Original Article

Predicting leaf wax *n*-alkane ^{2}H/^{1}H ratios: controlled water source and humidity experiments with hydroponically grown trees confirm predictions of Craig–Gordon model

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ABSTRACT

The extent to which both water source and atmospheric humidity affect δ^2H values of terrestrial plant leaf waxes will affect the interpretations of δ^2H variation of leaf waxes as a proxy for hydrological conditions. To elucidate the effects of these parameters, we conducted a long-term experiment in which we grew two tree species, *Populus fremontii* and *Betula occidentalis*, hydroponically under combinations of six isotopically distinct waters and two different atmospheric humidities. We observed that leaf *n*-alkane δ^2H values of both species were linearly related to source water δ^2H values, but with slope differences associated with differing humidities. When a modified version of the Craig–Gordon model incorporating plant factors was used to predict the δ^2H values of leaf water, all modelled leaf water values fit the same linear relationship with *n*-alkane δ^2H values. These observations suggested a relatively constant biosynthetic fractionation factor between leaf water and *n*-alkanes. However, our calculations indicated a small difference in the biosynthetic fractionation factor between the two species, consistent with small differences calculated for species in other studies. At present, it remains unclear if these apparent interspecies differences in biosynthetic fractionation reflect species-specific biochemistry or a common biosynthetic fractionation factor with insufficient model parameterization.

Key-words: compound-specific isotope analysis; hydrogen isotopes; stable isotopes.

INTRODUCTION

The stable isotope ratios of precipitation falling across landscapes vary as a function of geography and seasonality, with individual observations falling along a local meteoric water line (Dansgaard 1964). As plants generally take up meteoric waters from their roots with no isotopic fractionation (Ehleringer & Dawson 1992), the stable isotope ratios of plant xylem waters reflect the isotope ratios of precipitation, although evaporative processes in soils can modify the

isotope ratio of waters entering plants. Additionally, Craig–Gordon processes enrich leaf water during transpiration (Flanagan & Ehleringer 1991). Consequently, the isotope ratios of plant materials record information relating to the plant's original source water and the atmospheric environment to which the plant was exposed.

The oxygen (δ^{18}O) and hydrogen (δ^2H) isotope ratios of plant materials record the plant-water conditions at the time of biosynthesis (Roden & Ehleringer 1999b). Mechanistic models have been developed to describe the relationships between the δ^{18}O and δ^2H isotope values of plant waters and tissues and the surrounding environments at the time of material synthesis (Flanagan & Ehleringer 1991; Roden *et al.* 2000; Barbour *et al.* 2004; Farquhar *et al.* 2007; Kahmen *et al.* 2008; Ferrio *et al.* 2009). A fundamental component of mechanistic plant water models is a modified Craig & Gordon (1965) evaporative enrichment model that incorporates leaf boundary layers (Farquhar *et al.* 1989; Flanagan *et al.* 1991). These models use vapour pressures and the isotope ratios of source water and atmospheric water vapour, as well as kinetic and equilibrium fractionation factors to predict the δ^{18}O and δ^2H of leaf water (Flanagan & Ehleringer 1991). These mechanistic models can predict the isotopic composition of plant-water and organic matter that are applicable to ecological, physiological and geological questions.

Refractory plant tissues, such as tree-ring cellulose and leaf wax lipids, can be used to reconstruct past plant-water conditions, environmental change and climatic settings. Many studies have used variations in the δ^{18}O and δ^2H isotope values of tree-ring cellulose to reconstruct past changes in environmental and climatic conditions (Leavitt & Long 1991; Barbour *et al.* 2002; Leavitt *et al.* 2002; Roden *et al.* 2009). The δ^{18}O and δ^2H isotope values of tree-ring cellulose reflect the combination of source water isotope ratios and environmental parameters (i.e. humidity, temperature, atmospheric water isotope ratios). Tree rings are well suited to reconstruct past environmental change as some individual plants can live many thousands of years and growth rings relate to discrete periods of growth, allowing for seasonal and yearly cycles to be established (McCarroll & Loader 2004; Sternberg 2009). Thus, individuals and groups of individual trees can be used

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to reconstruct environmental variations at a given location over the lifespan of a tree (Loader *et al*. 1995; Hemming *et al*. 1998). Furthermore, multiple individual records can be spliced together to create longer chronologies allowing multi-centennial records to be established (Flanagan & Ehleringer 1991). However, additional plant isotope proxy records are required for palaeoclimate reconstructions at deeper geological intervals.

The hydrogen isotope ratios of long-chain *n*-alkanes have attracted increased interest as a plant-specific biomarker for deeper time palaeoclimate reconstructions (Sachse *et al*. 2012). Higher plant-derived *n*-alkanes are easily identified and isolated from a variety of geological matrices (i.e. soils, lacustrine sediments and marine sedimentary rocks). The hydrogen atoms of *n*-alkanes are bonded covalently to carbon atoms and have very slow exchange rates (10^4–10^8 years) in thermally immature sediments (Schimmelmann *et al*. 1999, 2006; Pedentchouk *et al*. 2006), making these leaf wax compounds very robust against diagenetic alteration. These characteristics make the δ^2H isotope values of leaf wax *n*-alkanes an ideal tool to interpret ancient hydrological characteristics and ecophysiological questions.

