S1). In addition, the concentrations of ferulic acid-based, luteolin-based and chrysoeriol-based SMs decreased from 3 L to 8 L across all [CO_2] treatments, whereas tricin-based and apigenin-based SMs increased at 680 ppm [CO_2] but remained relatively constant at 390 and 170 ppm [CO_2] (Supporting Information Fig. S1). Within roots, low [CO_2] tended to decrease DIMBOA-Glc (38%) more than HDMBOA-Glc (25%) (Supporting Information Fig. S2).

The response of SMs/NSC to [CO_2] differed between tissues and developmental stages (Fig. 6j–l). At 3 L and 6 L, leaf SMs/NSC was lower at 170 ppm [CO_2] than at 390 ppm [CO_2], but at 8 L there was no difference in leaf SMs/NSC across [CO_2] treatments (Fig. 6j). By contrast, stem and root SMs/NSC was higher at 170 ppm than at 390 and 680 ppm [CO_2] (Fig. 6k,l).

Proportional C allocation to respiration, structural growth, NSC and SMs

Proportional C allocation to R (R/A) remained relatively constant across [CO_2] treatments (Fig. 8). The proportion of C allocation to biomass (SG + NSC + SMs) was lower in plants grown at 170 ppm [CO_2] than at 680 ppm [CO_2], although this was not statistically significant (Fig. 8). Proportional C allocation to NSC was significantly higher in plants grown at 680 ppm than at 390 and 170 ppm [CO_2], and proportional C allocation to SMs significantly increased with increasing [CO_2] (Fig. 8). The unaccounted C pool (A − R − SG − NSC − SMs) showed large variations and no discernible trend with [CO_2].

Within-plant partitioning of biomass, NSC and SMs

Allocation of total plant biomass and NSC to leaves, stems and roots differed with [CO_2] (Fig. 9a–f). With increasing [CO_2], allocation of biomass and NSC to leaves decreased but allocation to stems and roots increased (Fig. 9a–f). However, allocation of SMs was not affected by [CO_2]; plants in all treatments showed the highest proportions in leaves and the lowest proportions in stems (Fig. 9g–i).

DISCUSSION

Continuous measurements of gas exchange combined with the recurrent quantification of whole-plant NSC and SMs allowed us to track C fluxes to different plant organs and functional pools. Manipulations of the whole-plant C balance provided insights into the response of plant C allocation to changes in C availability. Our study revealed that C allocation to respiration is maintained as a fixed proportion of net plant C gain, even over a large gradient of [CO_2]. With increasing C availability, the fraction of assimilation allocated to biomass (SG + NSC + SMs), in particular NSC and SMs, increased. Moreover, biomass and NSC increased more in stems and roots than in leaves with increasing C availability. However, under low C availability resource constraints favoured the establishment of a functional equilibrium, that is, biomass and NSC increased in leaves, the tissue responsible for the acquisition of the most limiting resource, CO_2. Increasing C availability released this constraint and allowed plants to invest proportionally more C into NSC and SMs.

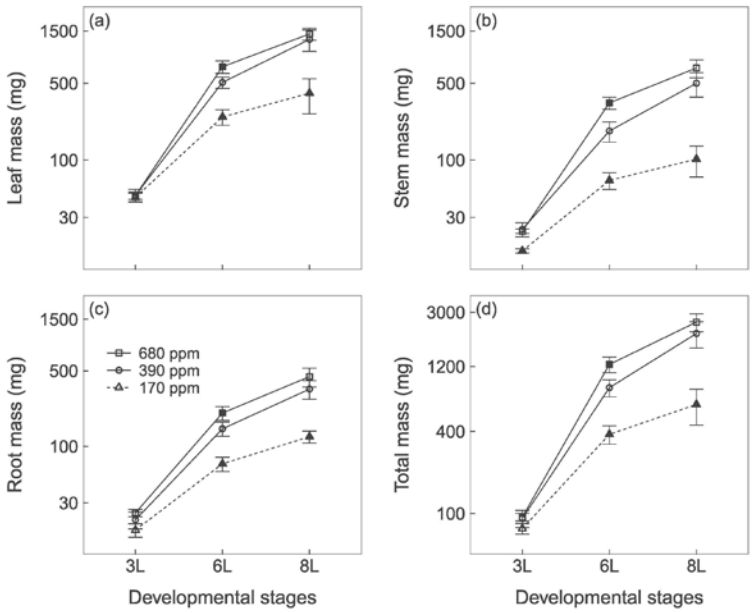

Figure 5. The dry mass of leaf (a), stem (b), root (c) and total mass (d) of winter wheat (*Triticum aestivum* cv. Genius) for the three [CO_2] treatments: 680 ppm [CO_2] (squares, black line); 390 ppm [CO_2] (circles, black line); 170 ppm [CO_2] (triangles, dashed line). Values are the means (mg) of three individual chambers; error bars represent ±1 SD. Significant differences between 680 and 170 ppm [CO_2] treatments compared to ambient [CO_2] (390 ppm) are indicated by filling of symbols ($P < 0.05$, Tukey's HSD). Note the dry mass is plotted on a log scale. Harvests were carried out after emergence of three, six and eight leaf sheaths; denoted as 3 L, 6 L and 8 L periods, respectively.

Minimizing carbon loss – with increasing C availability the proportion of C in biomass (in particular nonstructural components) increases but respiration does not

The higher relative allocation to respiration in the first weeks of the experiment (until 3 L) in the two lower [CO_2] treatments may be due to relatively greater carbon supply from seed storage than from photosynthesis. Because seed storage (~46 mg seed mass on average) was depleted rapidly over time, as indicated by strong increases in plant dry biomass (ca. 230 and 1150 mg at 3 L and 6 L at low [CO_2]), R/A decreased dramatically at low and ambient [CO_2] while it remained quite constant at elevated [CO_2]. Whole-plant R/A remained relatively constant across [CO_2] treatments afterwards, corroborating previously observed homeostasis of leaf-level R/A in response to changing [CO_2] in *Eucalyptus saligna* (Ayub *et al.*, 2011), *G. max* (Ayub *et al.*, 2014) and wheat (Gifford, 1995). The homeostasis of whole-plant R/A was also observed across a range of temperatures in two *Plantago* species (Atkin *et al.*, 2007) and at different irradiance levels in five herbaceous species (Pons & Poorter, 2014). However, all these studies assessed gas exchange during several hours or up to few days only, and hence our study, spanning many weeks, provides further insights into long-term responses of R/A to changes in [CO_2]. Note that our estimates of the R/A ratio (ca. 10–14%) were based on nighttime respiration (R) and daytime net photosynthesis (i.e. A = gross photosynthesis – daytime respiration), not on total respiration and gross photosynthesis as in other studies. Assuming daytime respiration rates equal those measured at night, the ratio of total respiration to gross photosynthesis would be ~30% and within the range of proportions previously reported (ca. 30–50%) (Atkin *et al.*, 2007; Gifford, 1995; Pons & Poorter, 2014).

Whole-plant assimilation and respiration are largely dependent on plant size and, therefore, do not represent photosynthetic or respiratory efficiency. By contrast, mass-based rates can indicate whether other factors than CO_2 availability, like leaf N or sugar concentrations, may constrain assimilation or respiration. Leaf mass-based net day-time assimilation declined over time and correlated negatively with leaf NSC concentrations but positively with leaf N concentrations in all [CO_2] treatments (Supporting Information Figs S3 & S4). Moreover, it decreased to a greater extent at elevated [CO_2] than at low [CO_2] as leaf NSC increased and N concentrations decreased to a greater extent at elevated [CO_2]. These findings support previously reported sugar- (reviewed in Long *et al.*, 2004) and Rubisco-mediated (Aranjuelo *et al.*, 2011; Pinto *et al.*, 2014) photosynthetic regulation under elevated [CO_2] but may also indicate proportionally greater light limitation at elevated than at low [CO_2] in the growth chambers.

The tight coupling between assimilation and respiration across [CO_2] treatments suggests that a large proportion of respiration serves to provide energy required during assimilation, for example, for processes like sucrose synthesis and phloem loading (Crous *et al.*, 2011). However, assimilation was not the only factor influencing respiration. In absence of

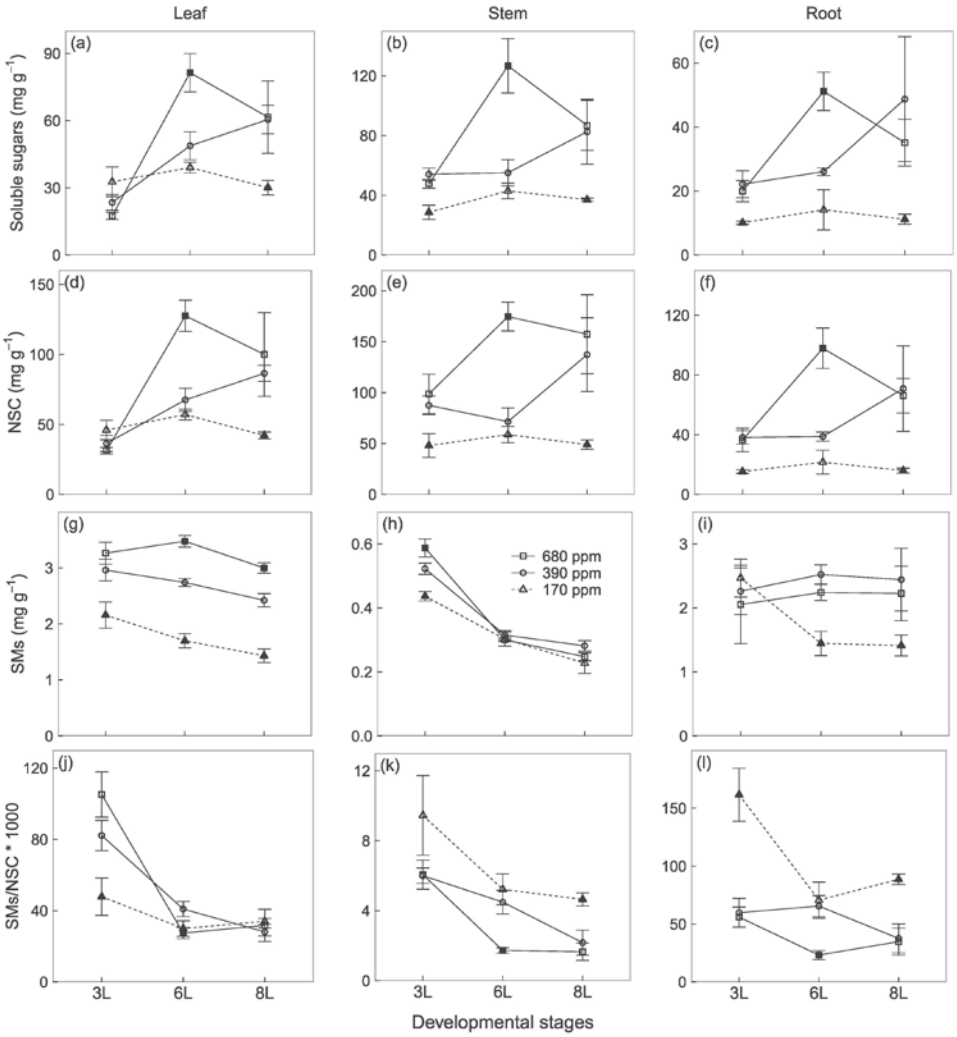

Figure 6. Concentrations of soluble sugars (mg g^{-1} dry weight, a–c), total non-structural carbohydrates (NSC, mg g^{-1} dry weight, d–e), secondary metabolites (SMs, mg g^{-1} dry weight, g–i) and ratios of SMs to NSC (j–l) in leaves, stems and roots of winter wheat (*Triticum aestivum* cv. Genius) for the three [CO_2] treatments: 680 ppm [CO_2] (squares, black line); 390 ppm [CO_2] (circles, black line); 170 ppm [CO_2] (triangles, dashed line). Total NSC concentrations were calculated as the sum of concentrations of soluble sugars, starch and fructans. Values are the means of three individual chambers; error bars represent ±1 SD. Significant differences between 680 and 170 ppm [CO_2] treatments compared to ambient [CO_2] (390 ppm) are indicated by filling of symbols ($P < 0.05$, Tukey's HSD). Note the different scales on *y*-axes. Harvests were carried out after emergence of three, six and eight leaf sheaths; denoted as 3 L, 6 L and 8 L periods, respectively.

assimilation, that is, during nights, respiration rates were quite low, but these may have been the result of low night-time temperatures in the greenhouse. The negative correlation between mass-based night-time respiration and whole-plant NSC concentrations (Supporting Information Fig. S3) indicates that substrate supply, at least at ambient and elevated [CO_2], was not a limiting factor for respiration.

Interestingly and contrary to our expectations, plants grown at high [CO_2] invested relatively more C in biomass (in particular in NSC and SMs) than plants grown at low [CO_2]. In these plants, a down-regulation of growth via modulation of phytohormones under stress (Achard *et al.*, 2006; Park *et al.*, 2007; Zhang *et al.*, 2009) could provide an explanation for the different allocation patterns. By contrast, at elevated [CO_2], photosynthesis was apparently light-, not C-limited, and plants tended to utilize more C for biomass. This is consistent with the growth rate hypothesis (Coley *et al.*, 1985; Endara & Coley, 2011) stating that plants in resource-rich environments generally grow faster in order to be able to compete for other resources such as water, nutrients or, as in our study, for light. Greenhouse light intensities were much lower than those occurring in the field. While this may put an upper limit on absolute resource fluxes, the general agreement of allocation patterns in our study with those observed in field-grown plants downplays the impact of greenhouse light limitation on allocation patterns.

