Research paper

A simple stable carbon isotope method for investigating changes in the use of recent versus old carbon in oak

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Stable carbon isotope ratios from early-wood (EW) and late-wood (LW) are used to test competing models of carbon storage and allocation, providing a cost-effective alternative to measuring and dating non-structural carbohydrates in mature temperate broad-leaf forest trees growing under natural conditions. Annual samples of EW and LW from seven mature oaks (*Quercus robur* L.) from Scotland, covering AD 1924–2012, were pooled, treated to isolate alpha-cellulose and pyrolysed to measure the carbon isotope ratios. Late-wood values are strongly correlated with summer temperature of the year of growth and EW contains the same signal offset by 1 year. After a warm summer, isotopic ratios of EW are similar to those of the preceding LW, but following cold summers they are relatively enriched. The results conflict with established models of isotopic variation within oak tree rings but support 'two-pool' models for storage of non-structural carbohydrates, with EW formation, which occurs prior to budburst, preferentially using young reserves accumulated in the previous summer. Under poor growing conditions trees access older reserves. Slight average isotopic enrichment of EW may be explained by preferential accumulation of reserves during warmer summers rather than by isotopic enrichment during starch formation in non-photosynthetic tissue.

Keywords: carbon cycling, carbon dynamics, carbon isotopes, non-structural carbohydrates, oak, *Quercus*, tree ring.

Introduction

The importance of including non-structural carbohydrates (NSCs) in models of the carbon dynamics of forest trees has become increasingly clear (Kozlowski 1992, Dietze et al. 2014, Palacio et al. 2014). Carbon cycle models that do not deal realistically with the way that NSCs are used are likely to be misleading because growth is not an inevitable consequence of carbon assimilation (Fatichi et al. 2014). The sugars and starch that mainly comprise NSCs are likely to serve multiple functions in trees. They may serve several osmotic purposes, such as maintaining turgor and long distance vascular integrity in xylem and phloem (Sala et al. 2012, Dietze et al. 2014). However, they are also used for maintenance respiration during winter (Ögren 2000) and to aid survival during periods of low accumulation or high sink demand resulting from, for example, environmental stress or structural damage (Dunn et al. 1990, Canham et al. 1999). Modelling carbon dynamics in forest trees is important

not least because of the potential impacts of climate change and the attendant feedbacks on the carbon and water cycles (Bodin et al. 2013). An understanding of carbohydrate storage is also essential for modelling the resilience of trees to climate change and extreme events. However, it is difficult to test models because of the paucity of field evidence on the size and age of NSC pools in mature trees (Barbaroux and Bréda 2002, Richardson et al. 2013). Measuring the amount of NSC stored by mature trees is difficult, and estimates based only on the stem wood may be misleading, because NSC is also stored in coarse roots, branches and leaves (Barbaroux et al. 2003, Hoch et al. 2003, Würth et al. 2005, Gough et al. 2009). Also measuring and dating the NSC of wood provides a static picture of NSC status, and monitoring over time is expensive and typically limited to a few years at most.

A cost-effective alternative to monitoring NSC dynamics is to use the carbon isotope ratios in different plant components as

tracers of carbon accumulation, storage and use. Recent work by Kimak and Leuenberger (2015), for example, suggests that carbohydrate storage strategies of broad-leaf trees are traceable by early-(EW) to late-wood (LW) carbon isotope differences. They report that under very harsh growing conditions, such as the European Little Ice Age, oak EW is dependent upon young reserves but that under normal or favourable conditions this dependence is not apparent. Under the good growing conditions of recent decades Kimak and Leuenberger (2015) found no significant correlation between the carbon isotope ratios of EW and those of the LW of the previous season. They argue that this is due to either spring growth becoming rapidly autotrophic (Keel et al. 2007), so that reserves contribute in a limited way to EW formation or to rapid mixing of carbon storage so that the signal of the previous year is not preserved. These results are in broad agreement with very detailed analysis of changes in carbon isotope ratios within individual tree rings (Helle and Schleser 2004), which also suggests that EW cells are generally formed from well-mixed stores of NSC, so that environmental signals are not carried over from one year to the next.

