Communication

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Shining a New Light on the Classical Concepts of Carbon-Isotope Dendrochronology

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Abstract: Retrospective information about plant ecophysiology and the climate system are key inputs in Earth system and vegetation models. Dendrochronology provides such information with large spatiotemporal coverage, and carbon isotope analysis across tree-ring series is among the most advanced dendrochronological tools. For the past seventy years, this analysis was performed on whole molecules and, to this day, ^{13}C discrimination during carbon assimilation is invoked to explain isotope variation and associated climate signals. Recently, however, it was reported that tree-ring glucose exhibits multiple isotope signals at the intramolecular level (see companion paper). Here, I estimated the signals' contribution to whole-molecule isotope variation and found that downstream processes in leaf and stem metabolism each introduce more variation than carbon assimilation. Moreover, downstream processes introduce most of the climate information. These findings are inconsistent with the classical concepts/practices of carbon-isotope dendrochronology. More importantly, intramolecular tree-ring isotope analysis promises novel insights into forest metabolism and the climate of the past.

Keywords: dendrochronology; tree rings; carbon stable isotopes; whole-molecule isotope analysis; intramolecular isotope analysis; water-use efficiency; paleoclimate reconstruction; plant carbon fluxes

Introduction

Tree rings are natural archives containing encoded information about plant metabolic processes, their environmental dependences, and the climate of the past. This information is (to a large extent) inaccessible to manipulation and monitoring experiments, and dendrochronologists strive to decipher it to contribute to a better understanding of the climate system, plant functioning, and biogeochemical cycles. Stable carbon isotope (^{12}C, ^{13}C) analysis across tree-ring series is among the most advanced dendrochronological tools available today. This tool has (*inter alia*) been used to reconstruct leaf intrinsic water-use efficiency (CO_2 uptake relative to H_2O loss, *iWUE*), air temperature, solar radiation, relative humidity, precipitation, and drought over past centuries at numerous locations worldwide (Cernusak & Ubierna, 2022; Gagen *et al*., 2022).

Seventy years ago, tree-ring ^{13}C/^{12}C ratios were measured for the first time (Craig, 1953, 1954). While early studies analysed whole-wood samples, most recent studies analyse cellulose, a glucose polymer extracted from tree rings to preclude error due to variation in wood composition (Helle *et al*., 2022). Note, arguments given below apply to glucose and cellulose but not necessarily to wood. Tree-ring cellulose ^{13}C/^{12}C data are commonly expressed in terms of ^{13}C discrimination, Δ_{trc}, denoting carbon isotope changes caused by physiological processes (Farquhar & Richards, 1984). Current data interpretation invokes a simplified mechanistic model of ^{13}C discrimination accounting for two processes: CO_2 diffusion from ambient air into leaf intercellular air spaces and carbon assimilation by rubisco (Farquhar *et al.*, 1982; McCarroll & Loader, 2004; Cernusak & Ubierna, 2022), combinedly termed diffusion-rubisco (DR) discrimination (Wieloch *et al.*, 2018).

Variation in DR discrimination depends on the ratio of intercellular-to-ambient CO_2 concentration (Farquhar *et al.*, 1982; Evans *et al.*, 1986; Voelker *et al.*, 2016). Intercellular CO_2 concentration, in turn, varies with the rate of CO_2 supply through leaf stomata and the rate of CO_2 assimilatory demand. Since stomata respond to moisture conditions, Δ_{trc} correlations with humidity parameters are generally assumed to derive from CO_2-supply-side effects on DR discrimination (Gagen *et al.*, 2022). By contrast, CO_2 assimilation responds to temperature and solar radiation, and corresponding Δ_{trc} correlations are generally assumed to derive from CO_2-demand-side effects on DR discrimination (Gagen *et al.*, 2022). Moreover, there is a mechanistic relationship between DR discrimination and *iWUE* (Farquhar *et al.*, 1982; Farquhar & Richards, 1984) which forms the basis of *iWUE* reconstructions from Δ_{trc} (Cernusak & Ubierna, 2022; Saurer & Voelker, 2022). *Nota bene*, all current Δ_{trc} interpretations assume DR discrimination governs Δ_{trc} variation (Gagen *et al.*, 2022). Discrimination downstream of rubisco, denoted post-rubisco (PR) discrimination (Wieloch *et al.*, 2018), is considered constant for any given species (Gessler *et al.*, 2014; Cernusak & Ubierna, 2022).