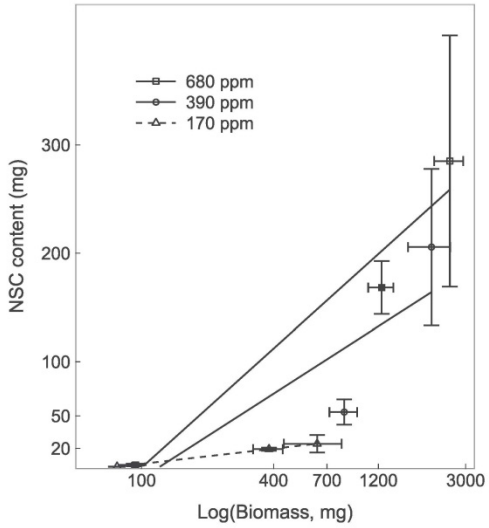

Figure 7. Correlations between whole-plant nonstructural carbohydrates (NSC) content and biomass in winter wheat (*Triticum aestivum* cv. Genius) for the three [CO_2] treatments: 680 ppm [CO_2] (squares, black line); 390 ppm [CO_2] (circles, black line); 170 ppm [CO_2] (triangles, dashed line). Values are the means (mg) of three individual chambers; error bars represent ±1 SD. Note the dry mass is plotted on a log scale. Significant differences in NSC content and biomass between 680 and 170 ppm [CO_2] treatments compared to ambient [CO_2] (390 ppm) are indicated by filling of symbols ($P < 0.05$, Tukey's HSD).

The unaccounted C pool showed relatively large variations across treatments, which may be attributed, at least partially, to changes in C export, like root exudation or the emission of VOCs. Plants release root exudates as a means to facilitate organic matter decomposition presumably for nutrient acquisition, and several studies found that root exudation was much lower in plants grown at ambient than at elevated [CO_2] (Johansson *et al.*, 2009; Phillips *et al.*, 2009). In our experiment, nutrients were provided and freely accessible (sand and nutrient solution) thus making nutrient uptake facilitation unnecessary. However, other studies showed that plants subjected to low [CO_2] always exhibited higher emissions of VOCs (Possell & Hewitt, 2011; Wilkinson *et al.*, 2009), likely for quenching reactive oxygen species (Harrison *et al.*, 2013; Loreto & Schnitzler, 2010) that are increasingly produced during inhibition of the photosynthetic electron transfer chain under limited CO_2 availability to the Calvin cycle (Brosché *et al.*, 2010).

Ensuring long-term survival – larger increases in NSC and SMs than structural growth with increasing C availability

How plants build up NSC remains a conceptual paradigm with potentially far-reaching implications for both understanding and predicting plant responses to environmental changes (Dietze *et al.*, 2014; Hartmann & Trumbore, 2016). There is substantial evidence suggesting that environmental stress limits growth faster than photosynthesis thus leading to an initial excess C accumulating as NSC (Palacio *et al.*, 2014). By contrast, trade-off allocation to NSC, that is, allocation of C to storage at the cost of growth, was directly assessed only in few recent studies (Gibon *et al.*, 2009; Hartmann *et al.*, 2015; Wiley *et al.*, 2013) but may represent an important insurance strategy against potential future threats like herbivory and thus promote long-term survival (Wiley & Helliker, 2012).

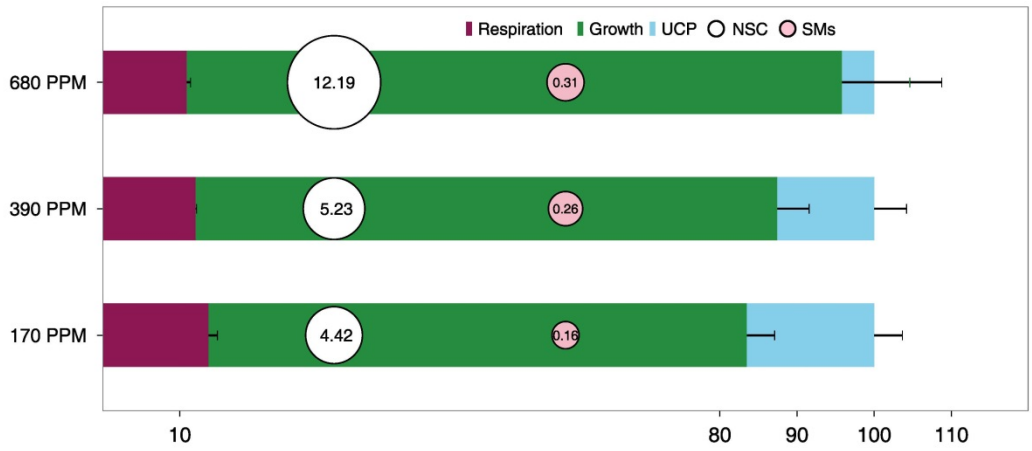

Figure 8. Percentage proportions of cumulative net carbon assimilation between 3 L and 6 L allocated to respiration (violet bars), growth (green bars), unaccounted carbon pool (UCP, blue bars), total nonstructural carbohydrates (NSC, white bubbles) and secondary metabolites (SMs, pink bubbles) of winter wheat (*Triticum aestivum* cv. Genius) for the three [CO_2] treatments: 680 ppm [CO_2]; 390 ppm [CO_2]; 170 ppm [CO_2]. All concentration data were scaled up to whole-plant content by multiplying them with tissue dry mass, and then converted to carbon content before calculating proportions. The numbers in the white and pink bubbles indicate the percentage of net carbon assimilation allocated to NSC and SMs, respectively. Note, however, that the size of these bubbles has been adjusted to allow numbers to be printed and is not linearly related to the pool sizes. Values are means of three individual chambers; error bars represent ±1 SE. [Colour figure can be viewed at wileyonlinelibrary.com]

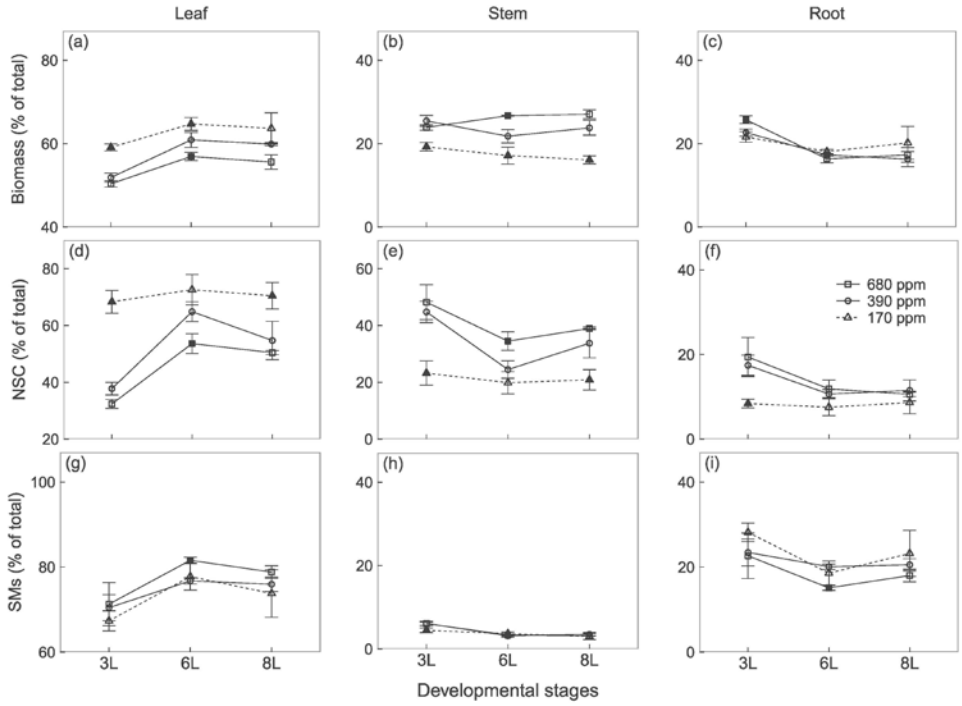

Figure 9. Percentage proportion of biomass (a–c), non-structural carbohydrates (NSC, d–e) and secondary metabolites (SMs, g–i) allocated to leaves, stems and roots of winter wheat (*Triticum aestivum* cv. Genius) for the three [CO_2] treatments: 680 ppm [CO_2] (squares, black line); 390 ppm [CO_2] (circles, black line); 170 ppm [CO_2] (triangles, dashed line). Values are the means of three individual chambers; error bars represent ±1 SD. Significant differences between 680 and 170 ppm [CO_2] treatments compared to ambient [CO_2] (390 ppm) are indicated by filled symbols ($P < 0.05$, Tukey's HSD). Note the different scales on *y*-axes. Harvests were carried out after emergence of three, six and eight leaf sheaths; denoted as 3 L, 6 L and 8 L periods, respectively.

As a result of positive C balance between 3 L and 6 L, total NSC (soluble sugars + starch + fructans) concentrations doubled at 390 ppm [CO_2] and increased by more than three times at 680 ppm [CO_2]. Therefore, greater C availability increased total NSC concentrations and whole-plant NSC content in proportions that were much higher than increases in total plant biomass. At ambient and elevated [CO_2], SG may be limited by environmental factors such as nutrients and water (Kirschbaum, 2011; Palacio *et al.*, 2014). The much higher C/N ratio at elevated than at ambient [CO_2] also suggests that SG was likely N-, rather than C-limited (Supporting Information Table S1). At 8 L, the subsequent decline in NSC concentrations at elevated [CO_2] may be due to photosynthetic acclimation of wheat plants to elevated [CO_2] (Aranjuelo *et al.*, 2011; Aranjuelo *et al.*, 2015). In addition, under elevated [CO_2], the size of the C pool in whole-plant NSC is equivalent to twice the whole-plant daily net assimilation at 6 L (1.5 times at 8 L). Thus, NSC at elevated [CO_2] may represent a multiple-day buffer pool for supporting metabolic activities.

Many manipulative studies have investigated the effects of elevated [CO_2] or of shading (to reduce C availability) on SMs (Lindroth, 2012), but little is known about the responses of whole-plant SMs over a large gradient of C availability. Leaf SM concentrations increased with increasing C availability, which is in accord with the GDBH and suggests that the SM pool is filled with C that is available in excess of growth demand (Herms & Mattson, 1992). Plants grown under low C availability may gain immediate benefits by reducing C investments into SMs. However, SMs can serve multiple functions, such as detoxification, primary metabolism and chemical defence (Neilson *et al.*, 2013). Large costs needed to repair and regrow plant tissues following stress conditions such as herbivory could reduce the long-term fitness of low [CO_2] plants with low SMs, as suggested by the growth rate hypothesis (Coley *et al.*, 1985). In addition, plants at 3 L had relatively more young leaves and higher leaf and stem SMs than plants at 8 L. Greater leaf and stem SMs in young vulnerable leaves that also have greater photosynthetic capacity may represent an investment insurance strategy.

N-rich SMs play important roles in ecological interactions, but very few studies investigated how they may respond to elevated [CO_2] (Lindroth, 2012). As expected, low [CO_2] decreased N-containing SMs (benzoxazinoid derivatives) in roots. This is consistent with the GDBH and suggests that limited C resources are preferentially invested in SG rather than SMs. Furthermore, low [CO_2] tended to decrease DIMBOA-Glc more than HDMBOA-Glc (Supporting Information Fig. S2), possibly because HDMBOA-Glc is more efficient against pathogens and insect herbivory (Glauser *et al.*, 2011) and represents

the better investment under C limitation. By contrast, concentrations of these compounds were slightly lower at elevated [CO_2] than at ambient [CO_2], likely because the production of these N-rich SMs (putrescine-based and benzoxazinoids) may have been limited by N supply rather than C supply (Supporting Information Table S1). This interpretation is in agreement with results from other studies showing that elevated [CO_2] inhibited nitrate assimilation in wheat (Bloom *et al.*, 2010). Given the defensive role of benzoxazinoid derivatives (Ahmad *et al.*, 2011), the responses observed in our study support previously reported increases in egg densities of western corn rootworm (*Diabrotica virgifera virgifera*) in the soil of soybean fields under elevated [CO_2] (Schroeder *et al.*, 2006).

The trade-off between allocation to NSC versus SMs was tissue specific and depended on the developmental stage of the plants. At 3 L, leaf SMs/NSC increased with increasing C availability, indicating that leaves tend to invest more NSC into SM production. By contrast, root SMs/NSC declined with increasing C availability, suggesting that plant roots grown at ambient and elevated [CO_2] increase NSC more than SMs, likely due to a potential N limitation on root SM production, as discussed above.

Achieving a functional equilibrium – with increasing C availability wheat plants prioritize allocation of biomass and NSC to stems and roots over leaves

Allocation patterns of biomass and NSC are consistent with the 'functional equilibrium' hypothesis (Poorter *et al.*, 2012). At low [CO_2], plants were apparently constrained to invest proportionally more biomass and NSC in leaves, while with increasing C availability biomass and NSC increased in stems and roots. Under low [CO_2], this optimization strategy allows plants to counteract C limitation, while a more balanced resource partitioning was required to support taller plants under elevated [CO_2]. Moreover, export of NSC from leaves to stems and roots in plants at elevated [CO_2] may help plants to build storage and avoid high NSC-induced negative feedback on assimilation (Long *et al.*, 2004). By contrast, proportional allocation to SMs across plant organs did not vary between treatments, likely because leaf SMs and root SMs differed in C and N cost.

OUTLOOK

While our experimental design allowed us to quantify several functional sinks of the plant C balance, it did not allow direct assessments of root exudates and VOCs and thereby prevented differentiating among C exports. The high variability in the unaccounted C pool in our experiment is likely due to the accumulation of uncertainties across different measurements and devices (Vaisala, Picarro, FTIR, elemental analyser, HPLC-PAD and HPLC-UV). Thus, future studies would benefit from direct assessment of 'export' C fluxes, including root exudates and VOCs, while keeping in mind that very short-lived or rapidly decomposed export may be detected in experimental set-ups as respiration (i.e. CO_2).

