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## Research paper

# Growth cessation uncouples isotopic signals in leaves and tree rings of drought-exposed oak trees

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An increase in temperature along with a decrease in summer precipitation in Central Europe will result in an increased frequency of drought events and gradually lead to a change in species composition in forest ecosystems. In the present study, young oaks (*Quercus robur* L. and *Quercus petraea* (Matt.) Liebl.) were transplanted into large mesocosms and exposed for 3 years to experimental warming and a drought treatment with yearly increasing intensities. Carbon and oxygen isotopic ( $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$ ) patterns were analysed in leaf tissue and tree-ring cellulose and linked to leaf physiological measures and tree-ring growth. Warming had no effect on the isotopic patterns in leaves and tree rings, while drought increased  $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$ . Under severe drought, an unexpected isotopic pattern, with a decrease in  $\delta^{18}\text{O}$ , was observed in tree rings but not in leaves. This decrease in  $\delta^{18}\text{O}$  could not be explained by concurrent physiological analyses and is not supported by current physiological knowledge. Analysis of intra-annual tree-ring growth revealed a drought-induced growth cessation that interfered with the record of isotopic signals imprinted on recently formed leaf carbohydrates. This missing record indicates isotopic uncoupling of leaves and tree rings, which may have serious implications for the interpretation of tree-ring isotopes, particularly from trees that experienced growth-limiting stresses.

**Keywords:**  $^{13}\text{C}$ , dual isotope approach,  $^{18}\text{O}$ , *Quercus petraea*, *Quercus robur*, water shortage, wood.

## Introduction

Due to increasing concentrations of atmospheric  $\text{CO}_2$ , temperatures will rise in the near future (CH2011 2011, IPCC 2012), prospectively resulting in changing species distribution and composition of forest ecosystems in Central Europe (Rebetez et al. 2006, Rigling et al. 2013). In addition, summer precipitation is predicted to decrease by 21–28% in large parts of Central Europe by the end of the century and the frequency of extreme heatwaves and severe droughts is likely to increase (Schär et al. 2004, IPCC 2012). Even though increasing  $\text{CO}_2$  concentrations might mitigate drought effects on trees by more efficient water use (Frank et al. 2015), drought has been recognized as a serious threat to forest ecosystems (Allen et al. 2010). Tree species sensitive to drought are expected to be

particularly affected by these climatic constraints, resulting in reduced productivities of these forest ecosystems and increased vulnerability to drought-induced mortality. The use of drought-tolerant tree species is, therefore, a promising strategy in future forestry to mitigate the effects of climate change on forest ecosystems.

Quantitative and qualitative analyses of tree-ring growth can provide useful information about the drought tolerance of tree species as tree rings integrate drought responses from different levels of plant organization and functioning. Although tree-ring growth is very sensitive to different stressors (Dobbertin 2005), it is often difficult to distinguish whether stress is due to drought, cold, air pollution, insect infestations or other factors. Here, stable isotopes of carbon and oxygen in plant tissues, in

combination with growth and leaf physiological measures, are helpful since they are good indicators of the physiological responses of plants to changes in precipitation and temperature (Dawson et al. 2002, Loader et al. 2007, Arend et al. 2013, Fonti et al. 2013). Mechanisms leading to altered isotope signatures in photosynthetic and non-photosynthetic tissues of drought-stressed plants are well described and numerous studies have shown the effects of drought on stable isotopes in plant organs (e.g., McCarroll and Loader 2004, Brüggemann et al. 2011).

Under drought conditions, plants reduce their stomatal conductance to limit water loss via transpiration, inevitably leading to a decreased concentration of intercellular CO<sub>2</sub> (*c<sub>i</sub>*) relative to the concentration of ambient CO<sub>2</sub> (*c<sub>a</sub>*). Farquhar et al. (1989) derived a simplified but effective equation that describes the relationship between *c<sub>i</sub>/c<sub>a</sub>* and the carbon isotope ratio (δ<sup>13</sup>C) in plant tissues:

$$\delta^{13}\text{C}_{\text{plant}} = \delta^{13}\text{C}_{\text{atm}} - a + (b - a) \times \frac{c_i}{c_a}, \quad (1)$$

where *a* is the fractionation factor for diffusion of CO<sub>2</sub> in air (*a* = 4.4‰) and *b* is the fractionation factor of RuBisCO carboxylation (*b* = 27‰). The strong dependency of δ<sup>13</sup>C on *c<sub>i</sub>/c<sub>a</sub>* is widely used to characterize environmental effects on plant physiology, particularly on leaf photosynthesis. However, it is not possible to distinguish whether environmental constraints affect δ<sup>13</sup>C via stomatal or biochemical limitations (Scheidegger et al. 2000).

The oxygen isotope ratio (δ<sup>18</sup>O) can facilitate such a distinction between stomatal and biochemical limitations of photosynthesis as it depends only on the evaporative enrichment of source water at the sites of leaf transpiration (Dongmann et al. 1974) and the so-called Péclet effect. The latter takes into account that advective and diffusive transport of non-enriched xylem water and enriched leaf water inside the leaf dampens this enrichment effect (Barbour 2007). In contrast to δ<sup>13</sup>C, δ<sup>18</sup>O is not changed by the fractionation during carboxylation although it is tightly linked to stomatal conductance. It is, therefore, an ideal covariable to determine to what degree photosynthesis or stomatal conductance modify δ<sup>13</sup>C (Scheidegger et al. 2000). Both isotope signals are imprinted on the organic matter of plants, from leaves to tree rings, and their information can be exploited to relate physiological as well as long-term responses of plants to environmental changes (e.g., Eilmann et al. 2010). Due to a partial exchange of oxygen atoms of phloem sugars with xylem water at the site of cellulose synthesis, source water has a greater influence on the oxygen isotopic composition of tree rings than on the leaf tissue (Roden et al. 2000).

Current knowledge about the relationship of both isotopes to CO<sub>2</sub> and H<sub>2</sub>O gas exchange has been implemented in the conceptual dual isotope approach of carbon and oxygen

(Scheidegger et al. 2000). Numerous recent studies used the information provided by stomatal-dependent variations in δ<sup>18</sup>O to explain changes in δ<sup>13</sup>C and therefore the plant's behaviour under environmental variations (Brooks and Coulombe 2009, Gessler et al. 2009, Barnard et al. 2012, Lévesque et al. 2013). A considerable number of studies revealed the influence of water availability on the isotopic composition in leaf tissues and tree rings (e.g., McCarroll and Loader 2004, Barbour 2007, Bowling et al. 2008). Furthermore, it has also been shown that changes in temperature may trigger alterations in the isotopic composition of carbon and oxygen (Libby et al. 1976, Loader and Switsur 1996). So far, the influence of drought and temperature on δ<sup>13</sup>C and δ<sup>18</sup>O has never been examined in a single study using the dual isotope approach along with gas exchange measurements and intra-annual tree-ring growth, which could strengthen the interpretation of the isotope pattern.

