Temperature-sensitive biochemical $^{18}$O-fractionation and humidity-dependent attenuation factor are needed to predict $\delta^{18}$O of cellulose from leaf water in a grassland ecosystem

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**Introduction**

The oxygen isotope composition of plant cellulose ($\delta^{18}$O$_{\text{cellulose}}$) and its enrichment above source water ($\Delta^{18}$O$_{\text{cellulose}}$) are thought to record environmental and physiological information of great interest to a range of scientific disciplines, including functional plant ecology and climate change biology (e.g. Barbour, 2007; Battipaglia et al., 2013; Gessler et al., 2014). In particular, $\delta^{18}$O$_{\text{cellulose}}$ or even more so $\Delta^{18}$O$_{\text{cellulose}}$ has been discussed as an integrated proxy of past stomatal conductance (e.g. Farquhar et al., 1998; Scheidegger et al., 2000; Barbour et al., 2000b) that can provide information about environmental or climate change effects on the processes regulating the water-use efficiency of $C_3$ plants. However, empirical evidence for a direct link between $\Delta^{18}$O$_{\text{cellulose}}$ and stomatal conductance is currently incomplete, which prevents the use of $\delta^{18}$O$_{\text{cellulose}}$ time series to interpret changes in plant water use efficiency. Fundamentally, all of the oxygen in cellulose is derived from water (DeNiro & Epstein, 1979; Liu et al., 2016). Oxygen exchange with water can occur in multiple steps in the photosynthetic carbon cycle up to the formation of sucrose (or other transport sugars), and during metabolism of these sugars in sink tissues, when cellulose is being formed (Hill et al., 1995; Farquhar et al., 1998; Barbour et al., 2005). Therefore, $\delta^{18}$O$_{\text{cellulose}}$ depends on the oxygen isotope composition of water in sink tissues, often approximated by the $\delta^{18}$O of plant source water taken up by the roots ($\delta^{18}$O$_{\text{source}}$), and on the $\delta^{18}$O of leaf lamina water ($\delta^{18}$O$_{\text{leaf}}$), respectively. During photosynthesis, leaf transpiration enriches isotopically leaf lamina water above source water ($\Delta^{18}$O$_{\text{leaf}}$ = $\delta^{18}$O$_{\text{leaf}}$ – $\delta^{18}$O$_{\text{source}}$). $\delta^{18}$O$_{\text{source}}$ can vary dynamically and is primarily controlled by the $\delta^{18}$O of meteoric inputs ($\delta^{18}$O$_{\text{rain}}$; Dansgaard, 1964; Bowen & Wilkinson, 2002), their mixing with soil water and the intensity of soil evaporation (Barnes & Allison, 1988). In addition, $\delta^{18}$O$_{\text{source}}$ is determined by the depth distribution of
roots and their specific uptake intensities as well as by the effect of transpiration and root water uptake on soil water emptying and refilling dynamics (Brinkmann et al., 2018; Hirl et al., 2019).

Arguably, the oxygen isotope enrichment of cellulose above source water (\(\Delta^{18}O_{\text{cellulose}} \approx \delta^{18}O_{\text{cellulose}} - \delta^{18}O_{\text{source}}\)) has the most important physiological interest because of its link with \(\Delta^{18}O_{\text{leaf}}\) and stomatal conductance. According to the Barbour-Farquhar model (Barbour & Farquhar, 2000),

\[
\Delta^{18}O_{\text{cellulose}} = \Delta^{18}O_{\text{leaf}} (1 - p_c p_s) + \epsilon_{\text{bio}}.
\]

Eqn 1

The product \(p_c p_s\) reflects the proportion of oxygen in cellulose derived from source water, where \(p_c\) is the proportion of exchangeable oxygen in the intermediates formed during cellulose synthesis from sucrose exported from leaves, and \(p_s\) is the proportion of source water at the site of cellulose formation. In other words, \(1 - p_c p_s\) represents the proportion of sucrose oxygen in isotopic equilibrium with \(\delta^{18}O_{\text{cellulose}}\) on the source water at the site of cellulose formation. \(\epsilon_{\text{bio}}\) is the average biochemical fractionation between the organic substrate for cellulose synthesis and water (Sternberg et al., 1986; Barbour, 2007).

Equation 1 relies on the assumption that \(\Delta^{18}O_{\text{leaf}}\) represents the \(18\)O-enrichment of water in isotopic equilibrium with leaf sucrose, as supported by two laboratory studies on castor bean (Barbour et al., 2000a; Cernusak et al., 2003). In those studies, conducted in climate-controlled, steady-state conditions, the \(18\)O-enrichment of sucrose above source water (\(\Delta^{18}O_{\text{sucrose}}\)) was well approximated by \(\Delta^{18}O_{\text{leaf}} + \epsilon_{\text{bio}}\) with \(\epsilon_{\text{bio}}\) set to 27.9%. However, a recent study by Lehmann et al. (2017) on two C3 grass species indicated that newly formed sucrose might not always be in isotopic equilibrium with average leaf lamina water. Moreover, in submerged aquatic plants (where \(\Delta^{18}O_{\text{leaf}} = \text{c. 0}\) \(\epsilon_{\text{bio}}\) was found to be inversely related to growth temperature, with a particularly strong temperature dependence below about 20°C (Sternberg & Ellsworth, 2011). Virtually the same temperature-sensitivity of \(\epsilon_{\text{bio}}\) was found in wheat seedlings during heterotrophic growth (Sternberg & Ellsworth, 2011). Although there is a likelihood that this result applies to autotrophic terrestrial plants, it is difficult to prove because it cannot be verified, as \(p_c p_s\) and \(\epsilon_{\text{bio}}\) cannot be measured simultaneously.

Probably, the least contentious parameter in Eqn 1 is \(p_s\), at least in nontranspiring tissue such as the leaf-growth-and-differentiation zones of grasses or the developing cells of tree trunks, where \(p_s\) has been shown to stay close to 1 (Cernusak et al., 2005; Liu et al., 2017a). Sucrose is the most common carbohydrate transported within plants (Lalonde et al., 2003) and the main substrate from which UDP-glucose, the immediate precursor of cellulose synthesis, is formed in heterotrophic tissue (Verbancic et al., 2018), such as the leaf growth zone of grasses (Baca Cabrera et al., 2020). Based on theoretical considerations of oxygen exchange between the metabolites of sucrose and water during cellulose formation and on observational data, the value of \(p_s\) is often assumed to be around 0.4 (Sternberg et al., 1986; Roden & Ehleringer, 1999; Barbour & Farquhar, 2000; Cernusak et al., 2005), but considerable variation in \(p_s\) has been suggested from other observational studies (Gessler et al., 2009; Song et al., 2014; Cheesman & Cernusak, 2017). As it cannot be measured in vivo, estimates of \(p_s\) must be obtained as a fitted parameter, whilst assuming values for \(p_c\) and \(\epsilon_{\text{bio}}\). Such estimations are sensitive to errors, including any effect of isotopic disequilibrium between \(\Delta^{18}O_{\text{sucrose}}\) and \(\Delta^{18}O_{\text{leaf}}\) (Lehmann et al., 2017). Higher isotopic disequilibria between leaf lamina water and sucrose have also been found when air relative humidity (RH) was lower (Lehmann et al., 2017). When interpreted with Eqn 1, this latter result suggests that \(p_c p_s\) may increase with increasing RH. A positive relationship between RH and \(p_c p_s\) was also suggested by the relationship between \(\Delta^{18}O_{\text{cellulose}}\) and \(\Delta^{18}O_{\text{leaf}}\) in the studies of Liu et al. (2016) and Helliker & Ehleringer (2002a) for a range of C3 and C4 grasses, perhaps pointing to isotopic disequilibria between \(\Delta^{18}O_{\text{sucrose}}\) and \(\Delta^{18}O_{\text{leaf}}\) also in these cases. Clearly, there remain important knowledge gaps on the effect of environmental conditions on \(\epsilon_{\text{bio}}\) and \(p_c p_s\) and, hence, their implication for physiological interpretation of \(\delta^{18}O_{\text{cellulose}}\) or \(\Delta^{18}O_{\text{cellulose}}\) from field studies.