Given that SMs can serve multiple functions, the role of C allocation to SMs as a strategic investment for defence can only be assessed by simultaneously evaluating responses of herbivores and/or pathogens. Such studies have been conducted on soybean under elevated [CO_2] (reviewed in Zavala *et al.*, 2013) and can guide future studies with low C availability ([CO_2]). Because our knowledge about the mechanisms controlling plant allocation is still very limited, future studies should also focus on biochemical and molecular regulation of growth (Claeys *et al.*, 2014) and explore regulatory mechanisms of other allocation sinks like storage and SMs.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Carbon (C) and nitrogen (N) concentrations and C/N ratios in leaves, stems and roots of winter wheat (*Triticum aestivum* cv. Genius) for the three $[CO_2]$ treatments: 680 ppm $[CO_2]$ 390 ppm $[CO_2]$ and 170 ppm $[CO_2]$. Values represent the means (±1 SD) of three individual chambers. Different letters indicate significant differences between $[CO_2]$ treatments ($P < 0.05$ Tukey's HSD)

Table S2. Molecular formula, molecular weight and C fraction of standards used for the quantification of secondary metabolites in leaves, stems and roots of winter wheat (*Triticum aestivum* cv. Genius).

Figure S1. Concentrations of ferulic acid-based (a), luteolin-based (b), chrysoeriol-based (c) and tricin-based (d) and apigenin-based (e) secondary metabolites in leaves of winter wheat (*Triticum aestivum* cv. Genius) for the three $[CO_2]$ treatments: 680 ppm $[CO_2]$ (squares, blue line); 390 ppm $[CO_2]$ (circles, black line); 170 ppm $[CO_2]$ (triangles, red line). Values are the means (mg g^{-1}) of three individual chambers; error bars represent ±1 SD. Significant differences between 680 and 170 ppm $[CO_2]$ treatments compared to ambient $[CO_2]$ (390 ppm) are indicated by filled symbols ($P < 0.05$, Tukey's HSD).

Figure S2. Concentrations of Putrescine-based (a), DIMBOA-Glc-based (b) and HDMBOA-Glc-based (c) secondary metabolites in roots of winter wheat (*Triticum aestivum* cv. Genius) for the three $[CO_2]$ treatments: 680 ppm $[CO_2]$ (squares, blue line); 390 ppm $[CO_2]$ (circles, black line); 170 ppm $[CO_2]$ (triangles, red line). Values are the means (mg g^{-1}) of three individual chambers; error bars represent ±1 SD. Significant differences between 680 and 170 ppm $[CO_2]$ treatments compared to ambient $[CO_2]$ (390 ppm) are indicated by filled symbols ($P < 0.05$, Tukey's HSD).

Figure S3. Correlations between leaf nonstructural carbohydrate (NSC) concentrations (mg g^{-1}) and leaf assimilation rate (nmol CO_2 g^{-1} s^{-1}) (a), and correlations between whole-plant NSC concentrations (mg g^{-1}) and respiration rate (nmol CO_2 g^{-1} s^{-1}) (b) in winter wheat (*Triticum aestivum* cv. Genius) for the three $[CO_2]$ treatments: 680 ppm $[CO_2]$ (squares, blue line); 390 ppm $[CO_2]$ (circles, black line); 170 ppm $[CO_2]$ (triangles, red line). Values are the means of three individual chambers; error bars are ±1 SD.

Figure S4. Correlations between leaf nitrogen (N) concentrations (mg g^{-1}) and assimilation rate (nmol CO_2 g^{-1} s^{-1}) in winter wheat (*Triticum aestivum* cv. Genius) for the three $[CO_2]$ treatments: 680 ppm $[CO_2]$ (squares, blue line); 390 ppm $[CO_2]$ (circles, black line); 170 ppm $[CO_2]$ (triangles, red line). Values are the means of three individual chambers; error bars are ±1 SD.

9.12. Chapter 12: Carbon and the works – orchestration of allocation

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RESEARCH PAPER

Increasing carbon availability stimulates growth and secondary metabolites via modulation of phytohormones in winter wheat

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Abstract

Phytohormones play important roles in plant acclimation to changes in environmental conditions. However, their role in whole-plant regulation of growth and secondary metabolite production under increasing atmospheric CO_2 concentrations ($[CO_2]$) is uncertain but crucially important for understanding plant responses to abiotic stresses. We grew winter wheat (*Triticum aestivum*) under three $[CO_2]$ (170, 390, and 680 ppm) over 10 weeks, and measured gas exchange, relative growth rate (RGR), soluble sugars, secondary metabolites, and phytohormones including abscisic acid (ABA), auxin (IAA), jasmonates, and salicylic acid (SA) at the whole-plant level. Our results show that, at the whole-plant level, RGR positively correlated with IAA but not ABA, and secondary metabolites positively correlated with JA and JA-Ile but not SA. Moreover, soluble sugars positively correlated with IAA and JA but not ABA and SA. We conclude that increasing carbon availability stimulates growth and production of secondary metabolites via up-regulation of auxin and jasmonate levels, probably in response to sugar-mediated signalling. Future low $[CO_2]$ studies should address the role of reactive oxygen species (ROS) in leaf ABA and SA biosynthesis, and at the transcriptional level should focus on biosynthetic and, in particular, on responsive genes involved in $[CO_2]$-induced hormonal signalling pathways.

Key words: Abscisic acid, auxin, elevated CO_2, jasmonic acid, low CO_2, salicylic acid, secondary metabolites, soluble sugars.

Introduction

Plants capture CO_2 from the atmosphere and convert it into sugars as essential building blocks for growth and substrates for metabolism (Hartmann and Trumbore, 2016). Atmospheric CO_2 concentrations ($[CO_2]$) has risen from ~170–200 ppm during glacial periods to the current 400 ppm, and are predicted to reach between 430 ppm and 1000 ppm by 2100 (Cubasch *et al.*, 2013). Understanding the mechanisms by which increasing $[CO_2]$ have influenced whole-plant growth and metabolism in the past will help to unravel mechanisms regulating plant responses to future elevated $[CO_2]$ but also to reduced carbon availability as may occur during shading, cold, or drought. Phytohormones play an important

role in plant acclimation to changing environmental conditions (Peleg and Blumwald, 2011), such as drought (Valluru *et al.*, 2016) and salinity (Albacete *et al.*, 2008). However, our understanding of how phytohormones, such as auxin, abscisic acid (ABA), jasmonic acid (JA), or salicylic acid (SA), are involved in the whole-plant regulation of plant gas exchange, growth, and secondary metabolite (SM) production under changing [CO_2] is still limited.

ABA plays a role in multiple physiological processes for stress acclimation. For example, ABA is the main regulator of stomatal responses to drought and salinity (Osakabe *et al.*, 2014). Elevated [CO_2] reduces stomatal conductance and density (Franks *et al.*, 2013), and this is often (Lake and Woodward, 2008; Chater *et al.*, 2015), but not always (Teng *et al.*, 2006; Merilo *et al.*, 2013) associated with increased leaf ABA concentration. Changes in carbohydrate availability have been proposed to be a sensing pathway by which plants may increase ABA biosynthesis at elevated [CO_2] (Chater *et al.*, 2014). Additional application of glucose can increase ABA biosynthesis (reviewed in León and Sheen, 2003), and the ABA-dependent signalling pathway is essential for sucrose-induced stomatal closure (Kelly *et al.*, 2013). Moreover, the activation of the ABA signalling pathway is involved in inhibition of root growth under osmotic stress (Achard *et al.*, 2006; Rowe *et al.*, 2016).

Auxin (indole-3-acetic acid; IAA) was recognized as an essential plant growth promoter >70 years ago (Enders and Strader, 2015), but much less is known about its role in modulating plant response to abiotic stress (Kazan, 2013). Elevated CO_2 increased carbohydrate and IAA concentrations and promoted growth in *Arabidopsis thaliana* (Teng *et al.*, 2006; Hachiya *et al.*, 2014) and tomato seedlings (*Solanum lycopersicum*; Wang *et al.*, 2009) but reduced IAA concentrations in roots of sweet pepper (*Capsicum annuum*; Piñero *et al.*, 2014). Niu *et al.* (2011) showed that the auxin-dependent signalling pathway is required for enhancing root development under elevated CO_2 in Arabidopsis. Moreover, accumulated soluble sugars may be key elicitors for increased IAA production under elevated [CO_2], as both glucose (Sairanen *et al.*, 2012) and sucrose additions (Lilley *et al.*, 2012) have been shown to stimulate IAA biosynthesis and resulted in higher growth rates in the latter study. In contrast, low [CO_2] reduces carbohydrate availability (Hartmann *et al.*, 2013, 2015) and limits plant growth (Gerhart and Ward, 2010), but whether IAA regulation is involved in these processes remains uncertain.

JA (Riemann *et al.*, 2015) and SA (Khan *et al.*, 2015) play key roles in regulating plant defence responses to abiotic and biotic stresses. The JA signalling pathway involves the isoleucine (Ile) conjugate of JA (JA-Ile), a phytohormone that activates the transcription of JA-dependent defence genes (Thines *et al.*, 2007). In contrast to JA-Ile, the hydroxylated derivatives of JA, such as 12-hydroxy-JA (12-OH-JA, tuberonic acid) may deactivate JA-dependent defence genes (Miersch *et al.*, 2008; Koo and Howe, 2012). Elevated [CO_2] has been shown to enhance SA-dependent defence and repress JA-dependent defence (Zavala *et al.*, 2013; Sun *et al.*, 2016), but how plants regulate JA and SA at low [CO_2] remains uncertain. Furthermore, changes in soluble sugars may regulate production of SMs via modulation of JA and SA. Sucrose (Loreti *et al.*, 2008) and glucose (Guo *et al.*, 2013) play a synergistic role with JA in anthocyanin and glucosinolate biosynthesis, respectively.

Shading and defoliation are common approaches for reducing plant sugar availability, but shading can activate IAA biosynthesis (Casal, 2013) and suppress JA biosynthesis via changes in phytochromes (Ballare, 2014), and defoliation itself is a wounding treatment that can trigger JA biosynthesis with cascading effects on other phytohormones (Erb *et al.*, 2012). Directly manipulating [CO_2] suffers less from such side effects and allows plants to regulate phytohormones under contrasting carbon (rather than light) availability.

Here, we present an analysis of whole-plant phytohormone dynamics in winter wheat (*Triticum aestivum*) grown along a gradient of atmospheric CO_2 concentrations (170, 390, and 680 ppm). We investigated correlations between phytohormone concentrations and stomatal conductance, relative growth rate (RGR), and soluble sugar and SM concentrations. In this study, we focused on ABA, IAA, SA, and JA as well as its derivatives JA-Ile and 12-OH-JA, although we are aware that other phytohormones such as cytokinin and ethylene also play important roles in the regulation of growth. Based on knowledge about hormonal regulation of growth and secondary metabolite production at the tissue/organ level, we explore whether the following relationships hold true at the whole-plant level: with increasing [CO_2], plants (i) reduce stomatal conductance via increasing ABA; (ii) promote growth via increasing IAA; (iii) increase biosynthesis of SMs via increasing JA, JA-Ile, and SA; and (iv) show higher carbohydrate concentrations that, in turn, induce the biosynthesis of phytohormones.

Materials and methods

Plant material

We used a cultivar of winter wheat (*Triticum aestivum* cv. Genius) adapted to Central Europe. On 7 January 2015, seeds were germinated on plates filled with sand and watered every day. After 6 d, we transplanted 10–12 seedlings with similar height into each pot (11 cm diameter, 24 cm height) pre-filled with quartz sand. Six pots were randomly placed in each growth chamber. All pots were irrigated with a continuous through-flow of a modified Hoagland solution (Hoagland and Arnon, 1950).

Growth chambers and treatments

Plants were grown in glass chambers (75 cm long×45 cm wide×80 cm high) flushed continuously with air at a flow rate of 14 l min^{-1} (for more details, see Hartmann *et al.*, 2013). Concentrations of the different CO_2 treatments were produced by first scrubbing all CO_2 from incoming air using a molecular sieve, and then injecting pure CO_2 (Schnyder, 1992). The [CO_2] of incoming air for the three [CO_2] treatments was measured with a Vaisala® (GMP 343) at intervals of 10 min. A micro-logger (Campbell® CR1000) compared these concentrations against pre-set values (170, 390, and 680 ppm) and adjusted them accordingly via mass flow controllers. For each [CO_2] treatment, three chambers were used to grow plants and one chamber was used to monitor [CO_2] reference levels.

In each chamber, we monitored air temperature and photosynthetic photon flux density (PPFD) continuously (for more details,

see Hartmann *et al.*, 2013). During the day, average temperature increased from ~12 °C at 06:30 h (local time) to ~20–24 °C and then decreased to ~16.5 °C at 22:00 h. Plants were grown in a light/dark regime of 16/8 h using supplemental greenhouse lamps. The average PPFD from 23 January 2015 to 20 March 2015 was 7.79 ± 0.92 mol m^{-2} d^{-1}.

Destructive harvesting

To rule out potential effects of plant development on hormone levels, we harvested plants in different treatments independently of calendar dates and when three, six, and eight leaf sheaths were completely developed, denoted as 3L, 6L, and 8L periods, respectively. The experiment was only conducted during the vegetative growth period (seedling growth and tillering stage). Plants grown in 390 ppm and 680 ppm [CO_2] chambers were sampled 3, 7, and 9 weeks after transplanting, whereas plants grown in low [CO_2] chambers were sampled 3.5, 8, and 10.5 weeks after transplanting. Harvests were always conducted between 16:00 h and 21:00 h to minimize light and temperature effects on hormones and metabolites. For each harvest, we removed one pot from each chamber. Plants were separated into leaves, stems, and roots. Leaf area was determined with a Li 3100A area meter (Li-Cor, Bad Homburg, Germany). All fresh tissues were weighed and frozen in liquid nitrogen and later transferred to a −80 °C freezer. Around 75% of the biomass was freeze-dried, weighed, and ground to fine powder using a ball mill (Retsch® MM400, Haan, Germany) and finally stored at −20 °C until further analysis. The rest of the samples were ground with liquid nitrogen using a mortar and pestle, and stored at −80 °C until analysis.