Originally, the dual isotope approach was introduced for photosynthetic tissue and only recently tested conceptually for the interpretation of tree-ring data (Roden and Farquhar 2012). The approach was valid for most of the applied treatments although there remained some unsolved issues and the authors highlighted the need for further research to make the concept operational under different scenarios. Additionally, Roden and Siegwolf (2012) listed 10 concerns and suggestions regarding the application and interpretation of the dual isotope approach. The commentary aimed to prevent false conclusions and highlighted areas of caution that have to be considered before applying this approach. One of these concerns was the missing isotopic signals in tree rings during periods of wood growth cessation which, for example, may occur when trees are exposed to severe drought. While leaf organic matter can still retain a drought-related isotopic signal, tree-ring tissue may reveal an isotopic pattern representing only less stressful periods due to the shut-down of tree-ring growth. The present study will test such a constellation in which the analysis of intra-annual tree-ring growth dynamics and gas exchange measurements facilitates the uncovering of contradicting isotope responses in leaves and tree rings.

In the present study, young trees of *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. were subjected to air warming and experimentally controlled summer droughts, with yearly increasing intensities of water shortage over a period of 3 years. Both *Quercus* species are commonly considered to be drought- and thermo-tolerant, even though they occupy different ecological habitats in terms of soil humidity (Ellenberg 2009) and show a different response of leaf photosynthesis to drought (Arend et al. 2013). Isotopic patterns of <sup>13</sup>C and <sup>18</sup>O were analysed in leaf tissue and tree-ring cellulose and linked to measures of leaf photosynthesis and intra-annual tree-ring growth to (i) study the influence of climate constraints on the relationships between isotopes, leaf physiology and tree growth and (ii) better explain the origin of isotopic signals in tree rings.

## Materials and methods

### Experimental design

The present study was carried out within the framework of the 'Querco' experiment that was conducted from 2007 to 2009 in the 16 large mesocosms of the model ecosystem facility MODEOK at the Swiss Federal Research Institute WSL, Birmensdorf, Switzerland (47°21'48"N, 8°27'23"E, 545 m above sea level, a.s.l.). Each mesocosm has a sliding roof at 3 m height allowing the exclusion of natural precipitation and encloses two concrete walled lysimeters with a depth of 1.5 m and a soil surface of 3 m<sup>2</sup>. The lysimeters were filled with acidic (acidic Haplic Alisol; loamy sand; pH 4.1) or calcareous (Calcaric Fluvisol; sandy loam; pH 6.9) forest soil. In spring 2006, three European oak species with four provenances each were transplanted into the mesocosms. Per mesocosm, two 2-year-old saplings of the selected species and provenance were planted into each soil compartment (Arend et al. 2011, Kuster et al. 2013). The present study focuses on the two oak species *Q. robur* and *Q. petraea* with one provenance each and on acidic soil only (Table 1, for biomass data of the two species, see Table S1 available as Supplementary Data at *Tree Physiology* Online and Arend et al. 2011).

Starting in 2007, the saplings were subjected to the climate treatments drought (D), air warming (AW) and their combination (AWD) as well as a control treatment (C) for three subsequent growing seasons. Each climate treatment was replicated in four mesocosms. The water regime in the mesocosms was controlled by an automated irrigation system and the sliding glass roofs closing automatically at the onset of rainfall. During the autumn and winter months, the roofs of all mesocosms were kept open to allow full replenishment of soil water reserves with natural precipitation (Figure 1). From spring to summer, C and AW mesocosms were irrigated with 10-mm deionized water every 2–3 days using six sprinklers at a height of 1 m. Deionized water was enriched with nutrients simulating the average composition of precipitation water for this site. In D and AWD mesocosms, drought was imposed by temporary interruption of the automated irrigation. Drought intensities were increased over the three consecutive years by reducing the number and duration of the irrigation events (Figure 1a). The air temperature inside the AW and AWD mesocosms was passively increased by varying the opening angle of the moveable mesocosm sidewalls. Air temperature and relative humidity in the control mesocosms were comparable to those of ambient stands (Figure 1b and c).

### Measurement of soil moisture, air temperature and relative humidity

Soil water content (SWC) was determined in each mesocosm manually every week using time domain reflectometry (TDR 100, Campbell Scientific Inc., Logan, UT, USA) at a soil depth of 38 cm (Figure 1a). Air temperature and relative humidity were measured automatically every hour at a height of 120 cm in each mesocosm using shaded EL-USB-2 data loggers (Lascar Electronics Ltd, Whiteparish, UK) (Figure 1b and c).

### Collection and preparation of leaves and tree-ring samples for isotope analyses

In early September of each experimental year (2007–09), five leaves representative for the whole tree foliage (regarding flush, colour, size and location in the tree canopy) were collected from each tree per species and treatment. Drought treatment in the D and AWD mesocosms reduced the number of flushes by ~20% in 2008 and 2009 (Kuster et al. 2014). Leaf samples of two individual trees per species and mesocosm were pooled, dried at 65 °C and ground to a fine powder using a ball mill (MM400 and MM2000, Retsch, Haan, Germany) prior to isotopic analysis. At the end of the experiment in October 2009, stems were cut ~15 cm above ground and stem discs of 0.5 cm thickness were produced. Tree rings of the years 2007–09 were dissected using a China blade under a stereomicroscope (Leica Wild M3Z, Leica Microsystems, Wetzlar, Germany). To eliminate the isotope signal of early-wood vessels, which are mostly formed from stored carbohydrates of the previous season, the first cell lines of each tree ring were discarded. Woody material was ground to a fine powder with an ultra-centrifugal mill (ZM 200, Retsch, sieve 0.5 mm) before cellulose extraction. For this purpose, 10–15 mg of the powdered wood material was weighed into Teflon bags and washed for 2 h in 5% NaOH solution at 60 °C to remove fats, hemicelluloses and phenols. Afterwards, the samples were washed with a 7% NaClO<sub>2</sub> solution (pH 4–5) for 32 h at 60 °C to remove lignins (Loader et al. 1997). Again, samples were dried for at least 48 h prior to isotopic analysis.