The carbon isotope results of Kimak and Leuenberger (2015) contrast with the results of Richardson et al. (2013), who monitored the seasonal dynamics of NSC in North American forests and also dated the NSC stored in the outer 2 cm of the stem wood using the radiocarbon bomb pulse. Whereas the carbon isotope studies suggest that under good growing conditions EW is produced from well-mixed reserves, so that there is no correlation between values obtained from the LW of one season and the EW of the next, the detailed monitoring study of Richardson et al. (2013) concludes that trees preferentially draw on very young reserves, so that the EW is formed primarily from reserves accumulated in the previous year. They argue for a 'two-pool' model of carbon storage, where an easily accessed pool dominated by very young reserves is linked to a well-mixed pool of older reserves that can be called upon at need.

A potential weakness of the stable isotope approach, as it has been applied so far, is that conclusions have been extrapolated from very detailed studies of just a few individual rings (Ogle and McCormac 1994, Loader et al. 1995, Helle and Schleser 2004) or by comparing EW and LW taken from a single tree (Switsur et al. 1995, Kimak and Leuenberger 2015). Here we use an alternative approach, adapted from methods developed for extracting strong climate signals from stable isotopes in tree rings (Gagen et al. 2012, Woodley et al. 2012, Young et al. 2012, 2015, Loader et al. 2013), and compare the EW and LW carbon isotope ratios of several trees by pooling the samples prior to analysis (Borella et al. 1998). We argue that although some intra-annual variability is lost by coarsely sub-dividing each tree ring into EW and LW, by measuring the average response of several trees over a long period we can efficiently provide a more representative picture of stand carbon dynamics and critically test the competing models of carbon storage and allocation in mature temperate broad-leaf forest trees.

Materials and methods

Fifteen straight-growing and mature oak trees (*Quercus robur* L.) were selected from a north facing slope 4 km south of Aviemore, Scotland (57° 9′ 15′′ N 03° 50′ 0′′ W, 300 m above mean sea level) and cored using standard methods to produce a ring width chronology. Further details of sampling strategy and the mean ring widths are included in the Supplementary Data at *Tree Physiology* Online. Climate records from Braemar (57° 0′ 40′′ N 03° 23′ 49′′ W, 325 m elevation), show that mean annual temperature is 6.5 °C and average annual precipitation is 900 mm for the period AD 1959–2012. Seven cores were chosen for isotopic analysis and their mean ring widths suggest they are representative of the full chronology (see Supplementary Figure SI available as Supplementary Data at *Tree Physiology* Online). Accurate separation of EW and LW was achieved by cutting each ring into thin slivers using a razor blade and dissecting microscope. The first 50 rings of each tree were not processed to avoid possible juvenile effects (McCarroll and Loader 2004, Labuhn et al. 2013, Kilroy et al. 2016), resulting in an 89-year chronology covering AD 1924–2012, constructed from a pooled series with a minimum sample depth of six trees. The wood was extracted to alpha-cellulose using standard methods (Loader et al. 1997), freeze-dried and 0.30–0.35 mg weighed into silver capsules and pyrolysed over glassy carbon at 1090 °C (Young et al. 2011). Pyrolysis was conducted using a Europa ANCA GSL elemental analyser interfaced with a Europa 20/20 isotope ratio mass spectrometer, with precision from a standard laboratory cellulose of 0.1‰ for $\delta^{13}C$ (σ_{n-1} $n = 10$).

The raw $\delta^{13}C$ series were corrected for changes in the atmospheric carbon isotopic ratio ($\delta^{13}C$) resulting from the burning of isotopically depleted fossil fuels by simple addition of the difference between smoothed atmospheric $\delta^{13}C$ values and a pre-industrial datum value of −6.4‰ for AD 1849 (McCarroll and Loader 2004). Regional meteorological data were obtained for northern Scotland from the UK Meteorological Office.