Whole-plant gas exchange

A Picarro® 2101-i (precision 0.01–0.4%, Picarro Inc., Santa Clara, CA, USA) was used to measure the [CO_2] and [H_2O] of air entering and leaving the growth chambers. The air coming from the 12 chambers and the reference air were measured sequentially at intervals of 6 min 40 s; the cycle was controlled by a micro-logger (Campbell® CR1000) connected to a custom-built valve switching unit, completing a whole cycle within 2 h. Transition periods after valve switching were excluded from analysis.

We assumed whole-plant gas exchange to be constant within the 2 h cycle. The instantaneous whole-plant assimilation (A) and transpiration (E) at hour j was calculated as:

$$[\mathrm{CO_2\,or\,H_2O}]_j\ (\mu\mathrm{mol\,s^{-1}}) = \frac{[\mathrm{CO_2\,or\,H_2O}]_{\mathrm{non-plant}} - [\mathrm{CO_2\,or\,H_2O}]_{\mathrm{plant}}}{M}\ (\mu\mathrm{mol\,mol^{-1}}) \times \frac{\mathrm{VFR}\,(1\,\mathrm{min^{-1}})}{22.4\,(1\,\mathrm{mol^{-1}}) \times 60\,\mathrm{s}} \quad (1)$$

where [CO_2 or H_2O]$_{non\text{-}plant}$ and [CO_2 or H_2O]$_{plant}$ is the [CO_2 or H_2O] of outgoing air from the reference chambers without plants and from chambers with plants, respectively. M represented the number of plants in the chamber, and VFR was the volumetric flow rate of air going through the chamber (14 l min^{-1}). The value of 22.4 l mol^{-1} is the molar volume of gas under normal conditions. Canopy conductance was then estimated as previously described in McDowell *et al.* (2008):

$$G_S = \frac{E}{\mathrm{VPD}} \quad (2)$$

where VPD (kPa) is vapour pressure deficit. Note that the relative humidity of incoming air was very low (<2%) therefore VPD is close to the saturation vapour pressure at chamber temperature. Leaf area-based (i.e. specific) net assimilation, transpiration, and stomatal conductance (G_s) were then calculated by dividing instantaneous whole-plant exchange by leaf area (m^2). To obtain a robust estimate of current whole-plant gas exchange, we averaged whole-plant gas exchange over the last few days prior to biomass sampling.

Analysis of soluble sugars

Concentrations of glucose, sucrose, and fructose were measured using the method of Raessler *et al.* (2010). Briefly, we added 1 ml (0.5 ml for small samples) of sterilized water to 50 mg (10 mg for small samples) of ground sample. The mixture was vortexed, incubated for 10 min at 65 °C, and then centrifuged for 10 min at 12 000 *g*. The supernatant was carefully collected and stored on ice, and the pellet was re-extracted twice. The supernatants were pooled and diluted at a ratio of 1:20 (1:8 for small samples) and stored at −20 °C before measurements. Sucrose, glucose, and fructose were determined by HPLC coupled with pulsed amperometric detection (HPLC-PAD), on a Dionex® ICS 3000 ion chromatography system equipped with an autosampler (Thermo Fisher GmbH, Idstein, Germany).

Analysis of SMs

A 500 µl aliquot (300 µl for small samples) of 95% methanol was added to 50 mg (30 mg for small samples) of freeze-dried tissues. The mixture was bead-beaten for 40 s at 6.0 m s^{-1} with a FastPrep Instrument (MP Biomedicals, Santa Ana, CA, USA), vortexed for 5 min, and then centrifuged at 13 000 *g* for 5 min. The supernatant was collected and the pellet was re-extracted. The supernatants were pooled and stored at 4 °C. Identification and quantification of SMs were achieved by HPLC coupled with MS and a UV Detector. Phenolic compounds were separated on a Nucleodur Sphinx RP18ec column (250 × 4.6 mm, particle size 5 µm, Macherey Nagel, Dueren, Germany) with two mobile phases 0.2% (v/v) formic acid (A) and acetonitrile (B) using the following elution profile: 0–28 min, 5–61% B in A; 28–30 min 100% B; and 30–35 min 5% B. Flow was diverted in a ratio of 4:1 before entering the mass spectrometer electrospray chamber. For identification, ESI-MS was operated at a negative mode scanning *m/z* between 50 and 1600 with an optimal target mass of 400 *m/z*. The MS conditions were: skimmer voltage, 60 V; capillary voltage, 4200 V; nebulizer pressure, 35 psi; drying gas, 11 l min^{-1}; gas temperature, 330 °C; capillary exit potential, −121 V. For quantification, the UV wavelengths 240, 260, 280, and 330 nm were monitored. Compounds of leaves and stems were identified by comparing the fragmentation patterns with previously reported wheat phenolic profiles (Moheb *et al.*, 2011; Wojakowska *et al.*, 2013). Root compounds were identified based on profiles of benzoxazinoids in grasses (Wouters *et al.*, 2014). All compounds were quantified by external standards.

Quantification of hormones

Concentrations of ABA, IAA, SA, and jasmonates, comprising JA, JA-Ile, and 12-OH-JA, were determined using the method of Vadassery *et al.* (2012) with modifications. Briefly, 250 mg of fresh samples were extracted with 1 ml of methanol containing 40 ng of D_6-ABA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 40 ng of D_5-IAA (Olchemin, Olomouc, Czech Republic), 40 ng of D_4-SA (Sigma-Aldrich), 40 ng of D_6-JA (HPC Standards GmbH, Cunnersdorf, Germany), and 8 ng of JA-[$^{13}C_6$]Ile conjugate as internal standards. JA-[$^{13}C_6$]Ile was synthesized using [$^{13}C_6$]Ile (Sigma-Aldrich) according to Kramell *et al.* (1988). The mixture was vortexed for 10 min and then centrifuged at 13 000 *g* for 10 min. A 800 µl aliquot of the supernatant was then collected and transferred into a 5 ml 96-well plate. The pellet was re-extracted with 500 µl of methanol using the same procedure, and 500 ml of supernatant was collected, pooled, and stored at −20 °C.

Hormone detection and quantification was accomplished with an Agilent 1260 HPLC system (Agilent Technologies,

Santa Clara, CA, USA) coupled to an API 5000 tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Turbospray ion source. Hormones were separated on a Zorbax Eclipse XDB-C18 HPLC column (1.8 µm, 50 × 4.6 mm; Agilent) at 25 °C, with two mobile phases consisting of 0.05% formic acid in water (solvent A) and acetonitrile (solvent B), at a flow rate of 1.1 ml min^{-1} using the following elution profile: 0–0.5 min, 10% B; 0.5–4.0 min, linear gradient from 10% to 90% B; 4.0–4.02 min, linear gradient from 90% to 100% B; 4.02-4.50 min, 100% B, 4.50-4.51 min, linear gradient from 100% to 10% B; and 4.51–7.00 min, 10% B. The parent ion and their fragments of jasmonates, SA, and ABA were analysed in negative mode by multiple reaction monitoring (MRM) (for more details, see Vadassery *et al.*, 2012). IAA was analysed in the positive ionization mode in a separate chromatographic analysis (same LC conditions as above for other phytohormones) with the following conditions: analyte parent ion→product ion: *m/z* 176→130 for IAA; *m/z* 181→134+*m/z* 181→133 for D_5-IAA. Collision energy (CE) was 19 V; declustering potential (DP) was 31 V. Q1 and Q3 quadrupoles were both maintained at unit resolution. Mass data were collected and processed using analyst 1.6 software (Applied Biosystems). Linearity in ionization efficiencies was confirmed by analysing serial dilutions of a standard mixture. The concentrations of ABA, IAA, SA, JA, and JA-Ile were determined relative to the corresponding internal standard. The concentration of OH-JA was determined relative to D_6-JA by analysing a mixture of OH-JA and D_6-JA at the same concentration. OH-JA was synthesized as described in Nakamura *et al.* (2011) and was kindly provided by Wilhelm Boland (MPI for Chemical Ecology, Jena, Germany).

Data analysis

Each growth chamber was treated as a biological replicate (n=3). We determined homogeneity of variances with the Levene test and log-transformed data when variance was not homoscedastic. Tukey's HSD (P<0.05) was used to detect significant differences between treatments. Weighted phytohormone concentrations were calculated by multiplying tissue-specific concentrations by tissue biomass and dividing their sum by whole-plant mass, as for weighted soluble sugars and SM concentrations. Phytohormones were reported on a fresh weight basis; therefore, to ensure consistency of units, the concentrations of soluble sugars and SMs were converted to fresh weight. We assessed the Pearson's correlation of phytohormones with gas exchange, RGR, soluble sugars, and SMs. All statistical analysis was conducted in R, version 3.23 (R Development Core Team, 2014).

Results

Gas exchange rate

Plants grown at 170 ppm [CO_2] exhibited lower assimilation rates but higher transpiration rates and higher stomatal conductance compared with plants grown at 390 ppm and 680 ppm [CO_2] (Fig. 1a–c). The difference between the two higher [CO_2] treatments, however, varied with developmental stages. At 3L, assimilation rates increased significantly at 680 ppm [CO_2] compared with 390 ppm [CO_2], while at 6L and 8L the increase disappeared (Fig. 1a). At 3L and 6L, transpiration rates and stomatal conductance remained relatively constant between 390 ppm and 680 ppm [CO_2], while at 8L they were higher at 680 ppm than at 390 ppm [CO_2] (Fig. 1b, c).

RGR, soluble sugars, and secondary metabolites

Similar to assimilation, plants grown at 170 ppm [CO_2] exhibited a lower RGR of all tissues than plants grown at 390 ppm [CO_2], across developmental stages (Fig. 2a–c). At 6L, RGR was higher in plants grown at 680 ppm [CO_2] than at 390 ppm [CO_2], but at 8L, plants grown at 390 ppm and 680 ppm [CO_2] exhibited a similar RGR of all tissues (Fig. 2a–c).

The [CO_2] response of soluble sugars varied with developmental stages and tissues. At 3L, all soluble sugar concentrations slightly increased at 170 ppm [CO_2] in leaves, but glucose and fructose concentrations significantly decreased in stems and roots, compared with 390 ppm [CO_2] (Fig. 3a–i). At 6L, there was no significant difference in soluble sugars across tissues between 170 ppm and 390 ppm [CO_2], but glucose and fructose concentrations significantly increased at 680 ppm [CO_2] in all tissues compared with 390 ppm [CO_2] (Fig. 3a–i). Interestingly, at 6L, we did not observe large differences in leaf sucrose concentration between the two higher [CO_2] treatments. From 6L to 8L, while soluble sugars of all tissues showed a declining trend at 680 ppm [CO_2], they accumulated at 390 ppm [CO_2] but slightly decreased at 170 ppm [CO_2] (Fig. 3a–i). Sucrose concentrations were

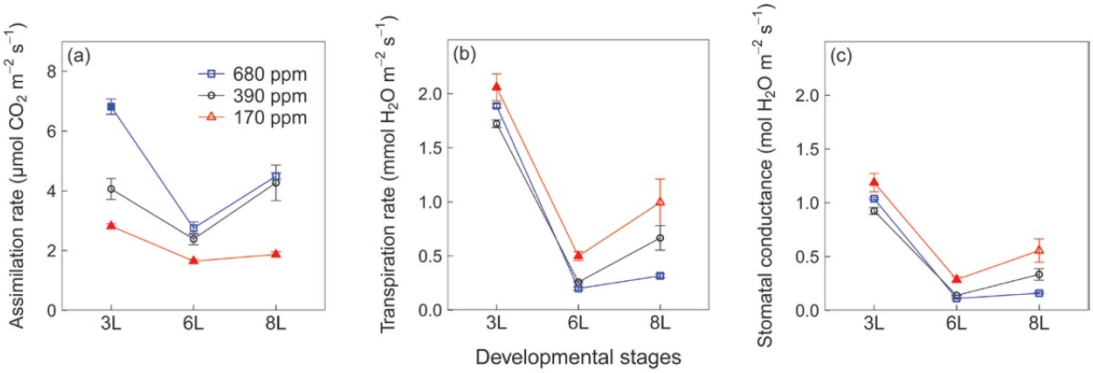

Fig. 1. Leaf area-based net assimilation rate (a), transpiration rate (b), and stomatal conductance of winter wheat (*Triticum aestivum*) for the three [CO_2] treatments (squares, 680 ppm; circles, 390 ppm; triangles, 170 ppm). Values are the mean ±SE of three individual chambers. Filled symbols of 680 ppm and 170 ppm [CO_2] treatments indicate significant differences compared with 390 ppm [CO_2] treatment (P<0.05, Tukey's HSD). We harvested plants after emergence of three, six, and eight leaf sheaths, denoted by 3L, 6L, and 8L, respectively. (This figure is available in colour at *JXB* online.)