The temporal integration of all processes involved in the making of leaf cellulose (i.e. assimilation, mobilization of stored substrate, allocation of substrate to growth, and cellulose synthesis) represents an additional challenge when interpreting \(\delta^{18}O_{\text{cellulose}}\) or \(\Delta^{18}O_{\text{cellulose}}\) (Hemming et al., 2001; Damesin & Lefarge, 2003; Gessler et al., 2009, 2014; Royles et al., 2013; Liu et al., 2017b). In intensively managed grassland, shoot biomass is mostly vegetative and consists of short-lived leaves (Lemaire et al., 2000). Leaf production during the growing season is continuous, with new leaf production occurring simultaneously with the senescence of older leaves (Schleip et al., 2013). Accordingly, the mean live leaf age (measured in growing-degree-days (GDD)) changes relatively little during the course of the vegetation period (Lemaire et al., 2000; Schleip et al., 2013). In a controlled environment study with Cleistogenes squarrosa (a perennial C4 grass), Liu et al. (2017b) found a close linear relationship between the fraction of remaining leaf elongation and the fraction of oxygen in cellulose assimilated following a change of RH in the growth environment, as inferred from \(^{18}O\)-abundance measurements. Similar results were previously obtained by Helliker & Ehleringer (2002b), highlighting the potential of grass leaves as recorders of environmental signals in \(\delta^{18}O_{\text{cellulose}}\). In light of this, allocation-and-growth models, coupled with isotope-enabled process-based models of soil–vegetation–atmosphere CO2 and H2O exchange, may be very useful tools for analysing the mechanisms and dynamics controlling \(\Delta^{18}O_{\text{cellulose}}\) and \(\delta^{18}O_{\text{cellulose}}\) in natural environments. Such models have already been applied to tree-rings (Roden et al., 2000; Barbour et al., 2002; Ogée et al., 2009; Keel et al., 2016; Lavergne et al., 2017), but are presently unavailable for grassland.

The present study explores the effects of environmental drivers on the parameters of the Barbour & Farquhar (2000) model (Eqn 1), used to predict \(\Delta^{18}O_{\text{cellulose}}\) and \(\delta^{18}O_{\text{cellulose}}\) of leaves produced during the growing seasons of multiple years (2007-2012) in a drought-prone pasture ecosystem. Specifically, we asked the following questions: What are the effects of environmental parameters (mainly RH, temperature and soil moisture
avAILability) on $\delta^{18}O_{\text{cellulose}}$ and $\delta^{18}O_{\text{cellulose}}$? Do environmentally driven adjustments of $p_{\text{es}}$ or $p_{\text{eb}}$ (temperature) improve predictions of observed $\Delta^{18}O_{\text{cellulose}}$ and $\delta^{18}O_{\text{cellulose}}$? Is canopy conductance reflected in $\Delta^{18}O_{\text{cellulose}}$ or $\delta^{18}O_{\text{cellulose}}$? To that end, we developed a new allocation-and-growth model suitable for grassland ecosystems (Fig. 1) that we incorporated into the $^{18}$O-enabled soil–vegetation–atmosphere transfer model MuSICA. Recently, we have parameterized MuSICA for the studied pasture ecosystem and used that model to explore and predict $\delta^{18}O_{\text{source}}$ and $\delta^{18}O_{\text{leaf}}$ in that system (Hirl et al., 2019). Here, we first validated the combined MuSICA and allocation-and-growth model by testing its ability to predict the labelling kinetics of autotrophic respiration observed by Gamnitzer et al. (2009), observed root:shoot C allocation (Schleip, 2013), and the observed $\delta^{18}O_{\text{cellulose}}$ of leaves at the study site, using integration times consistent with leaf growth dynamics observations. We then applied the model to explore the three questions developed above.

**Materials and Methods**

**Experimental site and sampling**

The study was conducted inside pasture paddock no. 8 of Grünschwaige Grassland Research Station near Freising, Germany (for details on site, vegetation and grazing management see Schnyder et al., 2006). Average air temperature in the study years 2007–2012 was 9.3°C, and mean annual precipitation was 753 mm (recorded at the Munich airport meteorological station). The mineral topsoil has a low water-holding capacity (66 mm plant-available field capacity) causing frequent and prolonged drought periods (Hirl et al., 2019). The pasture was continuously grazed by Limousin suckler cows during the growing seasons (from mid-April to beginning of November). Animal stocking density was adjusted periodically to balance grass production and consumption by the cattle (Lemaire et al., 2009), so that sward height was maintained at about 7 cm.

Two replicate leaf samples were collected at around midday (between 11:00 and 16:00 h Central European Summer Time) at approximately fortnightly intervals during the vegetation periods 2007–2012 (Hirl et al., 2019). Each sample consisted of a mixed-species collection of the codominant species: four C$_3$ grasses (Lolium perenne L., Poa pratensis L., Phleum pratense L., Dactylis glomerata L.), one rosette dicot (Taraxacum officinale F.H. Wigg.) and one legume (Trifolium repens L.). The sample included only the green, nonsenescing, fully expanded leaf blades, as well as the exposed part of the growing leaf (cf. Fig. 1 of Liu et al., 2017a) from 16 vegetative tillers of L. perenne, P. pratensis and P. pratense and two vegetative tillers of D. glomerata, as well as one half of a leaf blade of T. officinalis (with the midvein removed) and two trifoliate leaves of T. repens. Water in these leaf samples, along with source, soil, atmospheric humidity and rainwater samples had previously been analysed for $\delta^{18}$O, and presented and discussed in Hirl et al. (2019).

**Fig. 1** Scheme of the allocation-and-growth model for predicting carbon fluxes and cellulose isotope compositions in grassland. Photosynthetic assimilation ($F_{\text{assim}}$) supplies substrate with specific isotopic composition ($R_{\text{assim}}$) to a well-mixed metabolic pool ($W_{\text{pool}}$ with isotope ratio $R_{\text{pool}}$). Maintenance respiration ($F_{\text{maint,resp}}$) and allocation of substrate to growth ($F_{\text{growth}}$) are both effected from the metabolic pool and thus carry the isotope signal of the pool ($R_{\text{pool}}$). Partitioning between shoot and root growth ($F_{\text{shoot,resp}}$ and $F_{\text{root,resp}}$) is governed by xylem water potential ($\Psi_{\text{xylem}}$). The shoot and root structural biomass pools are represented as layered (or stacked, nonmixing) pools. The integration time (d) represents the maximum age of structural leaf biomass in a sample. The size (or ‘thickness’) and isotopic composition of a given daily layer (or stack) in the shoot or root is determined by shoot and root growth rates and by the isotope ratio of the pool on that day (for details see Materials and Methods section and Supporting Information Methods S2).
Cellulose extraction and isotopic analysis

Following the procedure of Brendel et al. (2000) as modified by Gaudinski et al. (2005), α-cellulose was extracted from a subsample (50 or 25 mg) of ground plant material (for details see Liu et al., 2017b). After redrying of the cellulose at 80°C for 24 h, 0.7 mg aliquots were weighed into silver cups (size: 3.3 × 5 mm, IVA Analysentechnik e.K., Meerbusch, Germany) and stored above Silica Gel orange (2–5 mm, ThoMar OHG, Lütau, Germany) in exsiccator vessels. Samples were pyrolysed at 1400°C in a pyrolysis oven (HTO, HEKAttech, Wegberg, Germany), equipped with a helium-flushed zero blank autosampler (Costech Analytical technologies, Valencia, CA, USA), interfaced (ConFlo III, Finnigan MAT, Bremen, Germany) to a continuous-flow isotope ratio mass spectrometer (Delta Plus, Finnigan MAT). A solid internal laboratory standard (cotton powder) was included after every third or fourth sample and used for V-SMOW scaling and instrument drift correction. Every sample was analysed in duplicate. All samples and the laboratory standard were measured against a laboratory working reference carbon monoxide gas, which had previously been calibrated against a secondary isotope standard (IAEA-601; accuracy of calibration ± 0.25‰ SD). The precision for the laboratory standard was < 0.3‰ (SD for repeated measurements). Oxygen isotope composition is expressed in per mil (‰) as δ18O = (Rsample/Rstandard − 1), with Rsample the 18O : 16O ratio of the sample and Rstandard that in the V-SMOW standard.