### Collection of drainage water in 2009

Drainage water was collected in closed containers from the bottom outflow of each lysimeter cumulatively at intervals of 3 weeks and stored at –20 °C for further analyses. At the end of the severe drought periods in the beginning of July and in the middle of August, drainage water could not be collected from all D and AWD mesocosms as there was no bottom outflow in these

Table 1. Geographic and climatic origin of the selected oak species. Acorns were harvested at the mother stands and seeded at WSL. Elevation of the mother stands (m a.s.l.), mean annual temperature (°C) and mean annual precipitation (mm) are shown. Climate data from the period 1961–90 are from meteorological stations (MeteoSwiss) near the collections sites.

Species	Acorn origin	Altitude	Temperature	Precipitation
<i>Q. robur</i> L.	Tägerwilen (47°38'N, 9°08'E)	510	8.7	929
<i>Q. petraea</i> (Matt.) Liebl.	Corcelles (46°51'N, 6°41'E)	550	9.0	893

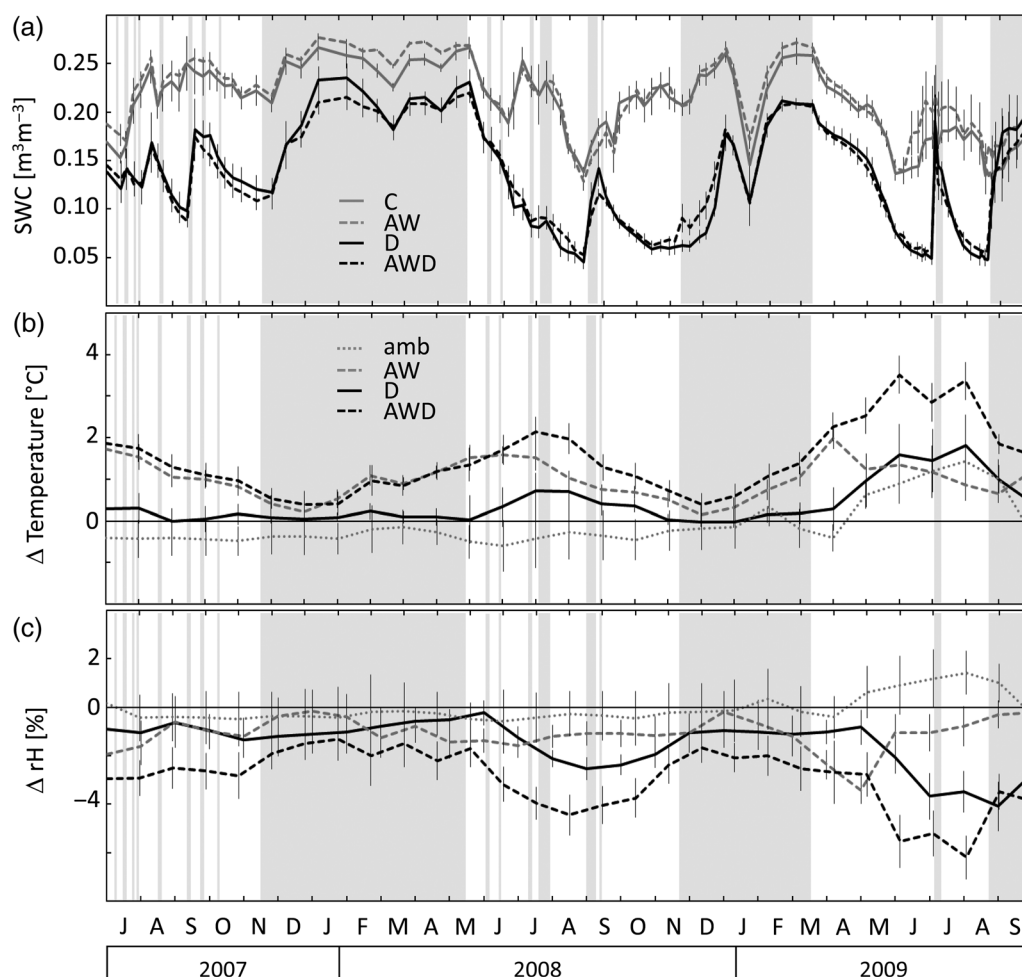


Figure 1. Soil water contents of all four treatments in 38-cm soil depth (a), differences in air temperature of the treatments and ambient plots without chamber relative to control (b) and differences in relative humidity ( $\Delta rH$ ) of the treatments and ambient plots without chamber relative to control (c) from 2007 to 2009. Grey shaded areas indicate water supply in the drought treatments D and AWD. Data are means of  $n = 4 \pm SE$  (data of (a) and (b) taken from Kuster et al. 2013 with permission of the publisher).

lysimeters due to the long-lasting irrigation stop (Figure 1a). This reduced the number of replicates for drainage water in the D and AWD mesocosms to  $n = 1$  on the respective dates, and no data are available for August in the AWD treatment. Prior to isotopic analyses of  $\delta^{18}O$ , samples were filtered with a syringe filter ( $0.45 \mu m$ , Rotilabo, Wetzlar, Germany) to remove soil particles. Drainage water consisted of two different water sources as plants were exposed to natural precipitation during the autumn and winter months and irrigated with deionized tap water enriched with nutrients during spring and summer. Deionized tap water was sampled for comparison every week and processed as described above. Precipitation water was considered to follow a clear seasonal variation with depleted  $\delta^{18}O$  in winter and enriched  $\delta^{18}O$  in summer months (Schürch et al. 2003).