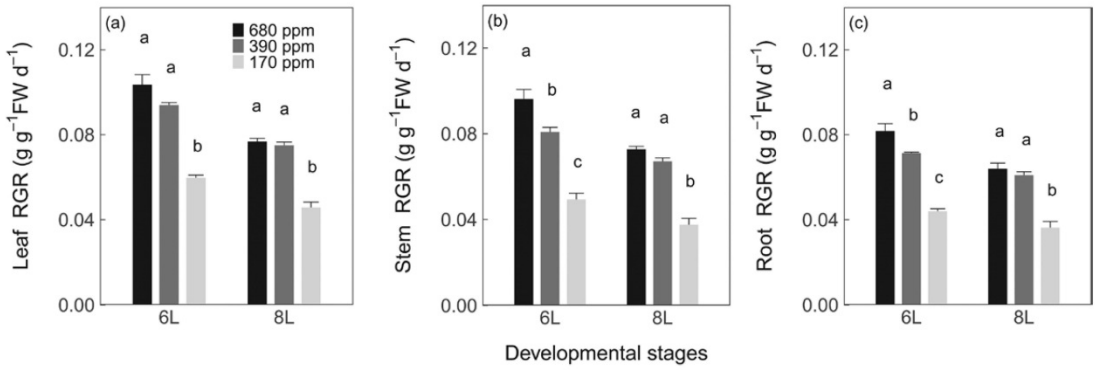

Fig. 2. Relative growth rate (RGR) of leaves (a), stems (b), and roots (c) of winter wheat (*Triticum aestivum*) for the three [CO_2] treatment. Values are the mean (g g^{-1} FW d^{-1}) ±SE of three individual chambers. Significant differences between [CO_2] treatments are indicated by different letters (P<0.05, Tukey's HSD). We harvested plants after emergence of three, six, and eight leaf sheaths, and RGR between three and six leaf sheaths and between six and eight leaf sheaths are denoted by 6L and 8L, respectively.

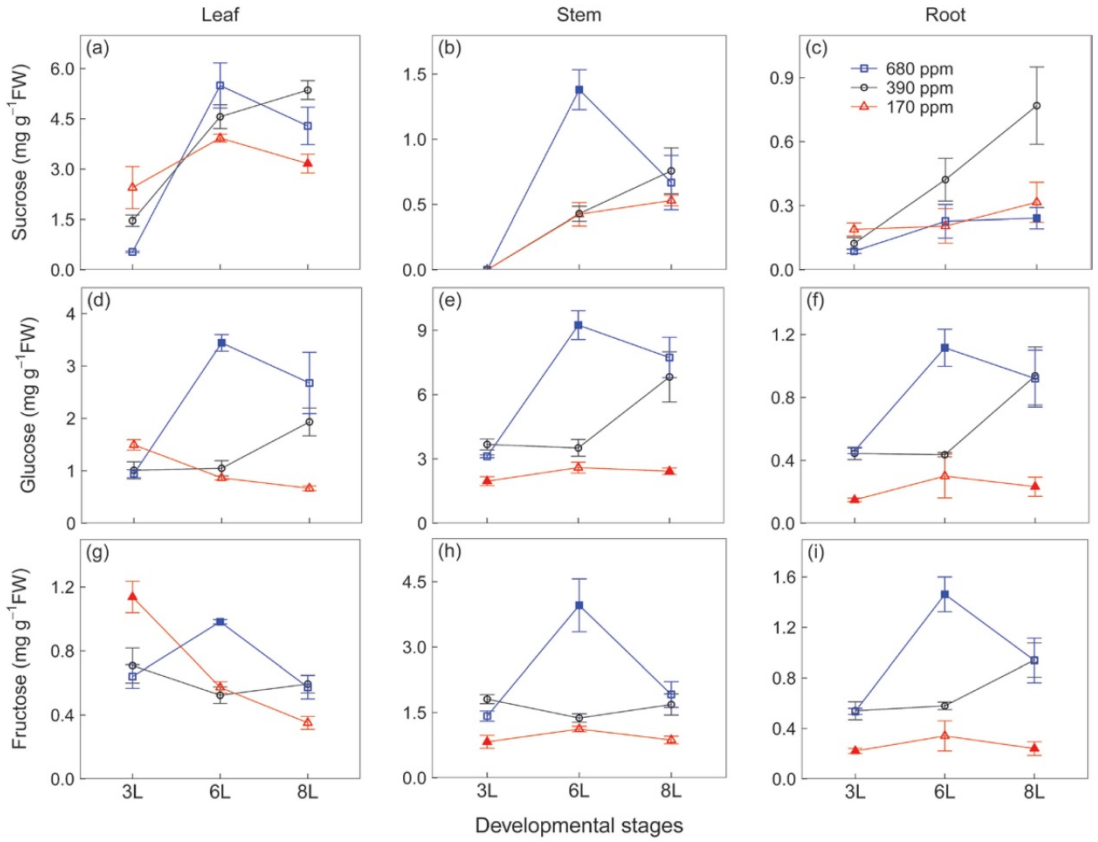

Fig. 3. Sucrose (a–c), glucose (d–f), and fructose (g–i) concentrations of winter wheat (*Triticum aestivum*) for the three [CO_2] treatments (squares, 680 ppm; circles, 390 ppm; triangles, 170 ppm). Values are the mean (mg g^{-1} FW) ±SE of three individual chambers. Filled symbols of 680 ppm and 170 ppm [CO_2] treatments indicate significant differences compared with 390 ppm [CO_2] treatment (P<0.05, Tukey's HSD). Note that the concentrations are expressed on a fresh weight basis and at different scales. We harvested plants after emergence of three, six, and eight leaf sheaths, denoted by 3L, 6L, and 8L, respectively. (This figure is available in colour at *JXB* online.)

much higher in leaves than in stems and roots, whereas glucose and fructose concentrations were generally higher in stems (Fig. 3a–i).

At 3L, plants grown at contrasting [CO_2] showed similar SM concentrations in leaves and roots. However, at 6L and 8L, leaf SM concentrations decreased with declining

[CO_2], but the decrease was greater at 170 ppm (compared with 390 ppm) than at 390 ppm (compared with 680 ppm). In contrast, at 6L and 8L, root SM concentrations remained unchanged between 390 ppm and 680 ppm [CO_2] treatments, but they were lower at 170 ppm than at 390 ppm and 680 ppm [CO_2], although not statistically significant at 8L (Table 1).

Hormonal profiling

At 6L and 8L, IAA concentrations decreased at 170 ppm [CO_2] in all tissues compared with 390 ppm and 680 ppm [CO_2], but the difference between the two higher [CO_2] treatments varied with developmental stages and tissues (Fig. 4a–c). IAA concentrations in stems were generally twice more than those in leaves and roots (Fig. 4a–c). In contrast to IAA, at 3L and 6L, ABA concentrations increased at 170 ppm [CO_2] in leaves but not in stems and roots, compared with 390 ppm and 680 ppm [CO_2] (Fig. 4d, e). From 3L to 8L, a consistent decrease in leaf ABA concentrations was observed at 170 ppm [CO_2], while the opposite was the case for 390 ppm and 680 ppm [CO_2] treatments (Fig. 4d). Note that ABA concentrations were much lower in roots than in leaves and stems (Fig. 4d, e).

Similar to IAA, at 6L and 8L, JA and JA-Ile concentrations were much lower in plants grown at 170 ppm than at 390 ppm and 680 ppm [CO_2], but at 6L there was no difference in leaves and stems between these two higher [CO_2] treatments (Fig. 5a–e). Plants grown at contrasting [CO_2] exhibited relatively similar 12-OH-JA concentrations in leaves and stems (Fig. 5g–i). Note that leaves and stems had lower concentrations of JA and JA-Ile but higher concentrations of 12-OH-JA compared with roots, in particular at 390 ppm and 680 ppm [CO_2] (Fig. 5a–i).

In contrast, plants grown at 170 ppm [CO_2] had higher leaf SA concentrations than at 390 ppm and 680 ppm [CO_2] (Fig. 6a). However, at 3L and 8L, there were no differences between the two higher [CO_2] treatments, and only at 6L were leaf SA concentrations lower at 390 ppm [CO_2] than at 680 ppm [CO_2] (Fig. 6a). SA concentrations remained relatively constant in stems and roots across [CO_2] treatments (Fig. 6b, c). Note that SA concentrations were relatively lower in stems and roots than in leaves (Fig. 6a–c).

Correlations of phytohormones to RGR, stomatal conductance, SMs, and soluble sugars

As expected, the decline in whole-plant RGR with decreasing [CO_2] was positively correlated with weighted IAA concentrations (R^2=0.84, P=0.01) (Fig. 7a). In contrast, RGR was negatively correlated with weighted ABA concentrations (R^2=0.15), but not statistically significantly (Fig. 7b). There was no correlation between stomatal conductance and leaf ABA concentrations (Fig. 7c). Weighted SM concentrations were also positively correlated with weighted JA concentrations (R^2=0.79, P=0.02) as well as concentrations of its bioactive derivative JA-Ile (R^2=0.51, P=0.11), but not with weighted SA concentrations (Fig. 7d, f). We also found a significant positive correlation between weighted soluble sugar concentrations and weighted IAA and JA concentrations, but not weighted ABA and SA concentrations (Fig. 8a–d).

Discussion

Manipulating whole-plant carbon availability along a gradient of [CO_2] combined with whole-plant hormonal analysis allowed unravelling of the mechanisms by which plants cope with abiotic stresses. As summarized in Fig. 9, our study revealed that low [CO_2] increased ABA and SA concentrations in leaves, probably in order to cope with potential oxidative stress from excess light excitation energy. With increasing C availability, wheat plants increased growth and

Table 1. *Secondary metabolite concentrations in leaves, stems, and roots of winter wheat (*Triticum aestivum*) for the three [CO_2] treatments: 680 ppm [CO_2], 390 ppm [CO_2], and 170 ppm [CO_2]*

Values are mean (μg g^{-1} FW) ±SD of three individual chambers. Note that the concentrations are expressed on a fresh weight basis.

Development	CO_2 ppm	Leaf					Stem	Root		
		Ferulic acid	Luteolin	Apigenin	Chrysoeriol	Tricin	Ferulic acid	Putrescine	DIMBOA-Glc	HDMBOA-Glc
3L	680	88.8 (6.1) a	59.8 (9.8) a	155.9 (15.5) a	43.5 (3.7) a	47.0 (3.9) a	55.9 (2.2) a	16.0 (4.3) a	46.3 (10.7) a	47.9 (6.5) a
	390	92.5 (10.2) a	51.3 (10.6)ab	163.1 (13.7) a	45.1 (4.5) a	48.3 (3.0) a	53.0 (4.7) a	20.3 (4.9) a	48.9 (6.2) a	44.5 (7.6) a
	170	87.9 (1.9) a	29.4 (5.5) b	134.2 (17.4) a	35.4 (4.8) a	43.3 (4.2) a	42.5 (3.7) b	18.2 (4.2) a	61.5 (24.4) a	58.0 (3.4) a
6L	680	57.9 (2.8) a	56.1 (4.7) a	193.8 (4.5) a	45.1 (2.5) a	70.7 (6.9) a	34.5 (0.5) a	32.1 (6.6) ab	59.4 (8.9) a	31.2 (2.4) a
	390	53.9 (6.3) ab	36.7 (3.2) b	164.4 (7.8) b	39.1 (3.0) b	50.2 (2.4) b	30.5 (2.5) b	45.6 (6.8) a	65.7 (13.4) a	27.8 (2.3) ab
	170	45.6 (2.6) b	11.8 (2.6) c	112.0 (11.3) c	25.4 (1.2) c	38.4 (4.0) b	29.0 (0.3) b	20.6 (4.3) b	44.0 (3.6) a	22.8 (2.3) b
8L	680	40.6 (3.4) a	39.8 (2.9) a	179.2 (8.8) a	39.5 (2.6) a	67.5 (7.4) a	29.2 (1.7) a	48.4 (5.6) a	56.1 (6.9) a	26.1 (4.3) a
	390	43.7 (1.5) a	26.9 (1.0) b	158.0 (10.7) a	33.4 (1.7) a	53.0 (7.4) b	31.4 (0.8) a	54.5 (12.9) a	57.4 (5.3) a	22.8 (1.3) a
	170	37.9 (4.7) a	6.1 (2.1) c	101.5 (10.7) b	17.7 (3.2) b	35.9 (2.5) c	23.6 (5.1) a	32.5 (10.9) a	44.8 (7.6) a	21.7 (7.4) a

Different letters indicate significant differences between [CO_2] treatments (P<0.05, Tukey's HSD).
We harvested plants after emergence of three, six, and eight leaf sheaths, denoted by 3L, 6L, and 8L, respectively.
DIMBOA-Glc, 2-(2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose; HDMBOA-Glc, 2-(2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose.

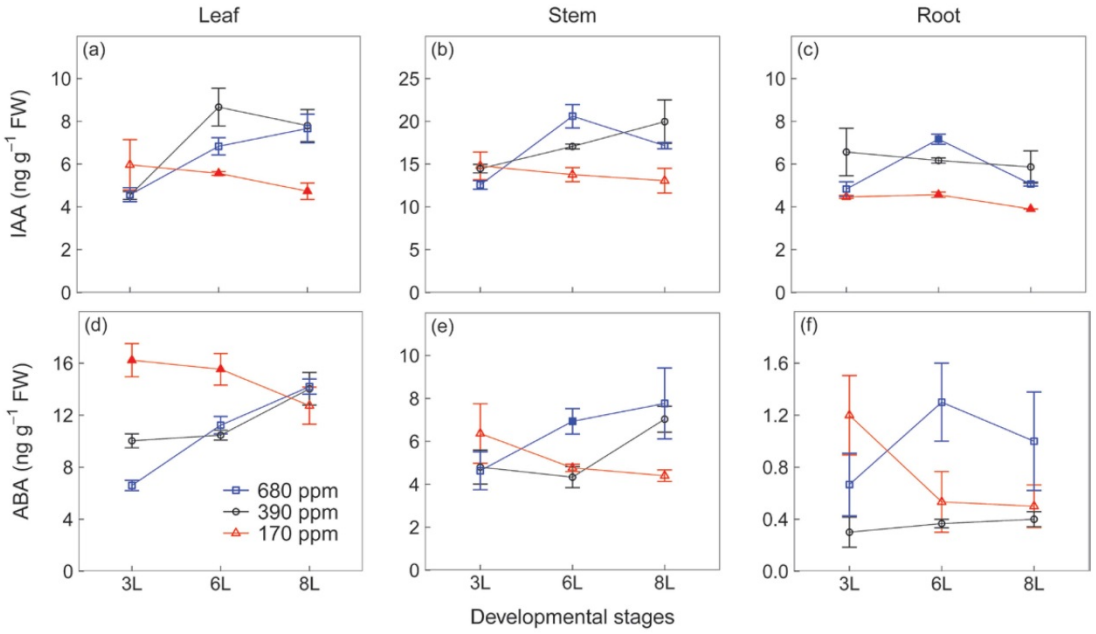

Fig. 4. Auxin (IAA) (a–c) and abscisic acid (ABA) concentrations of winter wheat (*Triticum aestivum*) for the three [CO_2] treatments (squares, 680 ppm; circles, 390 ppm; triangles, 170 ppm). Values are the mean (ng g^{-1} FW) ±SE of three individual chambers. Filled symbols of 680 ppm and 170 ppm [CO_2] treatments indicate significant differences compared with 390 ppm [CO_2] treatment (P<0.05, Tukey's HSD). Note that the concentrations are expressed on a fresh weight basis and at different scales. We harvested plants after emergence of three, six, and eight leaf sheaths, denoted by 3L, 6L, and 8L, respectively. (This figure is available in colour at *JXB* online.)