Model

The integral model was composed of the 18O-enabled soil–vegetation–atmosphere transfer model MuSICA (Ogée et al., 2003, 2009; Wingate et al., 2010; Gangi et al., 2015), parameterized as in Hirl et al. (2019), and a new allocation-and-growth model (Fig. 1). MuSICA is a multilayer multileaf model that simulates CO2, water and energy redistribution and isotopic exchange processes in an ecosystem based on current mechanistic understanding (see Supporting Information Methods S1). MuSICA requires half-hourly climate data at a reference level above the vegetation (0.5 m here) as well as the isotopic composition of rainfall, CO2 and water vapour at the same reference level. Meteorological variables were obtained from the meteorological station at Munich airport at about 3 km from the study site (wind speed, precipitation, air temperature, RH, air pressure), from two other meteorological stations at 10 and 12 km distance (radiation), and from an eddy flux station installed at the experimental site (CO2 concentration) (Hirl et al., 2019). For the precipitation and water vapour isotopic input data (δ18Orain and δ18Ovapour), we used data collected at the site whenever available, and gap-filled the data with offset-corrected IsoGSM data (Yoshimura et al., 2011) as detailed in Hirl et al. (2019). δ18O and δ13C data of atmospheric CO2 (δ18OCO2 and δ13CCO2) were obtained from NOAA/CMDL latitudinal products (J. Miller, pers. comm.). MuSICA parameterization included soil and vegetation properties that described the pasture system in terms of structural and hydrological characteristics, leaf gas exchange, and root distribution and hydraulics. MuSICA, in its ‘standard parameterization’ (Hirl et al., 2019), performed well in predicting δ18Oleaf, δ18Osource (termed δ18Osource in Hirl et al., 2019) and δ18Osoil at different soil depths throughout seven growing seasons (2006–2012). The model also predicted the dynamics of transpiration, canopy conductance (gcanopy), root water uptake and plant-available soil water (PAW) (Hirl et al., 2019). MuSICA parameters and parameters of the allocation-and-growth model were based on singular measurements (e.g. parameters of the soil water retention curve) or on measurements performed at intervals during the study period (e.g. canopy height, leaf area index). In the latter case, average observed values were used for the standard simulation, in agreement with the constant stand-state objective that ruled pasture management (see above). Missing parameters were taken from the literature (e.g. photosynthetic parameters) (see table S1 in Hirl et al., 2019).

A detailed description of the new allocation-and-growth model (Fig. 1, Table 1) is provided in Methods S2. Briefly, the model includes three compartments: one well-mixed metabolic pool (Wpool) and two structural (i.e. nonmetabolic) compartments representing the aboveground shoots (Wshoot) and belowground roots (Wroot), similar to Ostler et al. (2016). Current assimilates replenish Wpool with rate (Fasim, gross primary production) and 13C and 18O signatures (δ13Cassim and δ18Oassim, see below) predicted by MuSICA (Fig. 1). Maintenance respiration (Fmaint,resp) is directly supported by Wpool, while growth respiration (Fgrowth, resp) is a constant fraction of growth and included in the flux of substrate allocated to the growth of shoots and roots (Fgrowth). Fmaint,resp is an exponential function of soil surface temperature and is assumed to be proportional to total plant carbon mass and negatively related to Wpool when the latter decreases below a target value (Thornley & Cannell, 2000). Growth (and growth respiration) occurs only if the metabolic pool is replenished to its target value. Substrate for growth is allocated between shoot (Fshoot,growth) and root (Froot,growth) depending on root xylem water potential (Ψxylem), to account for the drought-sensitivity of leaf growth (e.g. Durand et al., 1995), and the fractional allocation to the shoot (Fshoot,growth) is greater in spring than summer/ autumn. New assimilates carry the isotopic signal of current assimilation, while the substrate supplied to growth and respiration carries the isotopic signal of the metabolic pool, assuming no isotopic fractionation during respiration or growth.

The 18O : 16O ratio of cellulose in a sample (Rcellulose) was calculated similar to Ogée et al. (2009), assuming that cellulose synthesis was proportional to shoot growth. The integration time in days was computed for a set thermal time by summing up daily mean soil temperature above a base temperature of 4°C (Schleip et al., 2013). The biochemical fractionation (εbio) between water and substrate used in cellulose synthesis was calculated from daily mean air temperature using the empirical function found by Sternberg & Ellsworth (2011, their fig. 2). The pco2 was calculated from midday RH of air: pco2 = 0.016 RH − 0.393 (Fig. 2), derived by regressing the differences between Δ18Ocellulose predicted with a constant, nonbiased pco2 and observed Δ18Ocellulose against RH (Fig. 3a) as detailed in Methods S3. The resulting function closely resembled the relationship found by Lehmann
Table 1 Parameter values of the allocation-and-growth model used in Grünschwaige pasture paddock no. 8.

| Parameter                                           | Symbol | Value | Min. | Max. | Units  | Comment                                                                 |
|-----------------------------------------------------|--------|-------|------|------|--------|--------------------------------------------------------------------------|
| Total above- and belowground dry mass                | DM     | 270   | 210  | 350  | g m⁻²  | Measured                                                                 |
| Carbon content of dry mass                           | C      | 0.43  | 0.42 | 0.46 | g total C g⁻¹ dry mass                                                  | Measured                                                                 |
| Metabolic carbon content                             | wmetab | 0.3   | 0.25 | 0.33 | g metabolic C g⁻¹ total C                                               | Ostler et al. (2016)                                                      |
| Target metabolic pool size                           | W_pool_target | 34.8 | –    | –    | g metabolic C m⁻² soil                                                   | Calculated from DM, C and wmetab (see Materials and Methods section and Supporting Information Methods S2) |
| Specific maintenance respiration                     | rmaint_resp | 0.01 | 0.003| 0.03 | g respired C g⁻¹ biomass C d⁻¹ | Thornley (1998)                                                         |
| Depth below or above the soil surface used for maintenance respiration scaling | d_Mk | –6    | –20  | 200  | cm    |                                                                           |
| Q₁₀ for maintenance respiration scaling              | Q₁₀   | 2     | 1    | 3    | –      |                                                                           |
| Reference temperature for maintenance respiration scaling | T_ref | 20    | 15   | 25   | °C     | Tjoelker et al. (2001)                                                   |
| Varylen-sensitivity of allocation                    | Δt    | 1     | 50   | 600  | GDD    | Lötscher et al. (2004)                                                   |
| Integration time                                     | Δt    | 1     | 50   | 600  | GDD    | see Materials and Methods section, Fig. 4(c) and Methods S2              |
| Maximum rate of carboxylation at 25°C               | Vcmax | 60    | 20   | 140  | µmol m⁻² s⁻¹ | Rogers et al. (1998)                                                   |
| Potential rate of electron transport at 25°C         | Jmax  | 100   | 32   | 224  | µmol m⁻² s⁻¹ | Calculated from Vcmax following Medlyn et al. (2002)                     |
| Slope of the Ball–Woodrow–Berry stomatal conductance model | mgs | 10    | 7    | 25   | –      | Miner et al. (2017), and references therein; Wohlfahrt et al. (1998)    |

‘Value’ denotes the parameter value used in the ‘standard parameterization’ and ‘Min.’ and ‘Max.’ are the minimum and maximum values used in the sensitivity analyses.

et al. (2017) (Fig. 2). Alternatively, we also calculated the isotope composition of cellulose by keeping either p_cwp (0.556) or ε_bio (27‰) or both p_cwp and ε_bio constant.