Results

The corrected EW and LW $\delta^{13}C$ values (Figure 1a) reveal a clear temporal offset. Although the correlation between EW and LW within individual rings (Figure 1b) is positive and statistically significant ($r = 0.43$, $P < 0.01$), it is caused by the rising trend since the late 1980s in both time series. When all trends are removed by first-differencing (current year minus previous year) the correlation drops to zero (Figure 1d). When the EW $\delta^{13}C$ values are compared with the LW values of the previous year (Figure 1c) the correlation is much stronger ($r = 0.75$), and remains high ($r = 0.65$) after first-differencing (Figure 1e).
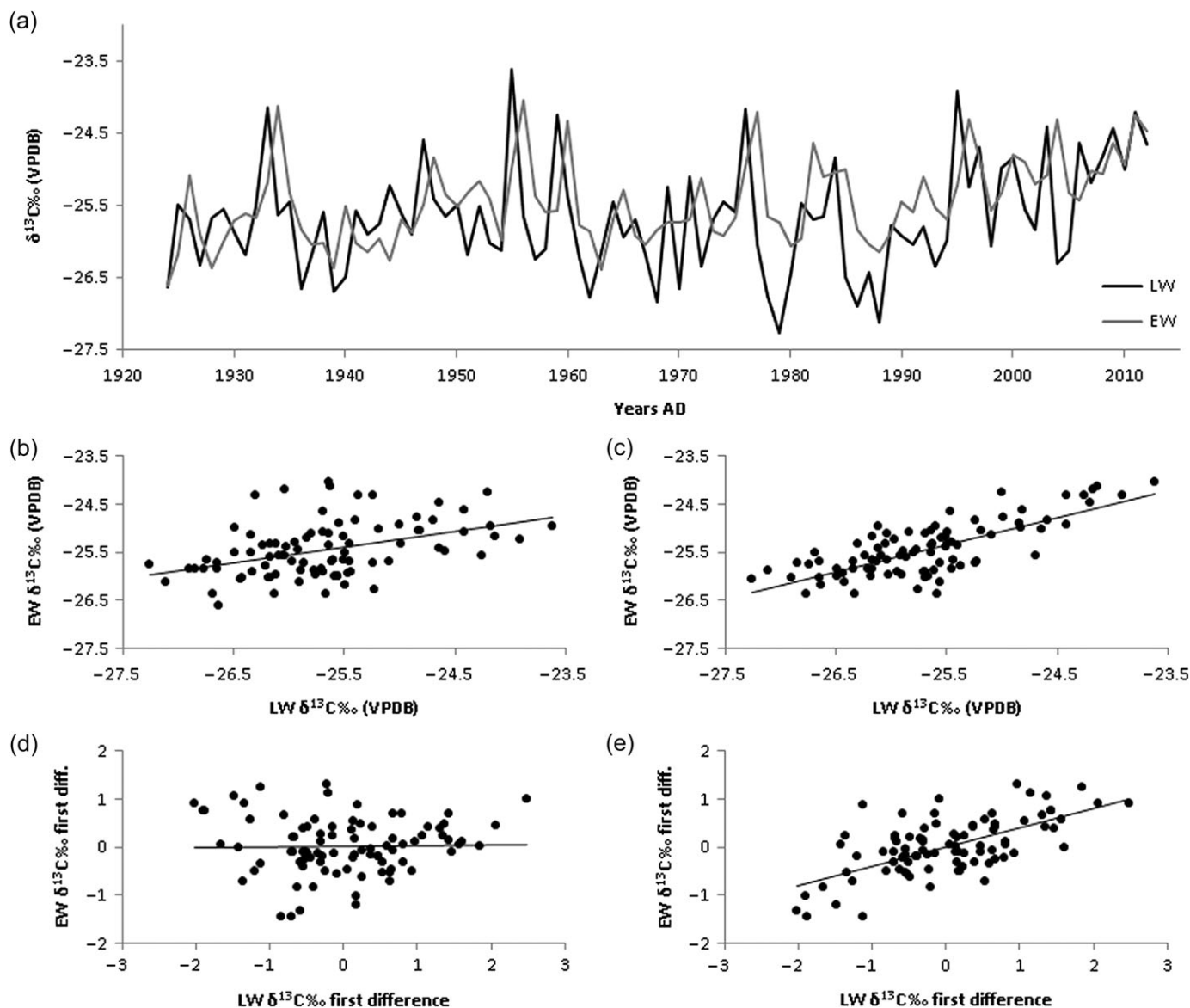
Figure 1. (a) Time series of early-wood (EW) and late-wood (LW) $\delta^{13}C$ of cellulose (relative to the Vienna Pee Dee Belemnite (VPDB) standard) obtained from a pool of seven oak trees. Note the clear 1-year offset. (b) Relationship between EW and LW $\delta^{13}C$ values obtained from the same ring. (c) Relationship between the LW $\delta^{13}C$ of 1 year and the EW $\delta^{13}C$ of the following year. (d) As for (b), but after first-differencing the data to remove all trends. (e) As for (c), but after first-differencing the data to remove all trends.