SM production via increases in IAA and JA (JA-Ile) levels and probably triggered by sugar signalling pathways.

Stomatal regulation

Transpiration rates and stomatal conductance were higher at low [CO_2] than at ambient and elevated [CO_2], and this difference increased over time. However, contrary to our expectation, stomatal conductance was not associated with leaf ABA concentrations under contrasting [CO_2]. Although it is well established that ABA can induce stomatal closure (Osakabe *et al.*, 2014), a lack of response to changes in bulk ABA cannot rule out ABA-dependent stomatal regulation, as mutants with only 10% of wild-type ABA concentrations showed stomatal closure under elevated [CO_2] (Merilo *et al.*, 2013). A recent study of ABA-deficient plants suggested that stomatal regulation under elevated [CO_2] requires increases in ABA in both stomatal precursor cells and guard cells, rather than in bulk ABA (Chater *et al.*, 2015). Furthermore, increasing leaf ABA concentration between 3L and 8L at elevated [CO_2] may be due to a down-regulation of stomatal density under elevated [CO_2] (Chater *et al.*, 2014).

Growth regulation

RGR increased in all tissues with increasing [CO_2] and were, at the whole-plant level, positively correlated with IAA but not with ABA, suggesting that IAA may be involved in growth regulation under low C availability. Growth modulation via IAA under elevated [CO_2] has also been observed in Arabidopsis (Hachiya *et al.*, 2014) and in tomato seedlings (Wang *et al.*, 2009), and during drought and low temperatures in rice (*Oryza sativa*) (Zhang *et al.*, 2009; Du *et al.*, 2013).

While leaves, stems, and roots showed similar RGR, IAA concentrations in stems were more than twice as high as in leaves and roots. IAA is synthesized mainly in the shoot apex and then transported to roots (Ljung *et al.*, 2001) so high IAA concentrations in stems may be an inactive transitory pool. Moreover, the basipetal transport of IAA from shoot to roots remained relatively constant even under low C availability, as the ratio of stem to root IAA concentrations remained relatively constant across [CO_2] treatments. In addition, at 8L, when RGR and soluble sugar concentrations at elevated [CO_2] declined in all tissues to ambient [CO_2] levels, we also observed a decrease in stem IAA concentrations. This decrease highlights the potential role of IAA in photosynthetic acclimation (Xu *et al.*, 2015), especially under elevated [CO_2].

We observed a weak relationship between RGR and ABA, and inconsistent temporal changes across tissues, suggesting that bulk ABA is not a main regulator for tissue growth. However, the ratio of leaf to stem ABA was, throughout the experiment, consistently higher at low than at ambient [CO_2], possibly because ABA is involved in photosynthetic acclimation to low [CO_2]. However, our data set cannot provide deeper insights into this mechanism, and assessments of other parameters, such as enhanced production of reactive oxygen species (ROS) from excess excitation energy at

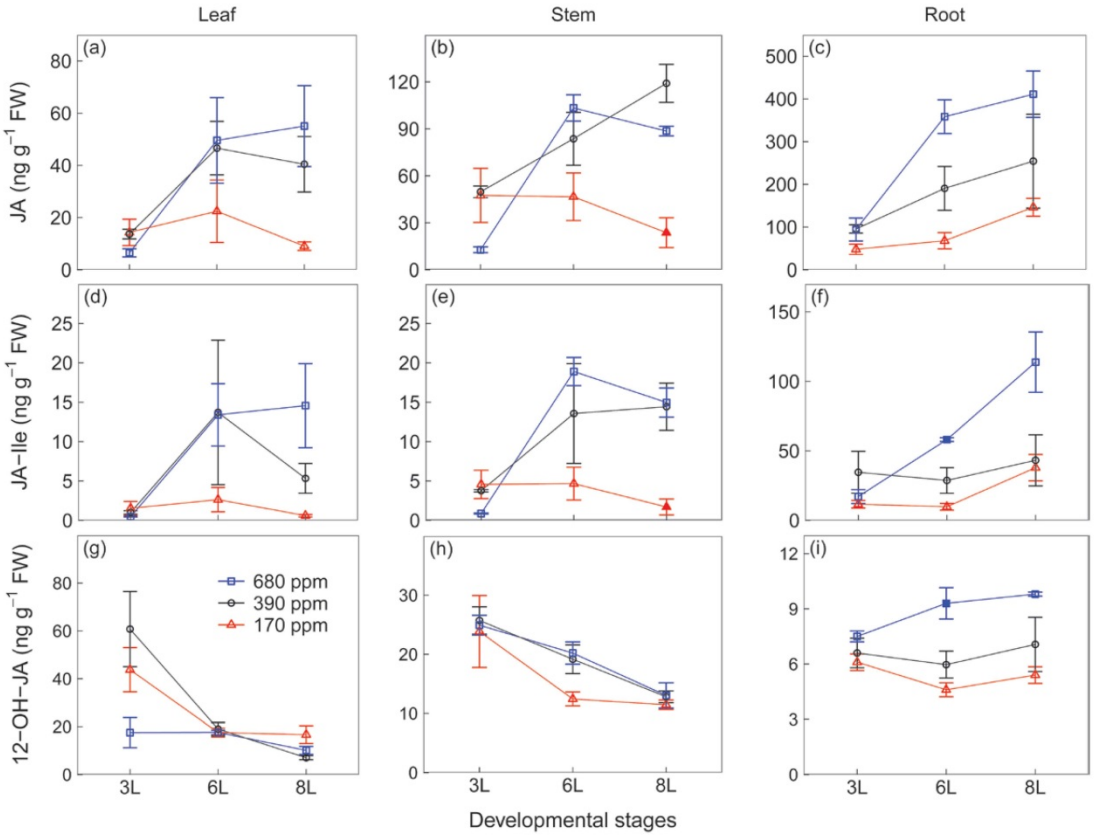

Fig. 5. Jasmonic acid (JA) (a–c), the isoleucine (Ile) conjugate of JA (JA-Ile) (d–f), and 12-hydroxy-JA (12-OH-JA) (g–i) concentrations of winter wheat (*Triticum aestivum*) for the three [CO_2] treatments (squares, 680 ppm; circles, 390 ppm; triangles, 170 ppm). Values are the mean (ng g^{-1} FW) ±SE of three individual chambers. Filled symbols of 680 ppm and 170 ppm [CO_2] treatments indicate significant differences compared with 390 ppm [CO_2] treatment (P<0.05, Tukey's HSD). Note that the concentrations are expressed on a fresh weight basis and at different scales. We harvested plants after emergence of three, six, and eight leaf sheaths, denoted by 3L, 6L, and 8L, respectively. (This figure is available in colour at *JXB* online.)

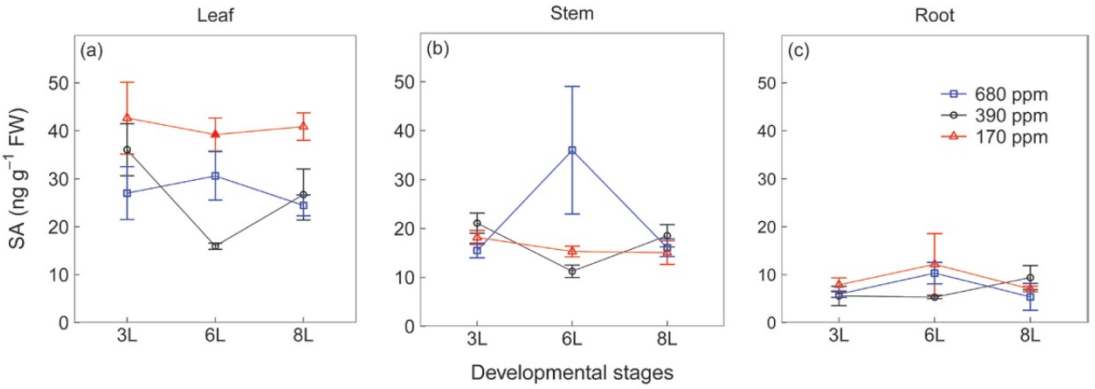

Fig. 6. Salicylic acid (SA) (a–c) concentrations of winter wheat (*Triticum aestivum*) for the three [CO_2] treatments (squares, 680 ppm; circles, 390 ppm; triangles, 170 ppm). Values are the mean (ng g^{-1} FW) ±SE of three individual chambers. Filled symbols of 680 ppm and 170 ppm [CO_2] treatments indicate significant differences compared with 390 ppm [CO_2] treatment (P<0.05, Tukey's HSD). Note that the concentrations are expressed on a fresh weight basis. We harvested plants after emergence of three, six, and eight leaf sheaths, denoted by 3L, 6L, and 8L, respectively. (This figure is available in colour at *JXB* online.)

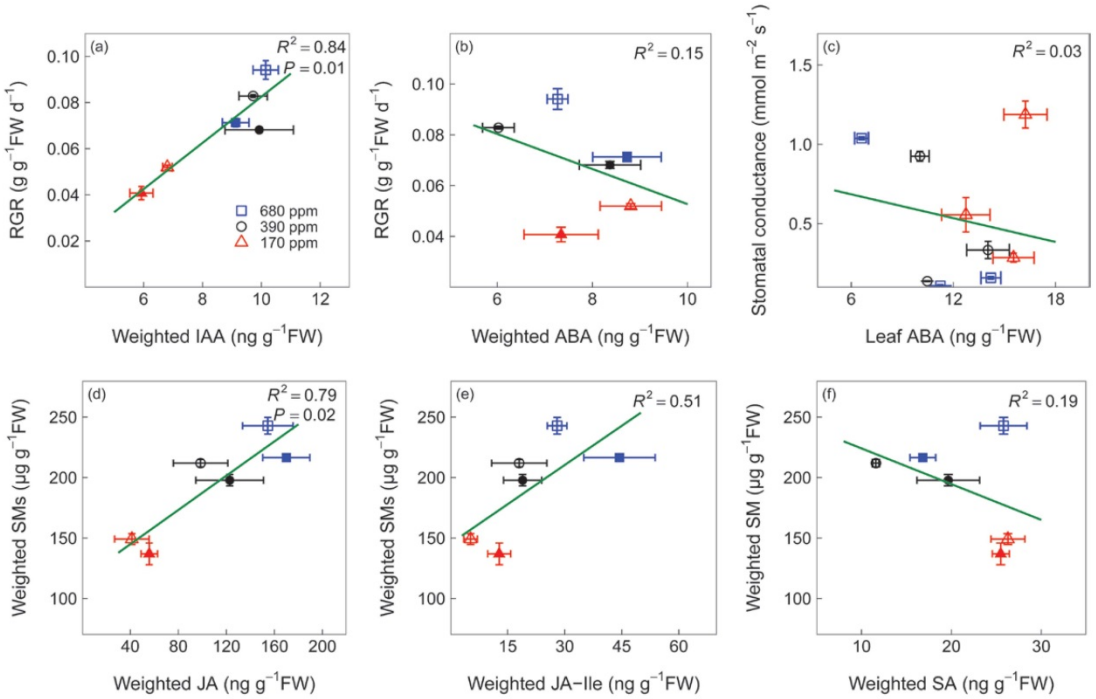

Fig. 7. Pearson's correlations of whole-plant relative growth rate (RGR) to weighted auxin (IAA) (a) and abscisic acid (ABA) (b) concentrations, between leaf area-based stomatal conductance and leaf ABA concentrations (c), and Pearson's correlations of weighted secondary metabolite (SM) concentration to jasmonic acid (JA) (d), isoleucine (Ile) conjugate of JA (e), and salicylic acid (SA) concentrations (f) in winter wheat (*Triticum aestivum*). The three [CO_2] treatments: squares, 680 ppm; circles, 390 ppm; triangles, 170 ppm. We harvested plants after emergence of three, six, and eight leaf sheaths, denoted by 3L, 6L, and 8L, respectively. However, before 3L, RGR and SM concentrations were also affected by seed storage, independent of [CO_2]; therefore, we only show RGR and SM concentrations of 6L and 8L, where open symbols represent 6L and filled symbols represent 8L. In contrast, we show stomatal conductance from 3L, 6L, and 8L, and all values are shown by open symbols. Values are the mean ±SE of three individual chambers. Note that the concentrations are expressed on a fresh weight basis and at different scales. (This figure is available in colour at *JXB* online.)

low [CO_2] (Galvez-Valdivieso *et al.*, 2009), are required to elucidate the role of ABA in stress mitigation (Mittler and Blumwald, 2015).