Statistical and sensitivity analysis

Model performance was evaluated by calculating the mean bias error (MBE = P – O, with P the mean predicted value and O the mean observed value) between observed and predicted δ¹⁸Ocellulose (or Δ¹⁸Ocellulose), the mean absolute error (MAE = ∑(P_j – O_j)/n), with P_j the predicted and O_j the observed value of sample j, and n the number of samples (Willmott & Matsuura, 2005), and R² values. Linear regression and correlation analysis was performed to investigate the relationship between δ¹⁸Ocellulose (or Δ¹⁸Ocellulose) and environmental parameters. All analyses were conducted in R, v.3.4.2 (R Core Team, 2017).

A sensitivity analysis was performed (1) to quantify and disentangle the effect of meteorological variables on Δ¹⁸Ocellulose and δ¹⁸Ocellulose and (2) to evaluate the responsiveness of δ¹⁸Ocellulose and Δ¹⁸Ocellulose to the parameters of the allocation-and-growth model. Two sensitivity runs were performed for each parameter or meteorological variable, using a minimum and a maximum value, based on the range of observed or expected values (see Table 1). In each sensitivity run, one parameter (or meteorological variable) at a time was changed while all other parameters (or variables) were held the same as in the standard simulation. Regarding (1) above, the incoming short-wave radiation and wind speed, two variables that are expected to affect the leaf energy budget, were halved and doubled for each time step. Regarding (2) above, the applied parameter ranges of the allocation-and-growth model were first derived from measurements at the experimental site conducted during the study period; if no measurements were available for a parameter, relevant ranges were taken from the literature (see Table 1). Quantification of parameter effects (sensitivities) followed the procedure outlined in Hirl et al. (2019). First, systematic effects were quantified based on the ‘mean sensitivity’, computed as the mean difference between the sensitivity and the standard run (Δsens,i) / n, where Δsens,i is the δ¹⁸Ocellulose,i (or Δ¹⁸Ocellulose,i) in a sensitivity run and δref,i is that in the standard run. Second, the variability of the parameter effect was depicted based on the standard deviation of the sensitivity, calculated from the differences between Δsens,i and δref,i. To disentangle an eventual contribution of a temperature-sensitive ε_bio from the effects of other processes on the temperature sensitivity of Δ¹⁸Ocellulose, we also performed a range of sensitivity analyses in which air temperature was decreased (or increased) by 1, 3 or 5°C for all half-hourly values.
Results

Metabolic pool turnover, carbon allocation and integration time

The turnover of the metabolic pool was validated by comparing the predicted $^{13}$C-labelling of $W_{\text{pool}}$ in shoot growth ($f_{\text{new}}$) to the fraction of labelled C in $W_{\text{pool}}$, forced by a step-change of $\delta^{13}$C$_{\text{CO2}}$ in the MuSICA input data, with the labelling kinetics of total autotrophic respiration observed at the same site in May 2007 (Gammizet et al., 2009; see their fig. 5). The predicted $f_{\text{new}}$ in $W_{\text{pool}}$ matched closely the observed fraction of labelled C in total autotrophic respiration ($\text{slope} = 0.99; \text{intercept} = 0.06; R^2 = 0.98; P < 0.001$; Fig. 4a).

Model predictions for the fraction of carbon allocated to shoot growth ($f_{\text{shoot, growth}}$) compared well with observations of $f_{\text{shoot, growth}}$ made by Schleip (2013) in the same pasture during May and September of 2007. The model predicted average $f_{\text{shoot, growth}}$ of 0.67 for spring and 0.54 for autumn 2007, while Schleip (2013) observed an average carbon allocation fraction to shoot growth of 0.65 ($\pm 0.05$ SE) and 0.5 ($\pm 0.05$ SE) over the same periods (Fig. 4b). The average allocation to aboveground biomass for all spring and summer/autumn periods 2007–2012 was 0.58 and 0.47, respectively.

To constrain the integration time of the $^{18}$O-signal in the leaf cellulose samples, we compared the $R^2$ between observed and predicted $\delta^{18}$O$_{\text{cellulose}}$ (Fig. 4c) for predictions of $\delta^{18}$O$_{\text{cellulose}}$ based on integration times of 50–600 GDD. Those calculations were made with the model in its standard parameterization (Table 1) and assumed leaf growth started on the 15 March every year. The $R^2$ between observed and predicted $\delta^{18}$O$_{\text{cellulose}}$ increased up to an integration time of 400 GDD ($R^2 = 0.57$) and decreased beyond that. This integration time of 400 GDD translated to time spans varying between 22 and 59 d in different periods, depending on thermal conditions (Fig. 4d) and was subsequently used as the standard integration time for all predictions of $\delta^{18}$O$_{\text{cellulose}}$ and $\Delta^{18}$O$_{\text{cellulose}}$.

Observed variation of $\delta^{18}$O$_{\text{cellulose}}$ and $\Delta^{18}$O$_{\text{cellulose}}$

$\delta^{18}$O$_{\text{cellulose}}$ displayed dynamic variation within and between years, with pronounced increases and decreases in some years (2007, 2010) and lower variability in others (2008, 2011; Fig. 5). The total range of observed $\delta^{18}$O$_{\text{cellulose}}$ was 5.1‰, and a clear seasonal pattern was not evident (right panel, Fig. 5a). Annual mean $\delta^{18}$O$_{\text{cellulose}}$ did not differ significantly between the individual years, except for 2010 when the mean was 1.5‰ lower than the average of the other years. The lower $\delta^{18}$O$_{\text{cellulose}}$ in 2010 was linked to a more negative $\delta^{18}$O$_{\text{rain}}$ and lower leaf water $^{18}$O-isotopic enrichment in that year (cf. Hirl et al., 2019).

The range of observed $\Delta^{18}$O$_{\text{cellulose}}$ was 50% greater than that of $\delta^{18}$O$_{\text{cellulose}}$ (compare Fig. 5a and b). Unlike $\delta^{18}$O$_{\text{cellulose}}$, average $\Delta^{18}$O$_{\text{Cellulose}}$ declined distinctively during the growing season at a rate of 0.83‰ per month ($R^2 = 0.90; P < 0.01$). This was related to an increasing trend of $\delta^{18}$O$_{\text{source}}$ over the growing season (Hirl et al., 2019). Overall, the observed variation
of $\Delta^{18}O_{\text{cellulose}}$ resembled only loosely that of $\delta^{18}O_{\text{cellulose}}$ ($R^2 = -0.18$ for the entire data set).