Stable carbon isotope ratios of LW cellulose from oak trees growing under moist conditions in the UK are strongly correlated with summer temperature and sunshine (Young et al. 2012), probably because photosynthetic rate, rather than stomatal conductance, dominates fractionation in the leaf. The relative strength of correlation with the temperature of different months (Figure 2) should therefore help to elucidate the controls on fractionation of the carbon that is incorporated into EW and LW. As expected, there is a strong correlation between LW $\delta^{13}C$ and temperature of the current summer, with July yielding the highest values for a single month ($r = 0.58$) and July and August as the strongest combination of months ($r = 0.63$). April temperatures are also positively and significantly

correlated with LW $\delta^{13}C$ ($r = 0.35$), but not with summer temperature.

The pattern of correlations for EW $\delta^{13}C$ (Figure 2) is virtually identical to that for LW, but the correlations are slightly lower and are with the temperatures of the previous year. The highest value for a single month is July ($r = 0.52$) and the highest for a combination of months is the mean temperature of July and August ($r = 0.57$). Spring temperatures of the current year are not correlated with EW $\delta^{13}C$.

As well as being offset by 1 year it is apparent (Figure 1) that the EW values are, on average, higher and less variable than those of the LW. The mean $\delta^{13}C$ values for the EW and LW series, respectively, are $-25.45‰$ and $-25.67‰$, and although

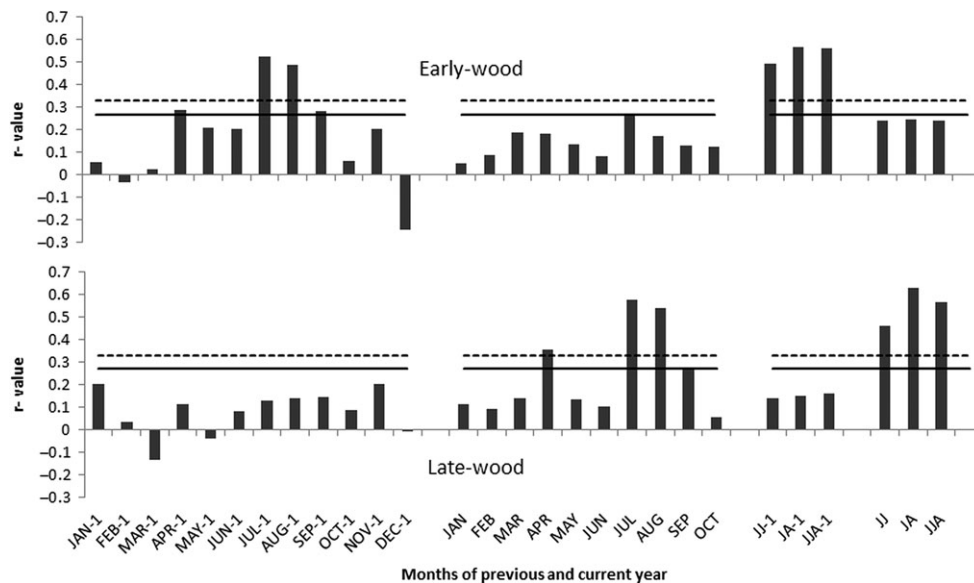Figure 2. Pearson's correlation coefficients (r-values) for EW and LW δ^{13}C compared with monthly mean temperatures for the current and previous years. The solid black horizontal line is the critical value for $P = 0.05$ for a single test and the dashed line represents the critical value 'Bonferroni' corrected to take account of family-wise error due to multiple testing.