Regulation of secondary metabolite synthesis

Low [CO_2] reduced leaf flavonoids and root putrescine-based compounds as well as root benzoxazinoid derivatives, and this response was strongly associated with reduced JA and its bioactive derivative JA-Ile, but not with its inactive derivative 12-OH-JA. Jasmonates are involved in the biosynthesis of a wide range of SMs including flavonoids (Gundlach *et al.*, 1992), putrescine (Horbowicz *et al.*, 2011), and benzoxazinoid derivatives (Oikawa *et al.*, 2002). That low [CO_2] reduced SM synthesis via down-regulation of JA-dependent pathways is corroborated by evidence that deficiencies in potassium (Troufflard *et al.*, 2010) and phosphate (Khan *et al.*, 2016) can enhance SM production via up-regulation of JA and JA-Ile in Arabidopsis (Troufflard *et al.*, 2010; Khan *et al.*, 2016). In contrast, SA concentrations were higher in leaves at low than at ambient [CO_2], indicating that low C availability reduces leaf SM production independent of the SA signalling pathway. Given that SA and JA are antagonistic (Zavala *et al.*, 2013; Sun *et al.*, 2016), it is possible that the up-regulation of SA signalling is associated with the suppression of JA signalling at low C availability.

In contrast, under elevated [CO_2], leaf JA and SA levels changed independently from each other across developmental stages but both may nonetheless play a role in regulation of SMs. At 6L, leaf flavonoids and SA concentrations increased under elevated [CO_2] but that was not the case for JA and JA-Ile, indicating that elevated [CO_2] may stimulate short-term leaf flavonoid synthesis via SA (Zavala *et al.*, 2013; Sun *et al.*, 2016). At 8L, however, plants grown at elevated [CO_2] had higher leaf JA and JA-Ile concentrations than at ambient [CO_2] and probably induced flavonoid production. These dynamics mirror a potential long-term acclimation of photosynthesis at elevated CO_2, as indicated by declines in RGR and soluble sugars in all tissues at elevated [CO_2]. Deeper insights into such long-term leaf acclimation to elevated [CO_2] via hormonal regulation require assessments of hormones and SMs over the entire plant developmental gradient.

In roots, however, while SMs and SA remained relatively constant, JA and JA-Ile concentrations were higher at elevated [CO_2] than at ambient [CO_2]. We suggest here that JA-dependent signalling for SM production in roots was probably constrained by N availability, given that root SMs are N-rich compounds and that assimilation of nitrate into

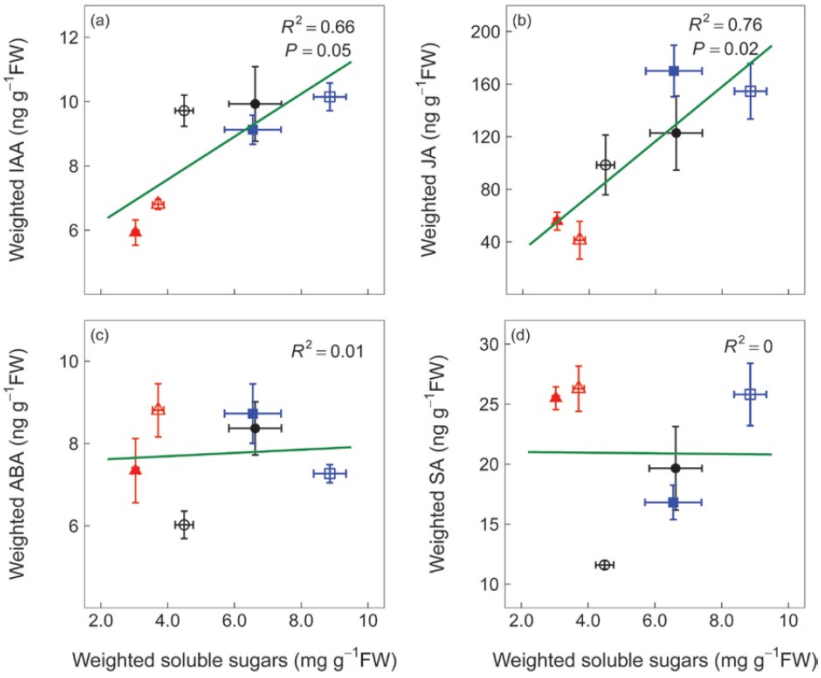

Fig. 8. Pearson's correlations of weighted soluble sugar concentration (mg g^{-1} FW) to weighted auxin (IAA) (a), jasmonic acid (JA) (b), abscisic acid (ABA) (c), and salicylic acid (SA) (d) concentrations (ng g^{-1} FW) in winter wheat (*Triticum aestivum*). The three [CO_2] treatments: squares, 680 ppm; circles, 390 ppm; triangles, 170 ppm. We harvested plants after emergence of three, six, and eight leaf sheaths, denoted by 3L, 6L, and 8L, respectively. However, before 3L, soluble sugar and SM concentrations were also affected by seed storage, independent of [CO_2]; therefore, we only show data of 6L and 8L, where open symbols represent 6L and filled symbols represent 8L. Values are the mean ±SE of three individual chambers. Note that the concentrations are expressed on a fresh weight basis and at different scales. (This figure is available in colour at *JXB* online.)

organic N compounds may be limited under elevated [CO_2] (Bloom *et al.*, 2010). Therefore, N availability, rather the JA- and JA-Ile-dependent signalling, determined the response of root SMs to elevated [CO_2].

Potential sugar signalling for IAA and JA synthesis

Soluble sugars are essential substrates for growth and metabolism (Hartmann and Trumbore, 2016), but also act as signalling molecules that interact with plant hormones to mediate plant stress responses (Rolland *et al.*, 2006; Lastdrager *et al.*, 2014). Our results revealed that accumulation of soluble sugars with increasing [CO_2] positively correlated with weighted IAA and JA concentrations, allowing our experiment to demonstrate nicely the interaction of soluble sugars and IAA and JA at the whole-plant level. It has been shown that glucose (Sairanen *et al.*, 2012) and sucrose (Lilley *et al.*, 2012) can both stimulate IAA biosynthesis and growth rates. In contrast, jasmonates reduced glucose and fructose concentrations in *Nicotiana attenuata* leaves (Machado *et al.*, 2015) and play a synergetic role with sucrose (Loreti *et al.*, 2008) and glucose (Guo *et al.*, 2013) in anthocyanin and glucosinolate biosynthesis. Hence, it is more likely that accumulation of soluble sugars at high [CO_2] stimulated whole-plant IAA and JA synthesis rather than vice versa. This sugar signalling pathway may also be a mechanism that triggers IAA and JA biosynthesis under cold, drought, and nutrient limitation, as these stresses decrease growth earlier than photosynthesis, thus allowing surplus carbon to be allocated to carbohydrates (Herms and Mattson, 1992; Palacio *et al.*, 2014). In contrast to IAA and JA, soluble sugars were not correlated with weighted ABA and SA concentrations, possibly because low [CO_2] may trigger leaf ABA and SA biosynthesis via signalling pathways independent of sugar availability, for example ROS accumulation from excess excitation energy and photorespiration (Galvez-Valdivieso *et al.*, 2009; Herrera-Vásquez *et al.*, 2015).

Light intensities (8 mol m^{-2} d^{-1}) were much lower in the greenhouse than usually occur in the field, and it might be that plants grown at ambient and elevated [CO_2] were light limited. However, the general agreement of plant responses in our study with results from field experiments downplays the importance of light limitation. Moreover, higher light intensities in the field are likely to amplify CO_2-induced responses, such as increases in assimilation, sugars, RGR, and secondary metabolites, as well as corresponding IAA and JA levels. Consequently, the suboptimal light conditions in the greenhouse underscore the robustness of our findings as the observed patterns are likely to be more pronounced in field-grown plants.

Outlook

Our study is an initial step towards unravelling whole-plant hormonal regulation of growth and defence in response to changing [CO_2] including low [CO_2]. We hypothesize that low

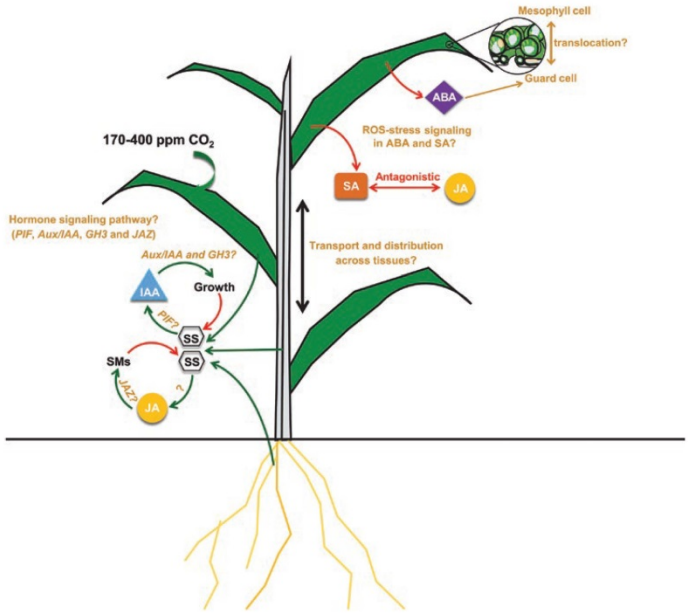

Fig. 9. Conceptual model and research needs of whole-plant hormonal regulation in response to changing [CO_2], derived from our experimental results and published literature. The leaf cross-section figure was modified from an original figure provided by Zephyris (Richard Wheeler) and Wikipedia. Increased [CO_2] stimulates photosynthesis and results in accumulation of soluble sugars at the whole-plant level which may elicit a cascade of downstream IAA and JA regulation of plant growth and defence, respectively. Increasing [CO_2] may dissipate the reducing power from the photosynthetic electron transfer chain and reduce photorespiration, which theoretically reduce ROS production (Galvez-Valdivieso *et al.*, 2009). Low [CO_2]-induced ROS accumulation may trigger ABA (Mittler and Blumwald, 2015) and SA biosynthesis (Herrera-Vásquez *et al.*, 2015), which in turn promote ROS accumulation or scavenging. Low [CO_2] could increase translocation of ABA from mesophyll chloroplast to guard cells and, as reported by Chater *et al.* (2015), the increase in guard cell ABA is sufficient to reduce stomatal conductance and density. Up-regulation of SA signalling may be associated with suppression of JA signalling (Zavala *et al.*, 2013; Sun *et al.*, 2016). Interactive signalling pathways via sugars for growth regulation and synthesis of secondary metabolites but also transport and distribution of phytohormones across tissues are still uncertain. Soluble sugars may stimulate IAA via the *PIF* family of transcription factors (Lilley *et al.*, 2012; Sairanen *et al.*, 2012). Furthermore, IAA may induce the *GH3* family which in turn catalyses the conjugation of IAA to amino acids (Sauer *et al.*, 2013). IAA and JA may stimulate growth and SM production via degradation of the repressor *Aux/IAA* (Enders and Strader, 2015) and *JAZ* (Riemann *et al.*, 2015), respectively. ABA, abscisic acid; IAA, auxin; JA, jasmonic acid; *JAZ*, jasmonate ZIM-domain protein; *PIF*, phytochrome-interacting factor; ROS, reactive oxygen species; SA, salicylic acid; SMs, secondary metabolites; SS, soluble sugars. Green arrows indicate positive, red negative, and brown uncertain.

[CO_2]-induced changes in ROS play important roles in leaf ABA and SA signalling, but this must be specifically addressed in future low [CO_2] studies. Hormones are highly interactive (Peleg and Blumwald, 2011), and therefore interactions between hormones, such as JA and SA, will help to establish a conceptual framework for the complex hormonal regulatory mechanisms of plant response to changing [CO_2] (Fig. 9).

Further progress in our understanding of hormonal whole-plant growth regulation can be achieved by combining measurements of bulk tissue hormone concentrations with investigations on hormone distribution and translocation across different tissues/organs. For example, root growth and development is regulated by auxin that has been synthesized in the shoot apex and transported to root tips to result in local auxin concentration peaks (Peer *et al.*, 2011). Similarly, ABA is stored mainly in the mesophyll cell, but a recent study shows that only ABA concentrations in guard cells control stomatal responses to elevated [CO_2] (Chater *et al.*, 2015). Experiments with mutants will provide more direct evidence for causality in hormonal regulation, and future studies at the transcriptional level should focus on biosynthetic and, in particular, responsive genes involved in hormonal signalling pathways. For example, soluble sugars may stimulate IAA via the phytochrome-interacting factor (PIF) family of transcription factors (Lilley *et al.*, 2012; Sairanen *et al.*, 2012). Auxin-responsive *GH3* (Du *et al.*, 2012, 2013) and *Aux/IAA* genes (Jung *et al.*, 2015), and the JA-responsive jasmonate ZIM-domain (JAZ) gene family (Du *et al.*, 2013; Riemann *et al.*, 2015) are signalling repressors and have been shown to be involved in drought and cold tolerance.

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10. Did I find all the answers to my questions?

The research described in this thesis has led to some interesting insights into plant functioning and hence allowed me to achieve most of the objectives I had set for my pre-habilitation qualification. My early studies on drought-induced mortality mechanisms have highlighted that the hydraulic framework proposed by McDowell *et al.* (2008) was too simplistic and not able to predict real-world response to drought. Reducing tree physiological responses to lethal drought to only species-specific leaf-level stomatal behaviour neglects other aspects of carbon dynamics, like translocation of carbon across plant organs via the phloem (Table 2, Hartmann, 2011).

Table 2 Main results for the research questions addressed in the research chapters.