Recently, nuclear magnetic resonance spectroscopy was used (for the first time in dendrochronology) to measure intramolecular ^{13}C discrimination, Δ_i', in glucose extracted across a series of tree rings from *Pinus nigra* Arnold (*i* denotes glucose carbon position C-1 to C-6; Supporting Information Notes S1) (Wieloch *et al.*, 2018). Data of Δ_1', Δ_2', and Δ_3' pertaining to 1961 to 1980 (early period) and 1983 to 1995 (late period) were analysed separately since these series exhibit a change point in 1980 (Wieloch *et al.*, 2024). Proposedly, the trees had access to groundwater during the early but not the late period (Wieloch *et al.*, 2022a) causing metabolism affecting Δ_1' to Δ_3' to move from a homeostatic to a climate-responsive state (Wieloch *et al.*, 2024). By contrast, no change point was detected in Δ_4', Δ_5', and Δ_6'. Based (*inter alia*) on multiple regression modelling, the dataset contains several ^{13}C signals (Tables 1 and S1, Figure S1). First, vapour pressure deficit (*VPD*) affects both Δ_1' and Δ_3' during the late period (Wieloch *et al.*, 2024). This relationship is thought to derive from DR discrimination. Additional leaf-level ^{13}C discrimination by phosphoglucose isomerase and/or glucose-6-phosphate dehydrogenase is thought to account for the stronger effect of *VPD* on Δ_1' compared to Δ_3'. Second, during the late period, Δ_1' and Δ_2' are related to ε_{met} denoting hydrogen isotope fractionation by metabolic processes at glucose H^1 and H^2, and ε_{met} can be substituted by precipitation (*PRE*) without losing much of the models' explanatory power (Wieloch *et al.*, 2022a, 2024). These relationships are thought to derive from ^{13}C discrimination by phosphoglucose isomerase and glucose-6-phosphate dehydrogenase in tree stems (Wieloch *et al.*, 2024). Note, the described Δ_1' to Δ_3' models do not work for the early period (Wieloch *et al.*, 2024). Third, global radiation (*RAD*) and temperature (*TMP*) affect Δ_4' to Δ_6' over the entire study period (Wieloch *et al.*, 2024). These relationships are thought to derive from leaf-level ^{13}C discrimination by glyceraldehyde-3-phosphate dehydrogenases affecting Δ_4' and enzymes modifying the carbon-carbon double bond of phospho*enol*pyruvate affecting Δ_5' and Δ_6' (Wieloch *et al.*, 2021, 2022b).

Table 1. Isotope-environment signals in Δ_i' and their proposed enzymatic origins (underlying Δ_i' models shown in Table S1; signal origins shown in Figure S1).

			Proposed origin of introduction		**Discrimination type**
Covariate	**Relationship**	**Period**	**Tissue**	**Enzyme**	
$\Delta_1' \sim \varepsilon_{met}^{(a)}$	negative	83 - 95	Stem	PGI, G6PD	**PR**
$\Delta_1' \sim VPD$	negative	83 - 95	Leaf	Rubisco$^{(b)}$, PGI, G6PD	**DR & PR**
$\Delta_2' \sim \varepsilon_{met}^{(a)}$	negative	83 - 95	Stem	PGI	**PR**
$\Delta_3' \sim VPD$	negative	83 - 95	Leaf	Rubisco$^{(b)}$	**DR**
$\Delta_4' \sim RAD$	negative	64 - 95	Leaf	p-GAPDH, np-GAPDH	**PR**
$\Delta_4' \sim TMP$	positive	64 - 95	Leaf		

$\Delta_{5\text{-}6}'$ ~ *RAD*	negative	64 - 95	Leaf	PEPC, PK, DAHPS, Enolase	**PR**
$\Delta_{5\text{-}6}'$ ~ *TMP*	positive	64 - 95	Leaf		