$\Delta^{18}O_{\text{cellulose}}$ showed statistically significant relationships with most meteorological variables averaged over the respective integration time (Table 2; Fig. 6). The correlation of $\Delta^{18}O_{\text{cellulose}}$ with midday RH ($r = -0.69$) was stronger than with daily mean air temperature ($r = -0.43$) and midday air temperature ($r = -0.37$). $\Delta^{18}O_{\text{cellulose}}$ was only weakly related to PAW in the rooting zone ($r = -0.24$; $P = 0.05$) and there were no statistically significant relationships with vapour pressure deficit (VPD) or annual precipitation (both $P > 0.05$). Correlation analysis also indicated connections between $\Delta^{18}O_{\text{cellulose}}$ and cumulative short-wave radiation ($r = 0.81$) and wind speed ($r = 0.56$). Yet, a sensitivity analysis in which the radiation input was halved or doubled demonstrated that cumulative short-wave radiation itself had only a very small effect on $\Delta^{18}O_{\text{cellulose}}$ (Fig. S1), indicating that the strong correlation with $\Delta^{18}O_{\text{cellulose}}$ was indirect and arose primarily from the relationship of short-wave radiation with midday RH ($r = -0.63$) and temperature ($r = 0.57$) (Table S1). Sensitivity analysis also indicated that wind speed itself had a negligible effect on $\Delta^{18}O_{\text{cellulose}}$ (Fig. S1).

$\delta^{18}O_{\text{cellulose}}$ also had a clear relationship with RH ($r = -0.45$). Unlike $\Delta^{18}O_{\text{cellulose}}$, $\delta^{18}O_{\text{cellulose}}$ was related to precipitation amount ($r = -0.40$) and PAW ($r = -0.58$), with the latter negatively related to $\delta^{18}O_{\text{rain}}$ (Fig. S2a). Other relationships between $\delta^{18}O_{\text{cellulose}}$ and meteorological variables were weak (short-wave downward radiation) or nonsignificant (daily mean or midday air temperature, VPD, wind speed) (Table 2; Fig. 6).

Prediction of $\delta^{18}O_{\text{cellulose}}$ and $\Delta^{18}O_{\text{cellulose}}$

The standard model, with RH-sensitive $p_{\text{ex}}p_{\text{ks}}$ and temperature-sensitive $\epsilon_{\text{bio}}$, was able to reproduce with great accuracy the
observed seasonal dynamics of $\delta^{18}O_{\text{cellulose}}$ ($R^2 = 0.57$) and $\Delta^{18}O_{\text{cellulose}}$ ($R^2 = 0.74$) over the entire 6-yr-long study period (Figs 5, 7d). The model error and bias were small (Table 3), as the model was also able to capture the short-term variations. By contrast, predictions of $\Delta^{18}O_{\text{cellulose}}$ made with constant $p_{\text{ex}}p_s$ and/or constant $\epsilon_{\text{bio}}$ degraded significantly the goodness-of-fit with observed $\Delta^{18}O_{\text{cellulose}}$ (Fig. 7a–c).

Variation of midday RH at the study site, averaged over the integration time, was significant (47–73%) and implied a large range of modelled $p_{\text{ex}}p_s$ (0.37–0.77; average: 0.59), with a generally increasing trend during the growing season (Fig. 5c). Meanwhile, daily mean air temperature varied considerably at the scale of integration times (between 7 and 22°C), leading to pronounced variation in $\epsilon_{\text{bio}}$ (from 26 to 30%; Fig. 5d). $\epsilon_{\text{bio}}$ generally decreased from spring to summer and increased towards late autumn. The ability of the model to reproduce the observed relationships between $\Delta^{18}O_{\text{cellulose}}$ (or $\delta^{18}O_{\text{cellulose}}$) and meteorological variables or PAW also depended on the inclusion of an RH-sensitive $p_{\text{ex}}p_s$ and a temperature-sensitive $\epsilon_{\text{bio}}$ (Table 2). In particular, the model was unable to reproduce the observed relationship between $\Delta^{18}O_{\text{cellulose}}$ and air temperature when a constant $\epsilon_{\text{bio}}$ of 27‰ was used instead of a temperature-dependent one (Fig. 5d–f).

Relationship between canopy conductance and $\Delta^{18}O_{\text{cellulose}}$ or $\delta^{18}O_{\text{cellulose}}$

Observed $\Delta^{18}O_{\text{cellulose}}$ was negatively related to midday canopy conductance predicted by the MuSICA model ($R^2 = 0.26$; $P < 0.001$; Table 2; Fig. 8), with a 100 mmol m$^{-2}$ s$^{-1}$ increase in $g_{\text{canopy}}$ connected to a 1.4‰ decrease of $\Delta^{18}O_{\text{cellulose}}$. Again, the relationship between midday canopy conductance and observed or predicted $\Delta^{18}O_{\text{cellulose}}$ was the same if the prediction was based on RH-sensitive $p_{\text{ex}}p_s$ and temperature-sensitive $\epsilon_{\text{bio}}$, but not if constants for $p_{\text{ex}}p_s$ or $\epsilon_{\text{bio}}$ were used.

The relationship between observed $\delta^{18}O_{\text{cellulose}}$ and modelled midday canopy conductance was also negative ($P < 0.05$), although the slope and $R^2$ for that relationship were much smaller than those for $\Delta^{18}O_{\text{cellulose}}$. The relationship between predicted $\delta^{18}O_{\text{cellulose}}$ and modelled canopy conductance was similar to the observations when predictions were based on RH-sensitive $p_{\text{ex}}p_s$ and temperature-sensitive $\epsilon_{\text{bio}}$, and became
insignificant when predictions used constant values for \( \epsilon_{\text{bio}} \) or \( p_{\text{ex,ps}} \) and \( \epsilon_{\text{bio}} \).

### Sensitivity to model parameters

Model predictions of \( \delta^{18}O_{\text{cellulose}} \) or \( \Delta^{18}O_{\text{cellulose}} \) were relatively insensitive to alterations of the parameters of the allocation-and-growth model (Table 1; Fig. S1), except for integration time (Fig. 4c). That is, both the mean sensitivity (i.e. the mean difference between the sensitivity run and standard run for predictions of \( \delta^{18}O_{\text{cellulose}} \) (or \( \Delta^{18}O_{\text{cellulose}} \)) as well as the standard deviation of the sensitivity was smaller than 0.2‰ for each parameter value (Fig. S1).

In the MuSICA model, a 2.5-fold increase of \( m_{\text{g,s}} \) (see Table 1) – the slope of the Ball–Woodrow–Berry stomatal conductance model (Ball et al., 1987) – caused an average reduction of 0.84‰ of the predicted \( \delta^{18}O_{\text{cellulose}} \) and \( \Delta^{18}O_{\text{cellulose}} \). For a particular situation (time), the size of that effect depended on the conditions prevailing during the respective integration time. This conditionality was reflected in the relatively high standard deviations between individual sensitivity and standard model runs for \( m_{\text{g,s}} \) (Fig. S1). Conversely, a reduction of \( m_{\text{g,s}} \) by one-third caused an average increase of 0.3‰, with the increase again dependent on prevailing conditions, as above. On the other hand, increasing \( V_{\text{max}} \) and \( J_{\text{max}} \) by 2.2-fold or reducing it by two-thirds had only small effects on the prediction of \( \delta^{18}O_{\text{cellulose}} \) and \( \Delta^{18}O_{\text{cellulose}} \) (mean sensitivity \( \leq 0.32\% \)).

In general, predicted \( \Delta^{18}O_{\text{cellulose}} \) decreased (increased) with increasing (decreasing) temperature in the sensitivity analysis (Fig. S4). If \( \epsilon_{\text{bio}} \) was allowed to vary with temperature (corresponding to the default parameterization), a 1°C increase (decrease) in temperature caused a 0.28‰ decrease (increase) in the predicted \( \Delta^{18}O_{\text{cellulose}} \). Conversely, if \( \epsilon_{\text{bio}} \) was set to be constant (at 27‰), the resulting temperature-sensitivity of predicted \( \Delta^{18}O_{\text{cellulose}} \) was only –0.11‰ °C⁻¹.