the difference is small, at 0.22‰, given the large sample size it is statistically significant even using a two-tailed test (independent samples t-test: $P = 0.03$). The variability of the EW values (SD = 0.57) is muted relative to LW (SD = 0.75) giving a variance ratio of 1.73, which is statistically significant (F-test for equality of variance, $P = 0.01$). First-order autocorrelation in the LW ($r = 0.21$) is identical to the first-order autocorrelation in the mean July–August (JA) temperature data. In the EW, by contrast, first-order autocorrelation is much higher ($r = 0.46, P < 0.001$). In the earliest 31 years of the time-series the first-order autocorrelation in the JA temperature data is zero and that in the LW is also very low ($r = 0.10$), whereas the EW still shows significant autocorrelation ($r = 0.42, P < 0.05$). Neither series shows significant autocorrelation at lags of more than 1 year.

The relationship between the LW values of 1 year and the EW values of the next appear to be related to the summer temperatures of the year of LW formation (Figure 3). In the warmest third of summers over the study period ($n = 29$, JA mean temperature 12.75–14.30 °C) the correlation between the LW and EW values is very strong ($r = 0.80, P < 0.0001$) and there is no tendency for EW to be enriched (14 of the 29 EW values were enriched while 15 were unenriched). The mean values of LW and EW are very similar (−25.17‰ and −25.13‰) and a paired t-test returns no significant difference (two-tailed $P = 0.67$). For the coolest one-third of summers (10.7–11.85 °C), in contrast, the correlation is much weaker ($r = 0.39, P = 0.04$) and in 24 of these 29 years the EW values are enriched. A paired t-test is strongly significant ($P < 0.0001$). For the intermediate summers the correlation is significant ($r = 0.68, P < 0.0001$) and so is the 0.25‰ offset (LW = −25.69‰, EW = −25.44‰, $P = 0.002$), with 22 of the 30 EW values showing enrichment. The relationship between

summer temperature and isotopic enrichment of EW is explored further in the Supplementary Data at *Tree Physiology* Online.

Discussion

Kimak and Leuenberger (2015) pooled material from four cores of one tree, arguing that 'the high natural variability of tree ring records might lead to cancelling effects of intra-annual variations when pooling across cores of multiple trees'. Our results, however, show a strong and consistent correlation between LW δ^{13}C and summer temperature, suggesting that pooling wood from several trees prior to isotope analysis produces reliable δ^{13}C time series (Borella et al. 1998, Dorado-Liñan et al. 2011, Woodley et al. 2012). Therefore, the concern that remobilization of stored NSCs might decouple trends in δ^{13}C in tree rings from the shorter-term trends predicted by leaf-scale fractionation models and thus overwrite seasonal influences of climate (Helle and Schleser 2004, Offermann et al. 2011, Gessler et al. 2014) appears to be unfounded. On the contrary, at this site, EW carries a signal virtually identical to that of the preceding LW.