#### Isotope analyses of plant samples and drainage water

Powdered material of leaves and tree-ring cellulose ( $\sim 0.5$  mg) was weighed into tin and silver capsules (Säntis Analytical,

Teufen, Switzerland) for stable C and O isotope analyses. For  $\delta^{13}C$ , the sample was combusted to  $CO_2$  with excess of oxygen at  $1020^\circ C$  in an elemental analyser (EA-1110, Carlo Erba Thermoquest, Milan, Italy), which was linked to a Delta S mass spectrometer with a CONFLO II (both Finnigan MAT, Bremen, Germany) operating in continuous flow mode. For  $\delta^{18}O$ , the samples were pyrolysed to CO at  $1080^\circ C$  in an elemental analyser (EA-1108, Carlo Erba Thermoquest) linked to a DELTA plus XP mass spectrometer via a variable open-split interface (both CONFLO-III, Thermo Finnigan, Bremen, Germany). Water samples were injected into a TC/EA Analyser (Thermo Finnigan) with a Combi Pal autosampler (CTC, Zwingen, Switzerland). At a temperature of  $1440^\circ C$ , the water was split into  $H_2$  and O, while the latter reacted with glassy carbon splinters in the reaction tube to CO. The gas components were carried in a He stream to the mass spectrometer DELTA plus XP via a variable open-split interface (both CONFLO-III, Thermo Finnigan). All isotope ratios are given in reference to their international standard



in the delta notation in ‰:  $\delta_{\text{sample}} = (R_{\text{sample}}/R_{\text{standard}} - 1)$ , with  $R_{\text{sample}}$  being the  $^{13}\text{C}/^{12}\text{C}$  or  $^{18}\text{O}/^{16}\text{O}$  ratio of the sample and  $R_{\text{standard}}$  being the ratio of Vienna Pee Dee Belemnite for C or Vienna Mean Standard Ocean water for O. Analytical precision was estimated as the standard deviation of laboratory cellulose standards, better than 0.10‰ for  $\delta^{13}\text{C}$  and 0.25‰ for  $\delta^{18}\text{O}$ .

### Measurement of leaf net photosynthesis

The seasonal course of instantaneous net photosynthesis ( $P_N$ ) was measured on four trees per species and treatment with a portable photosynthesis system equipped with a broadleaf cuvette (LI-COR 6400, LI-COR, Lincoln, NE, USA). Conditions in the cuvette were kept constant during the measurements at 380 p.p.m.  $[\text{CO}_2]$  and a photon flux density of  $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ , while the temperature was adjusted close to conditions outside the cuvette.

### Analysis of intra-annual tree-ring growth

Measurements of seasonal tree-ring growth were performed on four trees per species and treatment using the pinning technique (Wolter 1968, Fonti et al. 2013). Small wounds to the stem cambium were induced with a pin on 11 dates from April to October 2009 to retrospectively use the reaction in the wood to reconstruct the position of the ring formation at the pinning dates. After harvesting the trees, stem sections were analysed microscopically to determine the radial increment of wood tissue between the tree-ring border, formed at the end of the previous growing season, and the wound tissue formed in the growing season 2009. The relative growth was calculated as a proportion of the quantified intra-annual increment on the total ring width. A detailed description of the procedure is given by Fonti et al. (2013).

### Statistical analysis

Statistical calculations were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). Effects of treatments and their interactions were analysed as a three-factorial design (irrigation, temperature and species) by analysis of variance (ANOVA), with the factor species nested in the factors irrigation and temperature (nested ANOVA, general linear model). The factor year was highly significant; therefore, data sets were analysed separately for each year. The assignment of the plants to different mesocosms had no effect on the responses of the trees. Drainage water was analysed using a repeated-measures ANOVA with sampling date as the inner-subject factor and irrigation and temperature as between-subjects factors followed by Greenhouse–Geisser correction. Treatments and interactions among treatments were considered as significant when  $P < 0.05$ . Statistical calculations were performed with four replicates per species and treatment for leaf bulk tissue (pooled leaf samples from two trees) and eight replicates per species and treatment for tree-ring cellulose.

## Results

### Climate treatments

In the first experimental year 2007, volumetric SWC did not drop below  $0.1 \text{ m}^3 \text{ m}^{-3}$  in mesocosms with the drought (D) and the combination (AWD) treatments and remained high in the well-watered control (C) and the air warming (AW) mesocosms (Figure 1a). In 2008, soil water deficit became more severe in the drought-treated D and AWD mesocosms, as SWC nearly reached values of  $0.05 \text{ m}^3 \text{ m}^{-3}$ . During the growing season 2009, a severe drought was induced. SWC decreased to  $0.05 \text{ m}^3 \text{ m}^{-3}$  in D and AWD, while in C and AW, it remained between  $0.15$  and  $0.20 \text{ m}^3 \text{ m}^{-3}$  during the whole vegetation period (Figure 1a). Hence, drought intensity increased over the 3 years not only due to longer periods without irrigation in the growing season 2008 and 2009 but also due to increasing water demand of the developing canopies.

During the experimental period 2007–09, passive air warming led to an increase of mean monthly daytime temperatures (08:00–18:00 h, UTC + 1) during the growing seasons of  $1\text{--}2^\circ\text{C}$  in AW and  $1\text{--}3^\circ\text{C}$  in AWD mesocosms (Figure 1b), and only small differences in relative humidity were detected among the four treatments and ambient conditions (Figure 1c). In 2008, air warming in AWD was similar to that recorded in AW, while in 2009, the daily increase in air temperature was even higher in AWD than in AW. Moreover, in the D treatment, where no warming was applied, a drought-dependent increase in daytime temperature of nearly  $2^\circ\text{C}$  in 2009 was detected, which can be attributed to a decrease in evaporative cooling due to reduced transpiration of the drought-treated trees (Kuster et al. 2013).

### Isotopic patterns of $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in bulk leaf tissue and tree-ring cellulose

In the year 2007,  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  in leaf bulk tissue did not respond to the treatments D, AW and AWD (Figure 2a and d; Table 2). However, *Q. robur* had lower  $\delta^{13}\text{C}$  values compared with *Q. petraea*. In 2008, both species showed a highly significant difference in  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  as a response to the drought imposed in the D- and AWD-treated mesocosms (Figure 2b and e; Table 2). Compared with the control,  $\delta^{13}\text{C}$  increased by 1.5‰ in *Q. robur* and 1.3‰ in *Q. petraea*, while  $\delta^{18}\text{O}$  increased by 1.4 and 1.1‰ in *Q. robur* and *Q. petraea*, respectively (Figure 2b and e). Again, AW showed no significant effect on the isotopic ratios of C and O in leaf bulk tissue. While in 2007, the species did not differ in  $\delta^{18}\text{O}$ , significant species differences were observed in 2008, with *Q. robur* showing lower  $\delta^{18}\text{O}$  values compared with *Q. petraea*. In 2009, a relative increase in  $\delta^{13}\text{C}$  was found in the drought treatments compared with control, exceeding the increase in  $\delta^{13}\text{C}$  of the former season. Within the two oak species, *Q. robur* was more affected by the drought imposed in D and AWD treatments than *Q. petraea* (*Q. robur*:

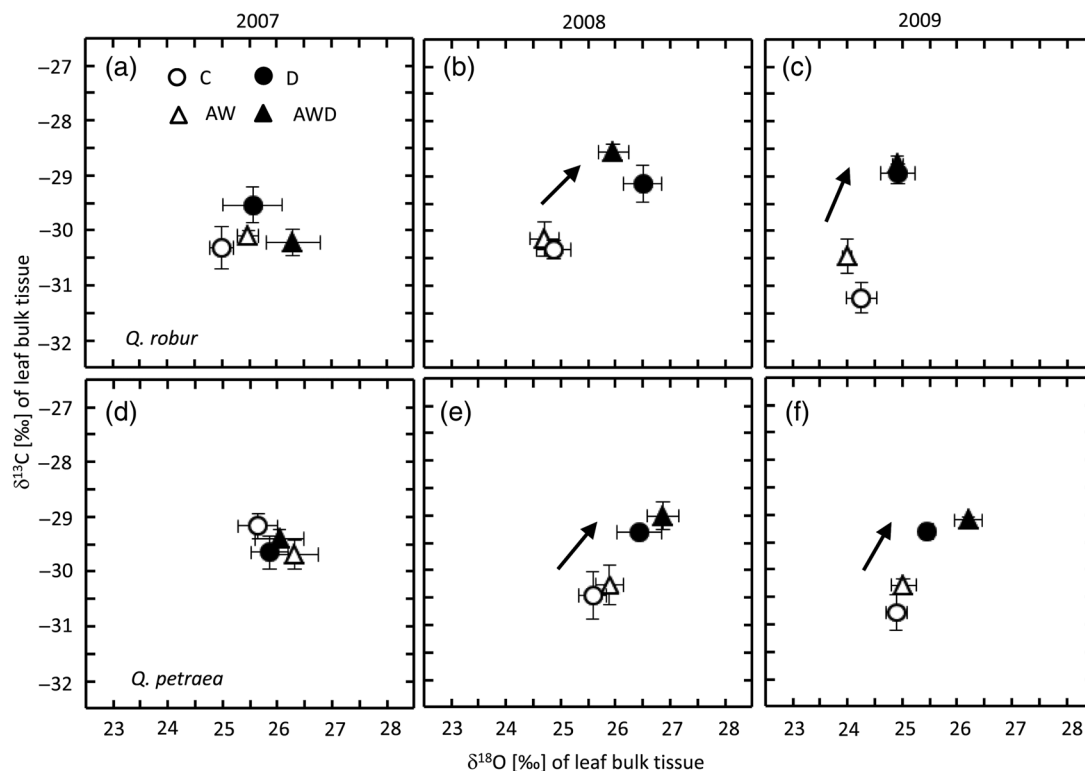


Figure 2. Patterns of carbon and oxygen isotopes in leaf bulk tissue of *Q. robur* (a–c) and *Q. petraea* (d–f) for the years 2007, 2008 and 2009. Symbols represent mean values of each treatment. Black arrows indicate the trend in isotopic patterns due to the drought treatments. Data are means  $\pm$  SE,  $n = 4$ .

Table 2. Effects of the treatments air warming (AW; control vs elevated air temperature), drought (D; continuous vs discontinuous irrigation) and species on  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  of leaf bulk tissue and tree-ring cellulose in three growing seasons 2007, 2008 and 2009. Bold numbers indicate a significant  $P$ -value as calculated with ANOVA according to the three-factorial test design (D, AW and Species). None of the two- and three-way interactions between factors (D  $\times$  AW; D  $\times$  Species; AW  $\times$  Species; D  $\times$  AW  $\times$  Species) was significant.

	Leaf bulk tissue						Tree-ring cellulose					
	2007		2008		2009		2007		2008		2009	
	$\delta^{13}\text{C}$	$\delta^{18}\text{O}$	$\delta^{13}\text{C}$	$\delta^{18}\text{O}$	$\delta^{13}\text{C}$	$\delta^{18}\text{O}$	$\delta^{13}\text{C}$	$\delta^{18}\text{O}$	$\delta^{13}\text{C}$	$\delta^{18}\text{O}$	$\delta^{13}\text{C}$	$\delta^{18}\text{O}$
AW	0.703	0.171	0.270	0.245	0.102	0.101	0.665	0.755	0.052	0.413	0.061	0.064
D	0.563	0.213	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	0.776	0.629	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.001</b>
Species	<b>0.007</b>	0.167	0.283	<b>0.003</b>	0.704	<b>0.000</b>	<b>0.011</b>	<b>0.002</b>	<b>0.040</b>	<b>0.012</b>	0.785	<b>0.001</b>

2.4‰, *Q. petraea*: 1.8‰, Figure 2c and f). In contrast to  $\delta^{13}\text{C}$ , the drought-mediated increase in  $\delta^{18}\text{O}$  was less pronounced in 2009 compared with the preceding year (*Q. robur*: 0.7‰, *Q. petraea*: 0.9‰), but still highly significant (Table 2). As in 2008, *Q. robur* showed lower  $\delta^{18}\text{O}$ , but this species effect became highly significant in 2009. Air warming in the AW and AWD treatments had no significant effect on the isotopic ratios of C and O in leaf bulk tissue of either species in any of the experimental years. It is worth noting that in leaf bulk tissue, the relative increase in  $\delta^{13}\text{C}$  observed in D and AWD mesocosms in 2008 and 2009 was related to a decrease in  $\delta^{13}\text{C}$  in C and AW mesocosms.