### Discussion

Estimates of isotopic signal integration time in cellulose agree with leaf lifespan and residence time of nonstructural carbon

This work presents a new allocation-and-growth model that generates realistic estimates for the turnover of the metabolic pool used to supply autotrophic respiration, the allocation of assimilates to shoot and root growth, and the integration (or residence time) of cellulose in growing leaf material in a drought-prone, temperate grassland ecosystem. When coupled to a recently published version of an \( ^{15} \)O-enabled soil–vegetation–atmosphere transfer model (MuSICA) parameterized for a grassland (Hirl et al., 2019), this allocation-and-growth model provided a faithful prediction of the fortnightly, seasonal and multi-annual variation of the \( ^{18} \)O composition of cellulose in mixed-species leaf samples from this pasture ecosystem. The coupled model also enabled an explicit evaluation of the current theory of \( ^{18} \)O-enrichment of cellulose (\( \Delta^{18}O_{\text{cellulose}} \)), namely the factors that determine the relationship between \( \delta^{18}O_{\text{cellulose}} \) and \( \delta^{18}O_{\text{source}} \) on the one hand and those of \( \delta^{18}O_{\text{leaf}} \) on the other. We found compelling evidence that: \( \epsilon_{\text{bio}} \) the average biochemical fractionation between the organic substrate used for cellulose synthesis and water in the field, is temperature-dependent, in close agreement with studies of submerged aquatic plants as shown by

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**Table 2** Regression equations for the relationships of observed and predicted \( \Delta^{18}O_{\text{cellulose}} \) and \( \delta^{18}O_{\text{cellulose}} \) with meteorological variables at Grünschwaige pasture paddock no. 8.

|                      | Observed          | Predicted          |                   |
|----------------------|-------------------|--------------------|------------------|
|                      | \( \Delta^{18}O \) | \( \delta^{18}O \) |                   |
| **Relative humidity**| \( RH \)          | \( RH \)           |                   |
|                      | \( \Delta^{18}O = 42.60 - 0.19RH; \) | \( \Delta^{18}O = 42.42 - 0.18RH; \) |                   |
|                      | \( R^2 = 0.48; P < 0.001 \) | \( R^2 = 0.51; P < 0.001 \) |                   |
| **Daily mean \( T_{\text{air}} \)** | \( \Delta^{18}O = 34.65 - 0.21T_{\text{air}}; \) | \( \Delta^{18}O = 35.65 - 0.26T_{\text{air}}; \) |                   |
|                      | \( R^2 = 0.19; P < 0.001 \) | \( R^2 = 0.32; P < 0.001 \) |                   |
| **VPD**              | \( \Delta^{18}O = 34.59 - 0.18T_{\text{air}}; \) | \( \Delta^{18}O = 35.66 - 0.22T_{\text{air}}; \) |                   |
|                      | \( R^2 = 0.14; P < 0.01 \) | \( R^2 = 0.25; P < 0.001 \) |                   |
| **Short-wave radiation** | \( \Delta^{18}O = 25.91 + 0.01R_{\text{ls}}; \) | \( \Delta^{18}O = 25.82 + 0.01R_{\text{ls}}; \) |                   |
|                      | \( R^2 = 0.65; P < 0.001 \) | \( R^2 = 0.83; P < 0.001 \) |                   |
| **Wind speed**       | \( \Delta^{18}O = 26.41 + 1.34u; \) | \( \Delta^{18}O = 26.73 + 1.30u; \) |                   |
|                      | \( R^2 = 0.32; P < 0.001 \) | \( R^2 = 0.34; P < 0.001 \) |                   |

Relative humidity (RH), midday air temperature (Midday \( T_{\text{air}} \)), vapour pressure deficit (VPD), plant-available water (PAW) and wind speed (\( u \)) all represent midday (± 3 h around noon) values averaged over the integration time \( \Delta t \) (400 GDD). Daily mean \( T_{\text{air}} \) represents daily average air temperature over \( \Delta t \), and precipitation amount (Precip) and short-wave radiation (\( R_{\text{ls}} \)) represent the precipitation sum and the total sum of incoming short-wave radiation within \( \Delta t \), not significant (\( P > 0.05 \)).
Sternberg & Ellsworth (2011); and $p_{ex}$, the proportion of oxygen in cellulose derived from source water, responded to RH in accordance with the findings of Lehmann et al. (2017) and our own investigations (unpublished data) with *L. perenne* in controlled environments.

To predict $\delta^{18}$O$_{cellulose}$ accurately, the integration time used in the model calculations was particularly important. Model-data comparison of $\delta^{18}$O$_{cellulose}$ indicated a best match with 400 GDD. This was 14% shorter than the average observed leaf lifespan (LLS; 463 $\pm$ 56 GDD) of the main species (*L. perenne*, *P. pratensis*, *T. officinale* and *T. repens*) at the same site (Schleip et al., 2013). A possible explanation is that Schleip et al. considered that LLS ended when a senescing leaf had 25% chlorotic tissue while leaf sampling for the present investigation comprised only fully green leaf blades and excluded any chlorotic leaf blades. Therefore, the collected leaves were probably not older than c. 320–450 GDD (compare with fig. 2 in Schleip et al., 2013), in close agreement with the observed best match for an integration time of c. 400 GDD (Fig. 4c). This value also agrees well with the mean residence time of structural carbon in above-ground biomass of the codominant species at the same site observed by Ostler et al. (2016).

First field-based evidence for a strong temperature effect on $\epsilon_{bio}$, in agreement with laboratory studies

Over the integration time, air temperature ranged from 7 to 22°C and midday RH from 47 to 73%. Importantly, however,

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**Fig. 6** Correlation coefficients for the relationships between (a) $\Delta^{18}$O$_{cellulose}$ and meteorological variables and (b) $\delta^{18}$O$_{cellulose}$ and meteorological variables: relative humidity (RH), air temperature ($T_{air}$), vapour pressure deficit of the air (VPD), plant-available water (PAW), precipitation amount (Precip), incoming (downward) short-wave radiation ($R_{s} \downarrow$) and wind speed (u). Red bars, observed data; blue bars, predicted data. RH, VPD and u represent midday ($\pm$ 3 h around noon) values averaged over the integration time $\Delta t$ (400 GDD); $T_{air}$ represents daily mean values averaged over $\Delta t$; and $R_{s} \downarrow$ and Precip represent the total sums of incoming short-wave radiation and the total precipitation within $\Delta t$. All predictions were made with the model in ‘standard parameterization’, including temperature-sensitive $\epsilon_{bio}$ and relative humidity-sensitive $p_{ex}$. Asterisks indicate the significance levels: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. 

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variations of RH and temperature were not significantly correlated at the integration time-scale \((P = 0.50)\), meaning that RH and air temperature varied largely independently from each other. This factor aided/enabled the identification and distinction of the RH effect on \(p_{\text{exp}}\) and of the temperature effect on \(\epsilon_{\text{bio}}\). 