Our multi-decadal results contrast with those based on high-resolution sampling across individual rings. Helle and Schleser (2004), for example, found a common tri-phase pattern typified by an early increase to maximum δ^{13}C values in EW, a decline that persists until close to the end of the LW and a final increase. The high values are attributed to remobilization of carbon stored as isotopically enriched starch. These results were based on only two oak rings from a single tree, though the general tri-phase pattern was replicated in two rings each of beech (*Fagus sylvatica*) and mulberry (*Morus alba*), and four rings of poplar (*Populus nigra*), all from central Europe.
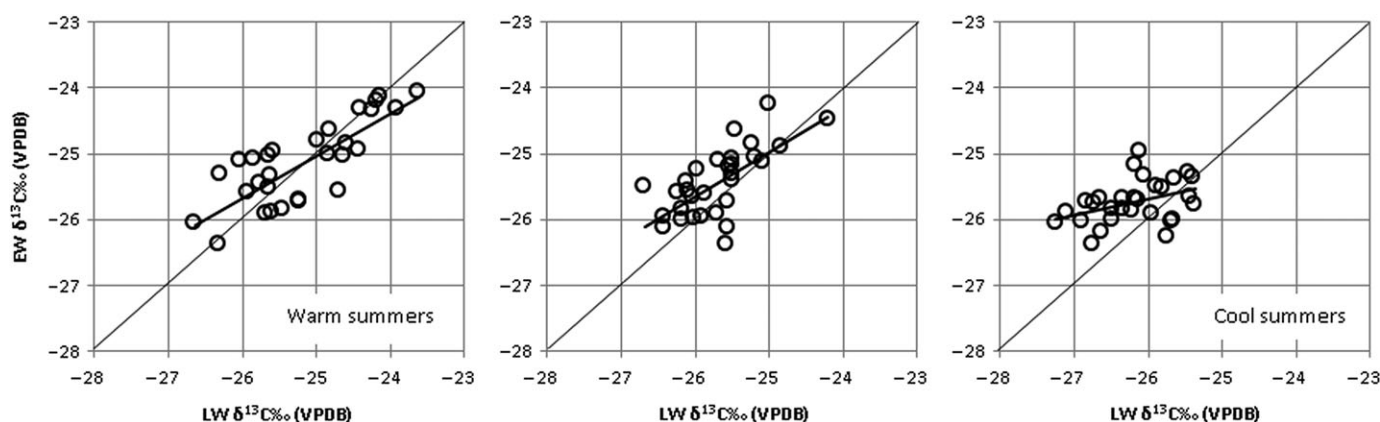
Figure 3. Correlation between the LW $\delta^{13}C$ value of 1 year and the EW $\delta^{13}C$ value of the next (relative to the Vienna Pee Dee Belemnite (VPDB) standard) separated according to summer temperature. In the warmest one-third of summers ($n = 29$) the LW and EW values are strongly correlated and there is no offset, whereas in the coolest one-third of summers the correlation is weak and EW values are isotopically enriched.

The tri-phase model prediction that LW $\delta^{13}C$ values should be consistently lower than those of the EW of the same ring is true in only 61% of rings in our study. The prediction that LW and the EW of the following year should not be correlated also conflicts with our results. It seems that at this coarser sampling resolution the tri-phase model, with EW consistently more enriched that LW, may not be as widely applicable as has been assumed.

It is unsurprising that oak EW $\delta^{13}C$ does not carry a climate signal from the current spring because, as a ring-porous species, EW growth in *Q. robur* is rapid and must begin in advance of budburst (Bréda and Granier 1996, Sass-Klaasen et al. 2011), because winter embolism of the large xylem cells requires the water flow pathway to be restored before the onset of transpiration (Essiamah and Eschrich 1986, Barbaroux and Bréda 2002, Michelot et al. 2012). In contrast to Kimak and Leuenberger (2015), who conclude that oak trees only rely on young reserves for EW formation when assimilation is low, our results clearly indicate that EW cellulose is formed preferentially from reserves accumulated in the previous growing season rather than from a well-mixed pool of older NSCs. The $\delta^{13}C$ values of EW are very strongly correlated with those of the preceding LW and carry an identical climate signal. Early-wood $\delta^{13}C$ values are, on average, slightly enriched, inter-annual variance is lower, and EW displays significant first-order autocorrelation, not seen in LW. These differences indicate that EW formation can, at need, access older isotopically-mixed reserves. The clear implication is that there are two pools of carbohydrate that can be drawn upon to support EW formation and that it is the younger of these pools that dominates, particularly under good growing conditions.