ε_{met}, Δ_i', and $\Delta_{5\text{-}6}'$ denote hydrogen isotope fractionation by metabolic processes at glucose H^1 and H^2, carbon isotope discrimination at glucose carbon position, *i*, and the arithmetic average of Δ_5' and Δ_6', respectively. DR and PR refer to diffusion-rubisco and post-rubisco discrimination, respectively. Glucose was extracted across an annually resolved tree-ring series of *Pinus nigra* from the Vienna Basin. Climate data series: *RAD*, April to September global radiation (data available from 1964); *TMP*, March to October air temperature; *VPD*, March to November air vapour pressure deficit. Enzymes: DAHPS, 3-Deoxy-D-*arabino*-heptulosonate-7-phosphate synthase; G6PD, glucose-6-phosphate dehydrogenase; np- and p-GAPDH, non-phosphorylating and phosphorylating glyceraldehyde-3-phosphate dehydrogenase; PEPC, phospho*enol*pyruvate carboxylase; PGI, phosphoglucose isomerase; PK, pyruvate kinase. [(a)] replacing ε_{met} by March to July precipitation results in models with only slightly reduced explanatory power [(b)] ^{13}C discrimination during CO_2 diffusion and assimilation by rubisco is introduced into carbon metabolism at rubisco

Here, the relative contributions of these intramolecular ^{13}C signals to whole-glucose ^{13}C discrimination (Δ_{glu}) were estimated by variance component analysis (Notes S2). Since glucose extracted from tree rings largely derives from cellulose, the results can be expected to also apply to tree-ring cellulose (Δ_{trc}). They are used for a critical assessment of the classical concepts and practices of carbon-isotope dendrochronology. Subsequently, the potential value of intramolecular ^{13}C analysis for constraining impacts of tropospheric ozone on forest metabolism and productivity is discussed. Lastly, it is tested whether intramolecular ^{13}C signals can also be extracted from whole-molecule (Δ_{glu}) data.

Components of Δ_{glu} Variation and Implications for Reconstructions of Leaf Intrinsic Water-Use Efficiency

Leaf *iWUE* is regarded as an important functional property of plant ecosystems and a key determinant in the response of biogeochemical cycles to climate change (Beer *et al.*, 2009). Retrospective assessment of *iWUE* relies on Δ_{trc} analysis which, in turn, relies on the assumption that DR discrimination governs Δ_{trc} variability (Ma *et al.*, 2021; Cernusak & Ubierna, 2022; Saurer & Voelker, 2022). Here, this assumption is critically examined.

-Figure 1A shows percent contributions of intramolecular isotope signals found by modelling and model residuals to Δ_{glu} variation for the more dynamic late period (Δ_{glu} variance = 1.47‰) (Wieloch *et al.*, 2024). Leaf ^{13}C discrimination accounts for *c.* 43.5% of the total Δ_{glu} variance while stem ^{13}C discrimination (related to ε_{met}) accounts for *c.* 19.5%. The rest is residual variance (Notes S3). PR discrimination at the leaf- and stem-level (*c.* 25.9% and 19.5%, respectively) each exceed the contribution of DR discrimination (*c.* 8.8%×2 = 17.6%).

-Similarly, Figure 1B shows percent contributions of intramolecular isotope signals found by modelling and model residuals to Δ_{glu} variation for the less dynamic early period (Δ_{glu} variance = 0.36‰). Evidently, the contribution of Δ_1' to Δ_{glu} is negligible. Moreover, measurement error can account for the entire variation in Δ_2' (Notes S3). Hence, Δ_1' and Δ_2' are not considered further. However, *c.* 50% of the total Δ_3' variance may be systematic unmodelled variance (Notes S3). If we assume this variation results from DR discrimination, then DR discrimination accounts for *c.* 7.5% of the total Δ_{glu} variance (*c.* 0.5×15) while PR discrimination accounts for *c.* 43.6%.

Hence, during both periods, DR discrimination is a comparably small contribution to total Δ_{glu} variation which argues against using Δ_{trc} for reconstructions of interannual *iWUE* variation. Since the *iWUE* signal is better resolved at the intramolecular level, Δ_i' analysis is expected to yield better estimates of *iWUE*.