Notably, daily mean temperatures over the integration time covered most of the temperature-sensitive range of \(\epsilon_{\text{bio}}\) (Sternberg & Ellsworth, 2011), explaining why the inclusion of a temperature-dependent \(\epsilon_{\text{bio}}\) was so critical for simulating \(\Delta^{18}O_{\text{cellulose}}\). Unfortunately, it is presently unknown how temperature influences the relevant biochemical mechanisms and their associated isotope effects that combine to yield the ‘average biochemical fractionation between substrate for cellulose synthesis and water’ \(\epsilon_{\text{bio}}\). In that regard, however, it is interesting and encouraging that the temperature-dependence of \(\epsilon_{\text{bio}}\) observed here matched closely the patterns observed by Sternberg & Ellsworth (2011) in aquatic and heterotrophic systems. To the best of our knowledge, convincing general, field-based evidence supporting the requirement of a temperature-sensitive \(\epsilon_{\text{bio}}\) has not been published so far. Still, a possible temperature-sensitivity of \(\epsilon_{\text{bio}}\) has been discussed in modelling studies on tree-ring \(\delta^{18}O_{\text{cellulose}}\) by Keel et al. (2016) and Lavergne et al. (2017) on tree species from different functional groups growing across a range of temperate to cold environments. Partial evidence in favour of a temperature-sensitive \(\epsilon_{\text{bio}}\) was presented by both groups, although overall evidence was inconclusive because of uncertainties in either the models or the climate and site data. Strong evidence for a temperature-sensitive \(\epsilon_{\text{bio}}\) is reinforced in our comprehensive model parameterization and validation study against an extensive field dataset. In particular, the leaf water modelling, partitioning of source \(\left(\delta^{18}O_{\text{source}}\right)\)
and enrichment effects ($\Delta^{18}$O$_{leaf}$) on leaf water isotope composition ($\delta^{18}$O$_{leaf}$) (Hirl et al., 2019) permitted a very robust modelling of $\Delta^{18}$O$_{cellulose}$ that could be contrasted with observations of $\Delta^{18}$O$_{cellulose}$ across a multiyear dataset using the same set of model parameters.

Sensitivity analyses of temperature effects on predictions of $\Delta^{18}$O$_{cellulose}$ demonstrated that c. 60% of the temperature effect in $\Delta^{18}$O$_{cellulose}$ was related to $\epsilon_{bio}$ with the remaining 40% connected to all other temperature-dependent processes influencing $\Delta^{18}$O$_{cellulose}$. Interestingly, the temperature sensitivity of $\Delta^{18}$O$_{cellulose}$ (~0.28% °C$^{-1}$) was similar in magnitude but opposite in sign to the temperature sensitivity of $\delta^{18}$O$_{rain}$ (+0.36% °C$^{-1}$; Fig. S2b). This caused a lack of correlation between $\delta^{18}$O$_{cellulose}$ and temperature, and highlights the need (and benefit) of studying both $\Delta^{18}$O$_{cellulose}$ and $\delta^{18}$O$_{cellulose}$. Previous studies found a significant correlation between $\delta^{18}$O$_{cellulose}$ (mainly from tree rings) and temperature. These studies used different temporal (decadal or century-scale changes in contrast to the seasonal patterns studied here), spatial (latitudinal or altitudinal, as compared to the stationary observations in the present study) or aridity gradients, and may have also differed in the temperature sensitivity of $\Delta^{18}$O$_{cellulose}$ relative to that of $\delta^{18}$O$_{rain}$ (e.g. Anderson et al., 1998; Saurer et al., 2002; Kress et al., 2010; Song et al., 2011).

Evidence for an increase of $p_{ex}p_{x}$ with air humidity, possibly linked to leaf water $^{18}$O-enrichment along leaf blades

Model-data comparison also strongly supported the inclusion of an RH-dependent $p_{ex}p_{x}$ in the computation of $\Delta^{18}$O$_{cellulose}$. This RH dependency of $p_{ex}p_{x}$ was derived from the relationship between RH and the model-data residuals when $\Delta^{18}$O$_{cellulose}$ was predicted with a constant $p_{ex}p_{x}$ of 0.556, combined with Eqsns S7 and S9 (see Methods S2 and S3). A similar agreement between predicted and observed data was obtained when using the RH-sensitivity of $p_{ex}p_{x}$ observed by Lehmann et al. (2017) in a controlled environment study with $L$. perenne and $D$. glomerata, two of the codominant species present in our system. The RH dependency of $p_{ex}p_{x}$ required to link $\Delta^{18}$O$_{cellulose}$ and $\Delta^{18}$O$_{leaf}$ has been discussed previously for a range of $C_3$ and $C_4$ grasses (Liu et al., 2016). Failure to predict $\Delta^{18}$O$_{cellulose}$ with a constant (i.e. RH-insensitive) $p_{ex}p_{x}$ was not related to erroneous MuSICA predictions of $\Delta^{18}$O$_{leaf}$ as these exhibited a virtually identical RH-response to observed $\Delta^{18}$O$_{leaf}$ (Hirl et al., 2019). Predictions of $\Delta^{18}$O$_{assim}$ by MuSICA were based on bulk leaf water $\Delta^{18}$O$_{leaf}$ and the common assumption that new assimilates are in isotopic equilibrium with $\Delta^{18}$O$_{leaf}$ ($\Delta^{18}$O$_{assim} = \Delta^{18}$O$_{leaf} + \epsilon_{bio}$) (Barbour, 2007). Lehmann et al. (2017) showed that this assumption may not always hold in grasses where RH was affecting the $\delta^{18}$O of sucrose ($\delta^{18}$O$_{sucrose}$) more strongly than $\delta^{18}$O$_{leaf}$. These results support the view that $\Delta^{18}$O$_{sucrose}$ is not always equal to $\Delta^{18}$O$_{leaf}$ + $\epsilon_{bio}$ in grasses, and that RH affects the divergence between $\Delta^{18}$O$_{leaf}$ and the $\Delta^{18}$O of the water in which assimilation and sucrose synthesis takes place ($\Delta^{18}$O$_{suc-water}$). This divergence between $\Delta^{18}$O$_{leaf}$ and $\Delta^{18}$O$_{suc-water}$ may be related to the gradient of $^{18}$O enrichment along leaf blades observed by Helliker & Ehleringer (2000, 2002a) and Gan et al. (2003), which is particularly steep at low RH. Uncertainties on $\Delta^{18}$O$_{suc-water}$ affect the calculation of $p_{ex}p_{x}$. $p_{x}$ is commonly calculated from a two-end-member mixing model, the end-members being the $\Delta^{18}$O of water at the site of cellulose synthesis ($\Delta^{18}$O$_{cell-water}$) and $\Delta^{18}$O$_{suc-water}$ with the latter approximated as $\Delta^{18}$O$_{leaf}$. $p_{x} = 1 - \Delta^{18}$O$_{cell-water}/\Delta^{18}$O$_{suc-water}$. A deviation between $\Delta^{18}$O$_{suc-water}$ and $\Delta^{18}$O$_{leaf}$ affects the calculation of $p_{x}$. However, for grasses, this error must be small, as the water in the leaf growth and differentiation zone of grasses is close to source water ($\Delta^{18}$O$_{cell-water}$ of c. 0) so that $p_{x}$ is c. 1 (Liu et al., 2017a). Accordingly, the divergence between $\Delta^{18}$O$_{suc-water}$ and $\Delta^{18}$O$_{leaf}$ noted by Lehmann et al. (2017) mostly affects the estimate of $p_{ex}$.