For tree-ring cellulose, no response of the isotopic ratios of carbon and oxygen to the climate treatments was detected in the

first experimental year 2007 (Figure 3a and d), but *Q. robur* showed significantly lower  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values than *Q. petraea* in 2007 (Table 2). In 2008, differences between trees in well-watered (C and AW) and drought-treated (D and AWD) mesocosms became highly significant for both isotopes. As in leaf bulk tissue,  $\delta^{13}\text{C}$  in tree-ring cellulose increased concomitantly with  $\delta^{18}\text{O}$  for D and AWD (*Q. robur*: 1.1‰  $\delta^{13}\text{C}$  and 0.8‰  $\delta^{18}\text{O}$ ; *Q. petraea*: 1.1‰  $\delta^{13}\text{C}$  and 0.7‰  $\delta^{18}\text{O}$ ; Figure 3b and e) in 2008. In 2009, an increase in  $\delta^{13}\text{C}$  in D and AWD mesocosms was observed for both species, *Q. robur* (Figure 3c: 2.1‰) and *Q. petraea* (Figure 3f: 2.5‰), which was even stronger than in the former year. In contrast,  $\delta^{18}\text{O}$  showed a decrease in tree-ring cellulose of  $-1.0$ ‰ in *Q. robur* and  $-0.5$ ‰ in *Q. petraea* compared with well-watered trees in 2009. In none of the years and

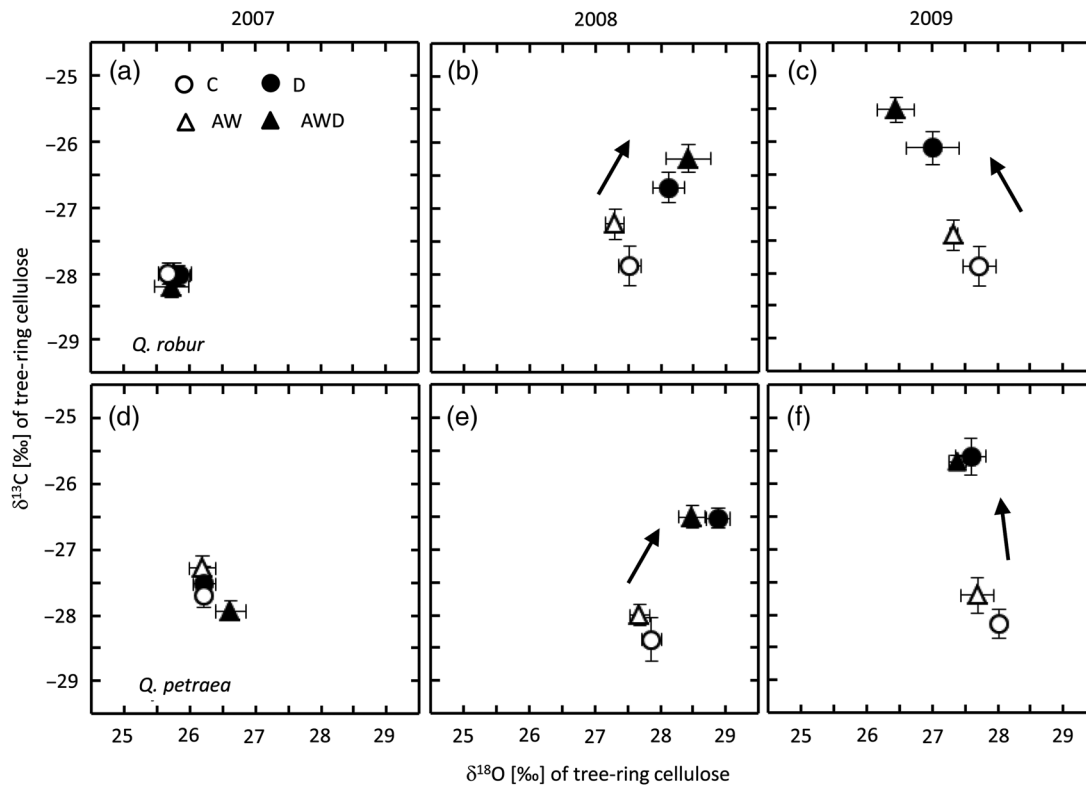


Figure 3. Pattern of carbon and oxygen isotopes in tree-ring cellulose of *Q. robur* (a–c) and *Q. petraea* (d–f) for the years 2007, 2008 and 2009. Symbols represent mean values for each treatment. Black arrows indicate the trend in isotopic patterns due to the drought treatments. Data are means  $\pm$  SE,  $n = 8$ .

species was a significant change in  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values due to air warming treatment alone detected.

### Seasonal net photosynthesis

Seasonal net photosynthesis ( $P_N$ ) was analysed in the last experimental year 2009 (Figure 4a and b; Arend et al. 2013).  $P_N$  was strongly affected by drought in D and AWD treatments and declined in both oak species with gradually decreasing soil moisture availability (Figure 1a).  $P_N$  in these treatments only recovered during the short re-watering period in July 2009 but decreased rapidly during the subsequent drought. Air warming in the well-watered AW treatment did not lead to a significant change in  $P_N$ , either in *Q. robur* or in *Q. petraea*.

### Intra-annual tree-ring growth

The severe drought applied in 2009 to the D and AWD mesocosms had a strong effect on the seasonal course of tree-ring growth as well as on its absolute values (Figure 4c and d, Table 3). While trees of *Q. robur* in the well-watered C and AW mesocosms completed 50% of their inter-annual tree-ring growth until the short re-watering period in July, trees in the drought-treated mesocosms already accomplished 75% of the entire annual growth and stopped tree-ring formation in early August. In contrast to *Q. robur*, *Q. petraea* maintained some growth activity in the D and AWD mesocosms until the end of

the season but grew much more slowly than well-watered trees. Comparing absolute tree-ring width of the two species, *Q. robur* produced wider tree rings than *Q. petraea* (Table 3,  $P = 0.002$ ). Both species suffered from the applied drought in the D and AWD mesocosms and responded with a reduction in tree-ring width ( $P < 0.001$ ). Compared with the control, the final reduction of intra-annual tree-ring growth was 59 and 66% for drought-treated *Q. robur* and *Q. petraea*, respectively, and 74 and 57% for *Q. robur* and *Q. petraea* in the AWD treatment, respectively (for more detailed information on intra-annual tree-ring growth, see Fonti et al. 2013 and for additional data on biomass, see Table S1 available as Supplementary Data at [Tree Physiology Online](http://www.treephys.oxfordjournals.org/)).