Research question(s)	**Main result**
Drought-induced tree mortality	
McDowell's hydraulic framework is incomplete	Carbon translocation as additional mechanism
Does drought cause carbon starvation?	Only local carbon starvation, likely from reduced carbon transport
Does reduced hydration prevent whole-tree carbon starvation?	Only well-watered trees can deplete all their carbon reserves
Does drought prevent carbon allocation from source to sink organs?	Not completely but enough to cause a net carbon depletion
Is carbon starvation during drought impossible?	Not enough data available to adequately respond to this question
General carbon relations in forest trees during drought	
How does elevated temperature affect whole-tree carbon balance during drought?	Carbon balance becomes negative in wetter soils at high temperature
Can tree use alternative substrate for respiration during drought?	Trees depend on stored carbohydrates during drought while they can switch to lipids under shading
How many growing seasons contribute to trees carbon reserves?	On average between 4 to 5 growing seasons
What is the role of carbohydrates in tree functioning?	New approaches are required to address processes of tree functioning
Carbon allocation within plants and with symbiotic partners	
Are plants or mycorrhiza in charge during symbiotic resource exchanges?	*Plantago* reduces carbon expenses without negative impact on nitrogen returns by mycorrhiza
Is carbon allocation to defence is constrained by carbon availability?	Yes, because allocation to secondary metabolites increases with carbon availability
How are whole-plant allocation patterns orchestrated by phytohormones?	Auxin and jasmonates regulate growth and secondary metabolites whereas abscisic and salicylic acid respond to oxidative stress

Our tissue-level study on carbon dynamics during lethal drought in Norway spruce (*Picea abies*, Hartmann *et al.*, 2013b) proved that within a given species, and irrespective of its stomatal behaviour, carbon dynamics are defined at the organ level, likely because drought-induced reductions in phloem functioning isolate organs from each other. In our study, root carbohydrate concentrations were very low when trees died, apparently from carbon starvation, while leaves (needles) senesced with high levels of carbohydrates, some even higher than in control trees. Roots

were not able to use the carbohydrates that were still available in above-ground tissues because they couldn't be transported via the phloem into the roots (Hartmann *et al.*, 2013b), or the transport was not sufficiently large to satisfy belowground demands (Hartmann *et al.*, 2015). This finding was corroborated during a starvation experiment, also on Norway spruce, which showed that a depletion of carbohydrate pools at the whole-tree level, i.e. carbon starvation, can only occur when plants were well hydrated (Hartmann *et al.*, 2013a). Our studies provided empirical evidence to explain why field observations have often shown inconsistent results with respect to carbon dynamics during drought, i.e. sometimes carbohydrate concentrations increase (Galvez *et al.*, 2011; Anderegg *et al.*, 2012) while in other instances they decrease (Galiano *et al.*, 2011; Galvez *et al.*, 2013; Poyatos *et al.*, 2013). A recent synthesis analysis of ~20 lethal drought experiments (Henry Adams, unpublished data) showed that carbohydrate depletion during drought mortality is not universal while strong reductions (> 60 %) in hydraulic conductivity are very common. Such observations have supported doubts over whether carbon starvation does actually happen in plants (Körner, 2015). Our results suggest that findings may differ depending on which organ is measured, and this may contribute to apparent inconsistencies in observations. However, as a carbon-starved root system will lead to tree death even if the above-ground organs are not starving, we may consider using the term local carbon starvation instead (Hartmann *et al.*, 2013b).

A major problem in drought research is the current inability to define drought severity as perceived by the plant. Soil water content is commonly used to infer treatment severity in drought manipulation experiments. However, our results show this may not be an indicative measure of whole-plant carbon balance, as drought can lead to a negative carbon balance at relatively high soil water content, depending on ambient temperature and the amount of soil water physiologically available to plants. In our study on Easter white cedar (*Thuja occidentalis*, Zhao *et al.*, 2013) the carbon balance became zero (daily assimilation < daily respiration) at a much higher soil water content (~20 % vs. 11%) when plants were grown at 35°C instead of 15°C.

Furthermore, we could show that carbon storage pools may be sufficiently large, at least in Sugar maple (*Acer saccharum*), to make up for several years of assimilation failure (like recurrent defoliations or multiannual drought, Muhr *et al.*, 2016). However, drought hampers not only carbohydrate translocation across organs and from sources to sinks (Ruehr *et al.*, 2009) but also carbohydrate metabolism (Fischer *et al.*, 2015). During drought young pine trees (*Pinus sylvestris*) were forced to rely on carbohydrates as the sole substrate source for respiration while under carbon limitation (via shading) they were able to switch to lipids as energy source – after using up most of the carbohydrates. Surprisingly, drought trees couldn't mobilize sufficient amounts of carbohydrates

to sustain metabolism and needed to strongly reduce respiration rates (Fischer *et al.*, 2015). The causes for this apparent paradox, i.e. the trees being unable to mobilize carbohydrates AND being unable to use alternative respiratory substrates at the same time, have not been resolved yet but further studies are planned to specifically address this question. Taken together our studies on carbon dynamics in trees suggest that carbon (carbohydrate) starvation during drought has not been sufficiently studied yet to discard it as a potential mortality mechanism (Hartmann, 2015). Carbohydrate concentrations, although currently used as indicator for carbon limitation (Hoch, 2015), are the result of many different processes and should not be mistaken as a plant function *per se* (Hartmann & Trumbore, 2016). Hence, future studies should focus more on the underlying processes which are determinate for plant function and survival – whether during drought or under normal conditions.

Consequently, my own most recent research has been redirected more towards the mechanisms that control plant functioning. For example, we were able to show that plants can modify interactions in resource exchange with symbiotic partners to their advantage. Using an advanced experimental design that separated plants from an essential resource, nitrogen, and forcing them into an obligate relationship with mycorrhiza to gain access to this resource, we were able to show that plantago plants (*Plantago lanceolata*) grown under carbon limitation could reduce carbon expenditure to fungal hyphae by ~ 60 % while still receiving the same amount of nitrogen from the fungus (Zhang *et al.*, 2015). Furthermore, in a study on winter wheat (*Triticum aestivum*) we showed that carbon allocation to respiration was a constant parameter and independent of carbon availability while partitioning to growth, storage and defence increased with increasing carbon availability (Huang *et al.*, 2016a). Hence, trade-offs between growth and defence, as claimed by the "Growth-Differentiation-Balance Theory", became questioned (*cf.* Matyssek *et al.*, 2012a; Matyssek *et al.*, 2012b). In the same species, we have been investigating control mechanisms of plant growth and allocation using whole-plant phytohormone analysis. We were able to show that auxin and jasmonates are involved in whole-plant regulation of growth and secondary metabolites whereas abscisic and salicylic acid may be associated with leaf oxidative stress in response to low carbon availability (Huang *et al.*, 2016b). These recent studies suggest an additional angle for future research that integrates our approach on carbon balance and tracing carbon through metabolic pathways to focus on questions of biochemical and molecular control mechanisms of plant carbon allocation.

11. What do I still want to know?

Much! Although research on plant carbon allocation has now a long history there are still many knowledge gaps. These gaps hamper our ability to predict plant responses to increasingly rapid environmental change. Mooney (1972) stated more than four decades ago that *"through a quantitative understanding of how different plants gain and* ***allocate their resources*** *it will be possible to make* ***predictions as to their success*** *in any given physical environment in combination with any competitor and predator. We are still* ***far from this reality****"*. Unfortunately, in the intervening 45 years, we have not gotten much closer to achieving this goal. Dynamic vegetation models have been developed to make predictions of terrestrial ecosystem responses and biogeochemical processes under rapid climate change (Cramer *et al.*, 2001). However, many of these models still do not represent carbon allocation or even tree mortality in a realistic way and especially the representation of carbon storage reserves in trees requires more realism (Dietze *et al.*, 2014). Forests are important because they make up most of the mass of terrestrial vegetation and play an essential role for the cycling of life-sustaining elements and compounds (e.g., water, carbon, nitrogen). As observations are accumulating that indicate an increasing climate change risk for forests (Allen *et al.*, 2010) there is a need to better understand the physiological responses of forest ecosystems to climate change (Allen *et al.*, 2015). Carbon allocation is the means by which plants adjust to environmental change and substantial advances are necessary to achieve a fundamental and mechanistic understanding of plant functioning in a rapidly changing environment and to predict future vegetation condition.

My postdoctoral research had focused on tree mortality mechanism and progressively migrated to general questions on tree carbon relations and carbon allocation patterns. These studies were done mainly under controlled environmental conditions in garden and greenhouse experiments and have contributed to improving our understanding of plant functioning. However, there are still many open questions regarding the role of carbohydrates in plant functioning and, in particular, regarding the role of carbon reserves in plant survival to stress (Hartmann & Trumbore, 2016). I enumerate a few:

(1) To what extent are older NSC stores available for metabolism?

(2) Do plants under stress shift sources of carbon substrates used in metabolism?

(3) Is allocation to storage in long-lived organisms like trees maintained even under carbon limitation and at the expense of allocation to other sinks like growth to ascertain long-term survival? And, most importantly;

(4) What controls storage?

My current challenges thus still address limitations of carbon allocation but will focus more on the underlying control mechanisms. A particular emphasis will lie on the regulatory processes of carbon storage in trees and other perennial plants and will involve both biochemical and molecular tools (Dietze *et al.*, 2014). So far investigations on control mechanisms of carbon reserve formation have been carried out in annual herbal model plants like Arabidopsis and for processes running on diel scales (Stitt & Zeeman, 2012). Given the importance of carbon reserves for preventing starvation, especially in species that can live for decades or even centuries, such studies should be extended to perennial species (Stitt & Zeeman, 2012). The insights gained in laboratory and greenhouse experiments must then be confronted with data from field observation and manipulations to test how fine-scale process understanding integrates at larger scales and at ecosystem level (Fig. 5).

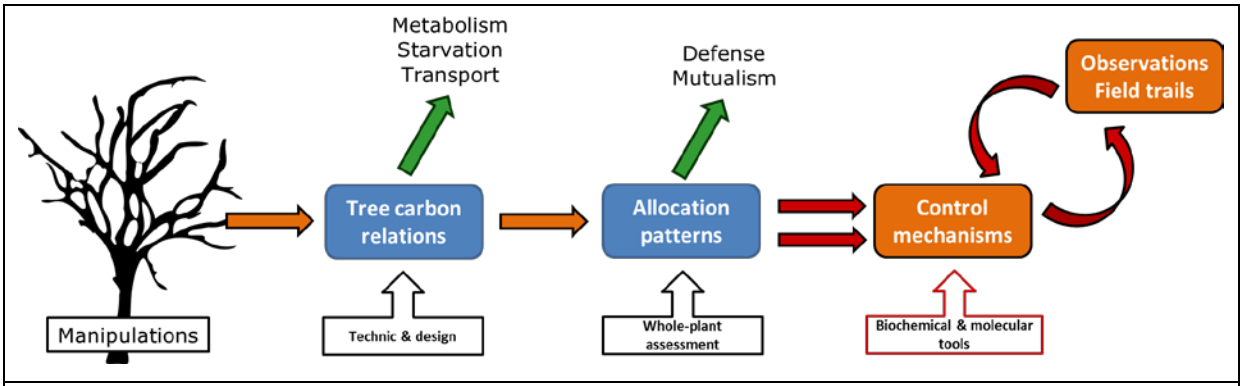

Figure 5 Research path during the last 7 years and current as well as future research challenges.
My postdoctoral research has started with manipulations addressing tree mortality mechanisms (left) and has then progressively migrated (orange arrows) toward investigations under controlled environmental conditions on general tree carbon relations. The more recent investigations on allocation patterns build upon whole-tree assessments of all three aspects of allocation (see section 6). The topics that have been addressed during that phase (green arrows) will be further investigated in the upcoming years (red arrows), with a particular focus on the underlying control mechanisms of carbon allocation. To do so, the application of additional biochemical and molecular tools (phytohormones, transcriptome analysis) will provide insights that can then be validated and refined with field trails and observations of plant and ecosystem responses to stress and environmental change.

Corroborative findings will then allow building more realistic predictive mechanisms for vegetation modelling while contradictory results may help refining objectives for ecophysiological research under controlled conditions.

Some of these investigations are already underway. To address question (1) listed above, we have girdled sugar maple and white birch (*Betula pendula*) trees in Canada and Finland, respectively. Both species mobilize carbon reserves in spring-time xylem sap ascent (see chapter 9.8) and we investigate potential shifts in $\triangle^{14}C$ signature of the mobilized carbon pool that would indicate the use of older carbon for metabolism. Question (2) on shifts in respiratory substrates will be addressed

in an Israeli-German collaborative project with Prof. Alon Angert of the Hebrew University of Jerusalem. Questions (3) and (4) are central elements of a running PhD project that manipulates carbon availability of spruce trees and that assesses diverse whole-plant allocation pools and fluxes as well as the underlying regulatory processes by using phytohormonal and transcriptome analysis. Finally, the application of this process understanding to field experimentations has been initiated in collaboration with Profs. Matyssek and Grams within the TUM KROOF rain-exclusion experiment where I am contributing by investigating carbohydrate dynamics in mature trees at Kranzberg Forest/Freising in response to drought. The next step will be a drought manipulation in the cerrado of south-western Brazil at the Tanguro farm in Mato Grosso. These forests are expected to be strongly affected by an anticipated substantial increase of the dry season length and will likely become hotspots of vegetation shifts and tree mortality. Using a variety of tools, including isotopic labelling, this experiment will become an ideal platform to test how environmental changes influence tree carbon relations and allocation patterns. The planning for this experiment, carried out in collaboration with our Brazilian (Amazon Environmental Research Institute, IPAM) and American partners (Woodshole Research Center) is running and a PhD student (David Andres Herrera Ramirez) and a postdoc (Daniel Marra) will start working on the implementation of our collaboration in March 2017. I am certain that many interesting new aspects identified during all my current and upcoming investigations will further enrich my future career path.

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