Other factors that have been related to variations of $p_{ex}$ have included $^{18}$O-exchange during phloem transport from the leaves to the growing tissue (Gessler et al., 2007; but see Gessler et al., 2013) and futile cycling of the growth substrate within the growing tissue (Barbour, 2007), potentially linked to cellular growth rate or other cellular anatomical features, such as the lumen area of cambial cells (Szejner et al., 2020). The rate of futile cycling has been related to the turnover of the water-soluble carbohydrate pool in $R$. communis (Song et al., 2014). However, we could not detect a significant relationship between the modelled mean residence time of substrate in the metabolic pool ($\tau_{pool}$) and $p_{ex}$, estimated from $p_{ex}p_{x}$ with $p_{x} = 1$ (Fig. S5), although statistical noise may have been a factor. Also, modelled integration time (in days) – which is a function of leaf appearance, growth and senescence rates that are all closely coordinated in grasses (Lemaire et al., 2000; Schleip et al., 2013) – did not correlate with estimates of $p_{ex}$. Lastly, in a recent study, we did not observe an effect of RH on the hydraulic conductance of $L$. perenne vegetative plants in controlled environments (Baca Cabrera et al., 2020), providing no (physiological) indication of an effect of RH on xylem lumen area. Clearly, there is a great need for detailed biochemical investigations of the factors underlying the variation of $p_{ex}$.

Predictions of canopy conductance from $^{18}$O-enrichment of cellulose required a temperature-dependent $c_{bio}$ and humidity-sensitive $p_{ex}p_{x}$

Finally, this work demonstrates a significant negative relationship between midday canopy conductance ($g_{canopy}$) and both $\Delta^{18}$O$_{cellulose}$ and $\delta^{18}$O$_{cellulose}$. Although used in multiple studies to interpret variation in $\Delta^{15}$C and intrinsic water-use efficiency, experimental or empirical evidence for the relationship between $\Delta^{18}$O$_{cellulose}$ (or $\delta^{18}$O$_{cellulose}$) and stomatal or canopy conductance is relatively scarce (Barbour et al., 2000; Grams et al., 2007; Sullivan & Welker, 2007; Moreno-Gutiérrez et al., 2012). In principle, the correlation between $g_{canopy}$ and $\Delta^{18}$O$_{cellulose}$ could be caused by any environmental factor that affects stomatal opening and consequently alters leaf temperature, leaf-to-air vapour pressure difference, and thus $\Delta^{18}$O$_{leaf}$ $\Delta^{18}$O$_{suc-water}$, $\epsilon_{bio}$ and ultimately $\Delta^{18}$O$_{cellulose}$. Correlations between $\Delta^{18}$O$_{cellulose}$ and stomatal conductance observed in previous studies were evoked by treatment differences in air CO$_2$ and O$_3$ concentration and by interplant competition (Grams et al., 2007), or by variation in soil temperature and soil water status (Sullivan & Welker, 2007).
In a previous study, we found a significant effect of both soil water content and RH on $\Delta^{18}$O$_{\text{cellulose}}$ and a significant relationship between soil moisture and canopy conductance (Hirl et al., 2019). Remarkably, however, we could not identify a drought-related increase of $\Delta^{18}$O$_{\text{cellulose}}$, which would indicate a drought-related decrease of stomatal conductance (Fig. S6). We believe that absence of a drought signal in $\Delta^{18}$O$_{\text{cellulose}}$ is caused by the low assimilation rate and deceleration (or complete cessation) of leaf growth under drought. By contrast, both $\Delta^{18}$O$_{\text{cellulose}}$ (Table 2; Fig. 6; Fig. S3a,c) and $\kappa_{\text{anop}}$ ($R^2 = 0.13; P < 0.01$) were significantly related to RH. The RH-sensitivity of $\Delta^{18}$O$_{\text{cellulose}}$ was even higher than that of $\Delta^{18}$O$_{\text{leaf}}$ ($-0.15\% \text{RH}^{-1}$), due to the relationship between RH and $p_{\text{ex}}$ (as already discussed). If $\Delta^{18}$O$_{\text{prec-water}}$ is more strongly related to evaporative conditions than $\Delta^{18}$O$_{\text{leaf}}$, the stomatal conductance signal in cellulose may in fact be more pronounced than previously expected solely from the climate-sensitivity of bulk leaf water. This lends further support to studies that aim to reconstruct stomatal conductance from $\delta^{18}$O$_{\text{cellulose}}$.

Conclusions and perspective

This study provides strong evidence for important climate-sensitive variation of the parameters $p_{\text{ex}}$ and $\epsilon_{\text{bio}}$ of the Barbour–Farquhar model in a terrestrial ecosystem. Elucidation of those sensitivities demanded and relied on the use of a carefully parameterized, $^{18}$O-enabled process-based soil–plant–atmosphere transfer model and the existence of a detailed multiseasonal observational data set of $^{18}$O signals in all relevant ecosystem water pools and leaf cellulose. As a result, our work demonstrates at the ecosystem scale the validity of earlier conclusions that were drawn from model-type, experimental studies conducted in steady-state, controlled environments, but have not been generally considered in the relevant fields of terrestrial ecosystem sciences. Our results have important implications for the interpretation of seasonal, interannual and geographical variations of $\Delta^{18}$O$_{\text{cellulose}}$ and $\delta^{18}$O$_{\text{cellulose}}$ in terms of plant physiology and climate. Existing datasets, for example on tree-ring $\Delta^{18}$O$_{\text{cellulose}}$ or $\delta^{18}$O$_{\text{cellulose}}$ from boreal to subtropical ecosystems, should benefit from a re-evaluation that considers the varying nature of biochemical fractionation and isotopic exchange with source and leaf water. In addition, there is a great need for more targeted studies investigating the mechanisms underlying the large observed effect of temperature on $\epsilon_{\text{bio}}$ and the RH-dependent variation of $p_{\text{ex}}$. Such knowledge will help us to better understand the physiological information that is imprinted in cellulose-$^{18}$O signals from past and present environments.

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Author contributions

RTH, JO and HS designed the research. RTH, UO and HS designed the allocation-and-growth model. RTH analysed the data and performed the modelling with guidance by JO and UO. RS performed the isotope analysis. HS, UO and IS designed and UO and IS performed the tracer experiment. HS and RTH planned and RTH, JABC and JZ performed the mesocosm experiment. RTH wrote the first draft. RTH, JO, UO, RS, JCB, JZ, IS, LW and HS contributed to the discussion and the revision of the manuscript.

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Data availability

The MuSICA model is written in FORTRAN 90 with scripts in PYTHON or R. The model code is currently on a private repository (https://bitbucket.org/musica_dev/musica/src/master/) accessible upon request and should very soon be moved to a public repository. The data and the R code for the allocation-and-growth model are also available upon request.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Sensitivity of modelled δ¹⁸Ocellulose and Δ¹⁸Ocellulose to the parameters of the allocation-and-growth model, to the responsiveness parameter of the Ball–Woodrow–Berry stomatal conductance model (m_p; Ball *et al.*, 1987), to maximum rate of carboxylation and potential rate of electron transport (V_cmax and f_max), and to incoming short-wave radiation and wind speed.

**Fig. S2** Relationship between the δ¹⁸O of rain collected at the Grünschwaige study site (see Hirl *et al.*, 2019) and plant-available water and air temperature.

**Fig. S3** Plot of Δ¹⁸Ocellulose vs midday mean relative humidity and daily mean air temperature.

**Fig. S4** Sensitivity of Δ¹⁸Ocellulose to air temperature, predicted based on a constant (ε_bio = 27‰) or temperature-sensitive biochemical fractionation.

**Fig. S5** Relationship between p_ex and the mean residence time of the metabolic pool (τ_pool,out), both averaged over the integration time (400 GDD).

**Fig. S6** Boxplots showing the effect of plant-available water on observed Δ¹⁸Ocellulose, predicted Δ¹⁸Ocellulose and modelled midday canopy conductance, g_canopy.

**Methods S1** The MuSICA model.
Methods S2 The allocation-and-growth model.

Methods S3 Derivation of the $p_{ex}p_{v}$--RH function for the standard simulation.

Table S1 Correlation between meteorological variables.

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