### $\delta^{18}\text{O}$ in drainage water

Drainage water, collected at regular intervals from the bottom outflow of the lysimeters, showed a clear seasonal variation in  $\delta^{18}\text{O}$  (Figure 4e), with depleted  $\delta^{18}\text{O}$  values in winter and enriched  $\delta^{18}\text{O}$  values in summer months ( $P = 0.001$ ). The drainage water originated from two different sources with different  $\delta^{18}\text{O}$  values: tap water used for irrigation in spring and summer and natural precipitation in autumn and winter. Tap water had a constant  $\delta^{18}\text{O}$  value of  $-11\text{‰}$ , while natural precipitation showed temperature-dependent seasonal variations, with a decrease in  $\delta^{18}\text{O}$  down to  $-15\text{‰}$  in winter (IAEA/WMO 2014,

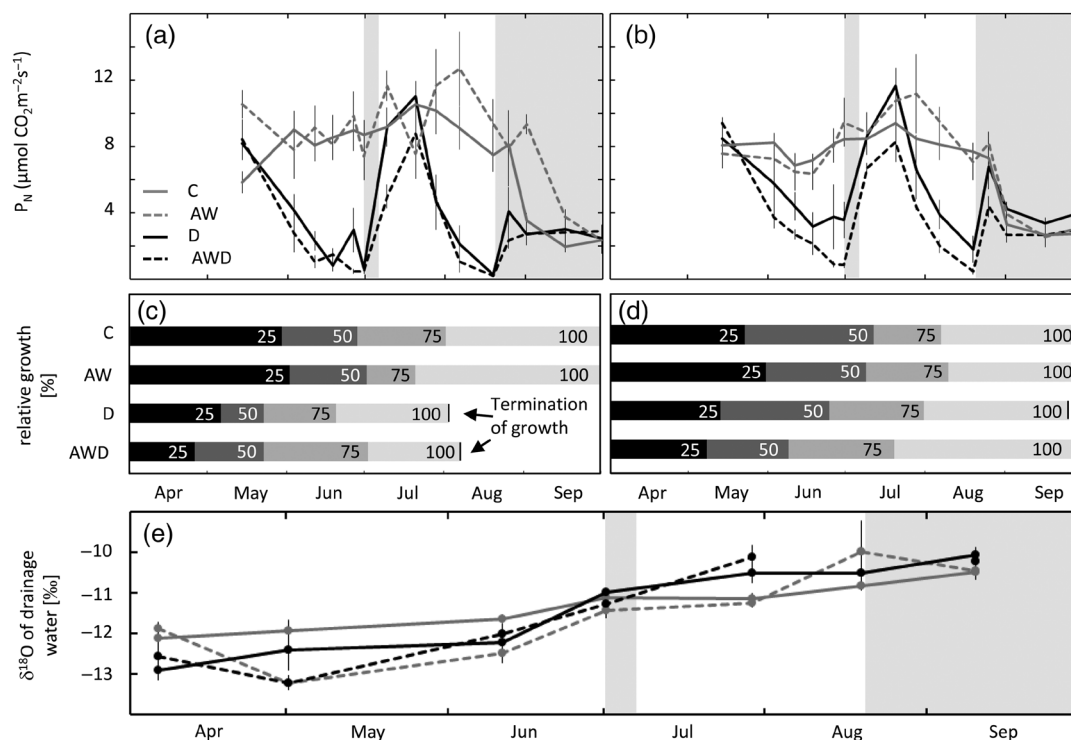


Figure 4. Seasonal course of net photosynthesis and relative tree-ring growth of *Q. robur* (a and c) and *Q. petraea* (b and d) and drainage water  $\delta^{18}\text{O}$  (e) for the year 2009. Grey shadings indicate water supply in the drought treatments. (a and b) Instantaneous net photosynthesis in AW, D and AWD treatment and C trees. Data are means  $\pm$  SE ( $n = 4$ ). (c and d) Relative tree-ring growth during the growing season, determined with the pinning technique. Different shadings of the bars indicate 25, 50, 75 and 100% of growth as a mean value for four trees per treatment. (e) Seasonal course of  $\delta^{18}\text{O}$  in drainage water in the four treatments ( $n = 4 \pm \text{SE}$ ; 1 July 2009: D and AWD  $n = 1$ ; 19 August 2009: D  $n = 1$  and AWD not available). Pattern of the drainage water  $\delta^{18}\text{O}$  is the same for the two species (photosynthesis data taken from Arend et al. 2013, with permission of the publisher).

Table 3. Tree-ring width of control (C), air warming (AW), drought (D) and combination (AWD) treated trees of *Q. robur* and *Q. petraea* in 2009 ( $\mu\text{m}$ ). Values are means  $\pm$  SE;  $n = 4$ .

	C	AW	D	AWD
<i>Q. robur</i>	2181 $\pm$ 374	2589 $\pm$ 214	908 $\pm$ 238	576 $\pm$ 14
<i>Q. petraea</i>	1552 $\pm$ 65	1586 $\pm$ 96	539 $\pm$ 45	680 $\pm$ 58

Schürch et al. 2003). This resulted in a  $^{18}\text{O}$  depletion in the drainage water in spring by 1–2‰ relative to the tap water used for irrigation of all treatments. The  $\delta^{18}\text{O}$  values of the drainage water increased over the course of the growing season due to progressive replacement of depleted winter precipitation water by more enriched tap water and additionally increased evaporative enrichment of soil water over summer. By mid-August, drainage water of all treatments was enriched by 1.5‰ compared with irrigation water.

## Discussion

Patterns of isotopic ratios of carbon ( $\delta^{13}\text{C}$ ) and oxygen ( $\delta^{18}\text{O}$ ) were analysed in leaves and tree rings of oaks (*Q. robur* and *Q. petraea*) subjected for 3 years to warming, drought and their combination. To better understand their physiological origin,

changes in  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  were linked to leaf net photosynthesis and inter-annual tree-ring growth. The applied drought treatments did not change  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  in leaves and tree rings in the first year 2007. This is not surprising as intermediate irrigation prevented the development of a severe and physiologically effective water deficit and trees were still small, with a rather low water demand for their developing canopies. The observed  $\delta^{13}\text{C}$  values in leaves and tree rings were comparable to data reported for well-watered oak trees (Picon et al. 1996, Zang et al. 2012), supporting the interpretation that our trees had not yet suffered from the applied drought. Also, the warming treatment, being rather small, did not affect  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  in leaves and tree rings in any year, probably due to the thermic plasticity of these thermo-tolerant oak species (Ellenberg 2009). This finding contradicts earlier studies that reported effects of increased temperature on  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  (Loader and Switsur 1996), which used  $\delta^{18}\text{O}$  to detect temperature shifts of 1.5 °C in annual average temperature (Libby et al. 1976). However, in our experiment, increased temperatures were only observed from May to October, while these former studies report an average deviation of the temperature over a whole year.