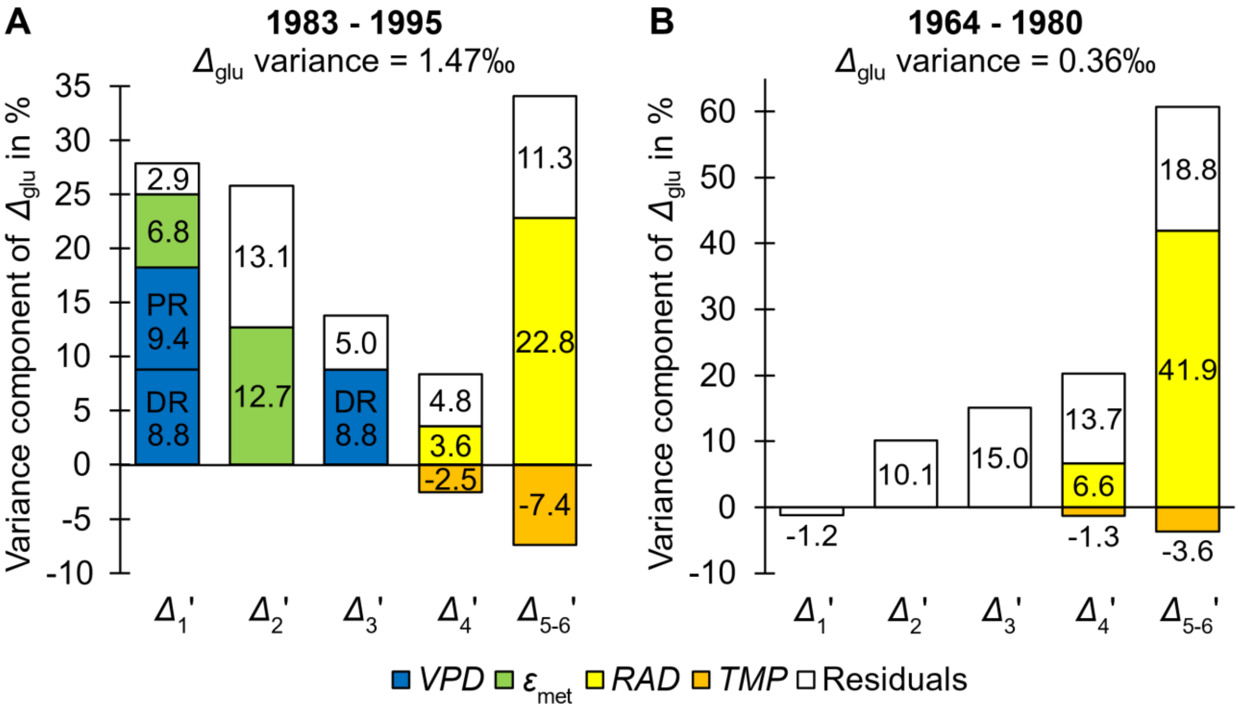

Figure 1. Percent contributions of intramolecular carbon isotope signals and model residuals to Δ_{glu} variation for the late **(A)** and early **(B)** period. According to current interpretation, the *VPD* signal goes back to both diffusion-rubisco (DR) and post-rubisco (PR) discrimination (blue bars). All other signals go back to post-rubisco discrimination (green, yellow, and orange bars). Model residuals are shown as white bars.

Physiological Interpretation of Climate Signals in Δ_{trc}

Currently, all reported Δ_{trc}-climate relationships are interpreted with respect to DR discrimination (Battipaglia & Cherubini, 2022; Churakova *et al.*, 2022; Gagen *et al.*, 2022; van der Sleen *et al.*, 2022). Thus, consideration is given only to two initial steps in the biosynthesis of tree-ring cellulose whereas ^{13}C discrimination by the numerous reactions downstream of rubisco (PR discrimination) is assumed to be constant (Figure S1). However, recent reports of multiple intramolecular isotope signals in tree-ring glucose (Table 1) call for a critical reassessment of this practice.

At the site discussed here, DR discrimination responds to *VPD* (for information about the site, see Notes S1 in Wieloch *et al.*, 2024). However, while DR discrimination accounts for *c.* 17.6% of the total variation of Δ_{glu} during the late period, *VPD*-dependent PR discrimination accounts for an additional *c.* 9.4% (Figure 1A). Hence, both DR and PR discrimination contribute to the *VPD* signal in Δ_{glu} and their combined contribution accounts for *c.* 27% of the total Δ_{glu} variance. Interestingly, simple linear regression between Δ_{glu} and *VPD* falsely suggests that *VPD* accounts for *c.* 54% of the total Δ_{glu} variance (Figure S2). This twofold overestimation of the actual *VPD* signal likely results from intercorrelation of *VPD* with other climate parameters that also affect Δ_{glu}. For instance, *RAD* affects tree-ring glucose C-5 and C-6 (Figure 1A), and there is intercorrelation between *RAD* and *VPD* ($r = 0.6$, $p < 0.05$, $n = 13$) which will result in overestimation of the *VPD* signal in *VPD*-Δ_{glu} simple linear regression.