These results strongly support the proposal by Richardson et al. (2013) that carbon cycle models should include a 'two-pool model structure' that takes account of NSCs. Using the FöBAAR (FOrest Biomass, Allocation, Assimilation and Respiration) model (Keenan et al. 2012) they demonstrated that model structures

that assumed either no pool of NSC or a single pool were inconsistent with the amount and age of NSC measured in tree stems and with measured changes in woody biomass increment. The best model assumed that spring growth preferentially accesses a 'fast pool', dominated by NSCs produced in the previous season, which exchanges with a 'slow pool' of much older carbohydrates. The older pool includes both starch and sugars, so they do not divide the two pools on this basis, preferring a 'last in first out' model of NSC dynamics whereby the most recently added starch molecules are the first to be converted back to sugars (Lacointe et al. 1993). Recent work supports the two pools concept and confirms that although the young pool is more readily used, much older reserves can still be accessed at need (Carbone et al. 2013, Richardson et al. 2015). Although Richardson et al. (2013) did not measure carbon isotope ratios, their two-pool model would predict the strong correlation between EW and LW of the previous year. The much smaller contribution from the well-mixed older pool explains the muting of the climate signal, reduction in variance and autocorrelation in the EW.

Although EW $\delta^{13}C$ values are, on average, slightly enriched (0.25‰) relative to LW, this may not represent the use of isotopically enriched NSC stored as starch. Leaf starch is isotopically enriched relative to leaf sugars, but Gessler et al. (2014) note that 'there is no evidence for kinetic or equilibrium effects for the reactions that produce starch from sucrose in non-photosynthetic tissue'. Although Gessler et al. (2014) go on to identify a biochemical pathway that might explain some fractionation, no such explanation is required here. The small offset between LW and EW carbon isotope ratios can be explained very easily using the two-pool model by assuming that the older pool of NSC is built mainly from reserves accumulated during favourable growth years, which in Scotland equates to warm summers. Simply assuming that the coldest one-third of summers do not contribute carbon to the older pool, and all others

contribute a similar amount, would give a mean isotope value for the long-lived pool that is equal to the mean isotope value for all of the EW. There is no need to invoke any fractionation due to storage. This conflicts with the conclusions of Eglin et al. (2010), who could only model oak EW $\delta^{13}C$ by including a fractionation factor during starch synthesis.

Conclusions

Our results demonstrate that carbon isotope ratios of EW cellulose are very strongly correlated with the values obtained from the LW of the previous year. Late-wood $\delta^{13}C$ values are strongly correlated with summer temperature, probably via variations in sunshine and therefore photosynthetic rate, and we see identical climate signals shifted by 1 year in the EW, with no evidence of response to current spring climate. The generally accepted 'triphase' model of $\delta^{13}C$ variation in broad-leaf tree rings, which suggests that EW is enriched relative to LW, due to reliance on well-mixed reserves derived from isotopically enriched starch, is not supported by our results. On the contrary, the environmental signals imparted in the LW of one season are carried very strongly into the EW of the next.

We find that under the good growing conditions of most of the 20th century, oak trees have consistently relied upon reserves produced in the previous summer to form EW cellulose, with no discernible contribution from young leaves. Following warm summers there may be no contribution from older carbohydrates, but following cold summers these isotopically well-mixed reserves become much more important. The small average offset between LW and EW can be explained by the effect of summer temperature on isotopic ratios of NSCs that are stored for more than 1 year. They are biased towards higher than average LW values because there is more surplus material available in warmer years. There is no need to invoke any fractionations during conversions between sugar and starch in non-photosynthetic tissues.

The new approach proposed here provides a powerful and cost-effective way of exploring the response of mature trees, growing under natural conditions, to a wide range of past environmental changes and extreme events. Measuring the carbon isotope ratios in EW and LW does not provide a quantitative measure of the amount of carbon that is stored, so it is not an alternative to measuring and dating NSCs within living trees, but it greatly extends the number of trees that can be studied and particularly the length of time over which measurements can be derived. The method could be used to compare the recent behaviour of trees with their behaviour under similar climate conditions before the rapid rise in carbon dioxide and in post-mortem studies, to compare, for example, the behaviour of a cohort of dying trees with the behaviour of exactly the same set of trees when they were in good health.

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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