As the number of irrigation events was considerably reduced in 2008 and 2009, we could observe the first response of the trees to the applied drought treatments. In both years, an



increase in  $\delta^{13}\text{C}$  was observed in leaf tissue and tree-ring cellulose, indicating a decrease in leaf internal  $\text{CO}_2$  concentrations (Farquhar et al. 1989). This is in line with studies on carbon isotope responses to drought where reduced water availability induced a decrease in stomatal conductance, leading to reduced internal  $\text{CO}_2$  concentrations and therefore to less negative  $\delta^{13}\text{C}$  ratios in photosynthetic and non-photosynthetic tissues of different oak species (Picon et al. 1996, Zang et al. 2012). Along with an increase in  $\delta^{13}\text{C}$ , an increase in  $\delta^{18}\text{O}$  was observed in 2008 and 2009 in leaf tissue and in the year 2008 in tree-ring cellulose. According to the dual isotope approach, the increase in  $\delta^{18}\text{O}$  indicates stomatal limitation of photosynthesis to be the main cause for the observed increase in  $\delta^{13}\text{C}$  (Scheidegger et al. 2000), which compares well with the physiological observations in our experiment and the gas exchange measurements by Arend et al. (2013).

The instantaneous drought effects on photosynthetic carbon assimilation and thus leaf sugar isotope composition become permanently recorded when sugars are transported to stems and incorporated in the tree-ring cellulose matrix (West et al. 2006, Gessler et al. 2009, 2014). It was surprising, however, that for drought-treated trees of *Q. robur*, a decrease in  $\delta^{18}\text{O}$  in tree-ring cellulose was observed in 2009, even below the level of well-watered control trees, while *Q. petraea* showed no clear response to the drought treatments. Applying the dual isotope approach to the observed isotope response, an increase in  $\delta^{13}\text{C}$ , together with a constant or decreasing  $\delta^{18}\text{O}$ , would suggest an increase in photosynthesis and a constant or increasing stomatal conductance despite the severe drought conditions in 2009. However, this clearly contradicts current plant physiological knowledge and is not supported by our observation of leaf gas exchange, growth data and PS II photochemistry (see also Arend et al. 2013).

Thus, here we have a situation, which Barbour and Song (2014) describe as the 'tree-ring stable isotope compositions remain somewhat unreliable as a record of tree carbon–water dynamics'. So, the question arises, what can explain these seemingly implausible results? One should note that in drought-treated oaks, 50% of the tree ring was formed during May/June, and growth came completely to a halt (*Q. robur*) or proceeded at a low rate (*Q. petraea*) in summer. Therefore, a relatively large proportion of the tree ring in drought-stressed trees was already formed in spring when the  $\delta^{18}\text{O}$  of the soil source water was probably still low due to winter rain. During the summer time, when no or only little growth was observed, the  $\delta^{18}\text{O}$  of the soil source water was higher and drought reduced stomatal conductance, resulting in an increase in  $\delta^{18}\text{O}$  in leaves. However, if there is no or only little growth, no isotopic signals from leaf water enrichment and from soil source water can be recorded in tree rings, probably explaining the much lower  $\delta^{18}\text{O}$  signals in 2009. One could argue that this pattern should be found in the  $\delta^{13}\text{C}$  signature of tree rings as well. This, indeed, might be the case if

there would be such a high seasonal variation in the atmospheric  $\delta^{13}\text{C}$  value as observed in soil source water  $\delta^{18}\text{O}$ , namely up to 3‰. However, the seasonal variation in  $\delta^{13}\text{C}$  at the latitude of the study (47°N) is negligible compared with  $\delta^{18}\text{O}$  (0.6‰; Troler et al. 1996).

This uncoupling in  $\delta^{18}\text{O}$  leads to an isotope pattern in tree rings that 'fakes' a counter-intuitive (and in fact non-existent) physiological response to drought. Interestingly, the  $\delta^{18}\text{O}$  isotope signal in tree rings of *Q. petraea* was less affected because growth stopped a few weeks later than that in *Q. robur*. Therefore, some of the summer isotope signals could still be recorded in the tree rings of *Q. petraea*, leading to higher  $\delta^{18}\text{O}$  values compared with *Q. robur*. However, although in *Q. petraea* a fake signal was recorded as well, the higher  $\delta^{18}\text{O}$ , together with a longer period of growth, suggests a more indifferent response to drought. Thus, our results clearly show the importance of intra-annual growth and soil source water when interpreting  $\delta^{18}\text{O}$  signals in tree rings, supporting other studies that provided evidence that seasonal variations in source water may have a strong impact on  $\delta^{18}\text{O}$  in tree rings (Offermann et al. 2011).

A similar result with missing  $\delta^{18}\text{O}$  signals in tree rings was also observed by Sarris et al. (2013) for pine trees growing in a dry Mediterranean summer climate. In this former study, an extended period of growth cessation was observed, along with depleted  $^{18}\text{O}$  values in tree rings. This depletion was explained by the use of different soil water sources (depleted ground water vs enriched precipitation water) but was not explicitly related to drought-induced growth cessation. In our study, we provide clear evidence (based on tree-ring growth assessments) that a drought-induced growth cessation leads to decreased  $\delta^{18}\text{O}$  values in tree rings as the isotopic signals from leaves and soil source water were not imprinted in the tree-ring matrix. Even a diminished tree-ring growth can lead to reduced  $\delta^{18}\text{O}$  values, as shown for *Q. petraea*, and therefore bias the interpretation of isotopic patterns in tree rings. The strong and concurrent reduction in leaf photosynthesis with increasing drought is in line with the observed decrease and cessation of growth. Although photosynthesis recovered rapidly due to intermittent irrigation events, the newly fixed assimilates were not sufficient to resume growth and thus no isotopic signals from leaves were recorded in the respective annual tree rings.

## Conclusion

In this study, we show that severe drought can uncouple the isotopic signals between leaves and tree rings. Once drought reaches an extent at which growth is halted or strongly reduced, e.g., detected by intra-annual tree-ring growth analysis, the isotopic signal imprinted on freshly formed leaf carbohydrates is no longer recorded in tree rings. Instead, the signal recorded before or potentially after the growth-limiting drought can be found later on in the isotopic tree-ring chronology. Whether isotopic signals of the environment are recorded at all depends on the amount

of mobile leaf carbohydrates that can be transformed to cell wall polymers in growing tree rings. Therefore, very dry (summer) conditions are most likely not recorded in tree rings and thus might get completely lost, since the major proportion of the tree ring is then probably formed in spring or autumn. As shown in our study, the dual isotope approach can be highly instrumental in detecting such missing records. This will have large implications on the physiological interpretation of any isotopic tree-ring chronology, particularly with respect to severely growth-limiting conditions, as projected for the future.

## 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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