More importantly, relationships of Δ_{glu} with *RAD* and *TMP* derive from leaf-level PR discrimination (Wieloch *et al.*, 2021, 2022b, 2024), and *RAD*-dependent PR discrimination alone exceeds the contribution of DR discrimination to Δ_{glu} variation (Figure 1, early period: *c.* 48.5% versus *c.* 7.5%, late period: *c.* 26.4% versus *c.* 17.6%). Similarly, relationships of Δ_{glu} with ε_{met} and *PRE* derive from stem-level PR discrimination (Wieloch *et al.*, 2024), and ε_{met}-dependent PR discrimination contributes similarly to Δ_{glu} variation as DR discrimination (Figure 1A; *c.* 19.5% and 17.6%, respectively).

Hence, *RAD*-, *TMP*-, *PRE*-, and a fraction of the *VPD*-dependent Δ_{glu} variation is not caused by DR discrimination and associated physiological processes. Instead, most of the climate information in Δ_{glu} derives from PR discrimination and associated physiological processes.

New Information from Old Archives—The Impact of Tropospheric Ozone on Forest Metabolism

As shown recently, tree-ring glucose carries numerous carbon (and hydrogen) isotope signals (Wieloch *et al.*, 2018, 2022a), and there is considerable interest as to their scientific value in plant ecophysiology and biogeochemistry. For instance, the *RAD*-dependent carbon isotope signal at tree-ring glucose C-5 and C-6 (Table 1) is thought to originate from ozone-induced metabolic adjustments (Wieloch *et al.*, 2022b). *RAD* promotes the photochemical formation of tropospheric ozone (Ainsworth *et al.*, 2012) which causes downregulation of rubisco and upregulation of PEPC (Saurer *et al.*, 1995; Dizengremel, 2001). Additionally, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase is expressed (Janzik *et al.*, 2005; Betz *et al.*, 2009). These biochemical adjustments can be expected to result in increased relative carbon flux into mitochondrial metabolism and the shikimate pathway (Figure S1; Dizengremel, 2001). Hence, the isotope signal at C-5 and C-6 can potentially be used to reconstruct tropospheric ozone concentration, and ozone effects on forest metabolism and productivity.

In 2100, ozone is predicted to cause forest productivity losses of 17% relative to preindustrial air which would have severe adverse effects on global carbon cycling and climate change (Wittig *et al.*, 2009). This estimate, however, relies strongly on short-term experiments on tree seedlings and saplings and may therefore not apply to mature natural forests (Emberson, 2020). The tree-ring isotope signal at glucose C-5 and C-6, on the other hand, can potentially be used to constrain ozone effects on mature natural forests across space and time. Similarly, other intramolecular carbon and hydrogen isotope signals detected in tree-ring glucose may help to advance our knowledge about other aspects of forest metabolism (Wieloch *et al.*, 2024).

Mining Whole-Molecule Data for Information Seen at the Intramolecular Level

Over the past decades, dendrochronologists have collected a wealth of (whole-molecule) Δ_{trc} data covering various forest biomes worldwide (e.g., Battipaglia & Cherubini, 2022; Churakova *et al.*, 2022; van der Sleen *et al.*, 2022). These data (*per se*) contain the same valuable information as (intramolecular) Δ_i' data. However, since Δ_{trc} has 6-fold lower resolution than Δ_i', clearcut extraction of Δ_i'-environment signals from Δ_{trc} data may not be feasible.

To test this, Δ_{glu} data of both study periods were modelled as function of all covariates known to significantly affect Δ_i' (*cf.* Tables 1 and S1). It was found that, during the late period, Δ_{glu} is significantly related with ε_{met} and *RAD* (Table 2, $p \leq 0.01$, $n = 13$), and close to significantly related with *VPD* and *TMP* ($p \leq 0.15$). By increasing the number of observations, all relationships might become significant. Moreover, the slope estimates of the Δ_{glu} model are not significantly different from those of the Δ_i' models (Figure S3). During the early period, Δ_{glu} is significantly related with *RAD* (Table 2, $p \leq 0.005$, $n = 15$) but not *TMP* ($p = 0.39$). Still, the slope estimates of the Δ_{glu} model are not significantly different from those of the Δ_i' model (Figure S4). Lastly, the change point separating the two study periods is detectable at both the intramolecular ($\Delta_{1\text{-}3}'$) and whole-molecule (Δ_{glu}) level (Wieloch *et al.*, 2024).

Table 2. Multiple linear regression models of Δ_{glu} as function of ε_{met}, *VPD*, *RAD*, and *TMP*.

$\Delta_{glu} \sim \varepsilon_{met} + VPD + RAD + TMP$, 1983-1995			
$R^2 = 0.86$, $adjR^2 = 0.79$, $p < 0.002$, $n = 13$			
	Estimate	**±SE**	**$p \leq$**
Intercept	25.0	5.2	0.001

ε_{met}	-0.0142	0.0042	0.01
VPD	-0.00753	0.00475	0.15
RAD	-0.00475	0.00142	0.01
TMP	0.686	0.411	0.13
Δ_{glu} ~ *RAD* + *TMP*, 1964-1980			
$R^2 = 0.5$, $adjR^2 = 0.42$, $p = 0.015$, $n = 15$			
	Estimate	**±SE**	**$p \leq$**
Intercept	21.2	3.8	0.0001
RAD	-0.00350	0.00102	0.005
TMP	0.242	0.269	0.39

Δ_{glu} and ε_{met} denote whole-molecule ^{13}C discrimination of glucose and average hydrogen isotope fractionation caused by metabolic processes at glucose H^1 and H^2, respectively. Glucose was extracted across an annually resolved tree-ring series of *Pinus nigra* from the Vienna Basin. Climate data series: *RAD*, April to September global radiation; *TMP*, March to October air temperature; *VPD*, March to November air vapour pressure deficit.

Taken together, in the present case, most of the isotope-environment signals evident in Δ_i' can also be extracted from Δ_{glu}. Hence, reanalyses of existing Δ_{trc} datasets based on recent insights into plant isotope fractionation may yield both more accurate estimates of ecophysiological properties linked to DR discrimination (such as *iWUE*) and novel information about ecophysiological properties linked to PR discrimination (such as metabolic responses to ozone). That said, in Δ_{trc} analysis, the intramolecular location of any isotope-environment signal will always remain unknown which adds a level of uncertainty regarding the signal's metabolic origin and process specificity.

Conclusions and Outlook

The picture emerging here is inconsistent with the classical (DR-discrimination-centred) concepts and practices of carbon-isotope dendrochronology. Evidently, processes downstream of rubisco in leaves and stems introduced most of the isotope signals and variation in the tree-ring series examined. Hence, most of the ecophysiological and climate information in this record relates to PR processes. This opens new and exciting research avenues. First, the isotope signal reflecting *iWUE* is better resolved at the intramolecular than at the whole-molecule level. Careful separation of this signal from other signals in Δ_i' or Δ_{trc} is expected to yield more accurate estimates of *iWUE*. Second, an isotope signal at tree-ring glucose C-5 and C-6 reports metabolic changes in response to tropospheric ozone. Ozone is known for its severe adverse effects on forest productivity, global carbon cycling, and climate change. Analysing the signal at C-5 and C-6 may help to constrain these effects in natural forest ecosystems. Third, Δ_i' analysis gives access to deconvoluted information about multiple climate parameters and is therefore expected to enable distinctly more comprehensive paleoclimate reconstructions than Δ_{trc} analysis, providing an improved baseline for climate predictions (Wieloch *et al.*, 2024). Fourth, recent and future insights into plant ^{13}C discrimination from Δ_i' analysis may enable extraction of information about multiple ecophysiological processes from existing Δ_{trc} datasets.

Taken together, Δ_i' analysis has significant disruptive potentials regarding the scientific development of the field of carbon-isotope dendrochronology. Unfortunately, measuring Δ_i' by nuclear magnetic resonance spectroscopy is labour-intensive and requires technology and know-how inaccessible to most dendrochronological laboratories. However, protocols enabling Δ_i' measurements by Orbitrap mass spectrometry are currently under development and may soon make Δ_i' data broadly accessible (Neubauer *et al.*, 2023; Dion-Kirschner *et al.*, 2023; Gessler *et al.*, 2024). Moving from whole-molecule to intramolecular tree-ring isotope analysis is comparable to using a more powerful microscope and promises novel information about metabolism and climate across space and time (Wieloch *et al.*, 2018, 2021, 2022b,a, 2024; Gessler *et al.*, 2024).

Data Availability: The author declares that the data supporting the findings of this study are available within the paper and its supporting information.

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