As with other plant tissues, the δ^2H isotope values of leaf wax lipids largely record the δ^2H isotope value of source water, which, in most cases, reflects precipitation (Sachse *et al*. 2012). Leaf waxes extracted from modern lake sediments and soils demonstrate a strong correspondence between the δ^2H isotope values of lipids and of modelled mean annual precipitation or lake water (Sauer *et al*. 2001; Huang *et al*. 2004; Sachse *et al*. 2004; Hou *et al*. 2007b; Polissar & Freeman 2010; Garcin *et al*. 2012; Tipple & Pagani 2013). These data suggest that at the ecosystem level, the δ^2H isotope values of leaf waxes strongly correlate with the average δ^2H values of annual precipitation falling on a landscape. This was foundational for reconstructing ancient precipitation δ^2H isotope values from measurements of refractory leaf waxes. Furthermore, these tools have shown great promise and have been used to reconstruct ancient climate and the plant-water environments on decadal (Douglas *et al*. 2012; Feakins *et al*. 2013) to millennial (Berke *et al*. 2012, Feakins *et al*. 2007, Pagani *et al*. 2006, Schefuβ *et al*. 2005, Tierney *et al*. 2010) and multi-million year timescales from geological archives (Tipple & Pagani 2010; Tipple *et al*. 2011).

While results from plant waxes extracted from sediments are extremely promising for palaeo-research, surveys of the δ^2H isotope values of leaf waxes from modern plants indicate that secondary parameters, such as soil evaporation and evapotranspiration, may or may not impart additional information on the δ^2H isotope values of leaf waxes (Smith & Freeman 2006; Hou *et al*. 2008; Feakins & Sessions 2010a; McInerney *et al*. 2011; Kahmen *et al*. 2013b). Specifically, the relative importance of evapotranspiration has been argued to be significant (Kahmen *et al*. 2013b), insignificant (Hou *et al*. 2008) or largely absent (McInerney *et al*. 2011) in shaping $^2H/^1H$ ratios of leaf waxes. In addition to environmentally controlled processes acting upon plants, lipid biosynthesis, plant physiology and phenology have been suggested to contribute to the δ^2H isotope values of modern leaf waxes (Hou

et al. 2007b; Feakins & Sessions 2010b; Gao *et al*. 2012; Sachse *et al*. 2012; Tipple *et al*. 2013). Secondary processes of particular interest and importance for the interpretation of the δ^2H isotope values of modern leaf waxes are the timing of wax synthesis, leaf morphology, source of H, plant growth form and photosynthetic pathway.

To resolve some of these issues and develop mechanistic insights for the interpretation of the δ^2H isotope values of leaf waxes, we measured the δ^2H isotope values of leaf waxes from angiosperm tree species grown for an entire growing season under climate-controlled greenhouse conditions. We grew two deciduous tree species hydroponically under combinations of two humidity conditions and six isotopically distinct source waters, ranging over 150‰ in δ^2H values. This isotopic range of source waters exceeded the entire range observed in nature, allowing for the effects of leaf water ^2H enrichment to be clearly illustrated. In addition, greenhouse humidity and temperature were controlled and measured. The δ^2H isotope values of atmospheric water vapour and source water were measured over a 5 month interval. Growing plants hydroponically under two controlled humidities and with a wide range of source water δ^2H values allowed us to eliminate the effects of soil evaporation and isolated the controls of atmosphere water vapour on the δ^2H isotope values of leaf waxes. This experimental design permitted the development of a mechanistic model for the interpretation of δ^2H variation in leaf waxes and estimate ε_{bio} for leaf wax synthesis.

MATERIALS AND METHODS

Plant materials and greenhouse growth conditions

Two- to three-year-old saplings of water birch (*Betula occidentalis* Hook) and cottonwood (*Populus fremontii* Wats) were obtained from local nurseries. All saplings were dormant or in the earliest phase of leaf flush prior to the start of the experiment and were grown for over 5 months (see Roden & Ehleringer 1999a). The trees were grown hydroponically in 190 L tanks, with aquarium pumps and airstones providing oxygen to the roots. Each tank contained replicates of each species that were fixed in position by a 5.5-cm-thick closed cell foam attached to a wooden frame with the foam fitting tightly on the tank to prevent evaporation. Nutrients were provided to the roots as a 1/10 strength Hoagland's solution. The trees were grown in one of six source water treatments: −120‰/−15‰ ($\delta^2H/\delta^{18}O$), −60‰/−10‰, 0‰/−5‰, +60‰/0‰, +120‰/+5‰ and +180‰/+10‰ (Fig. 1). Plants were grown in two greenhouses maintained at near 25 °C. One greenhouse was operated at ambient humidity and the second at a higher relative humidity by adding water vapour with a misting humidifier (Fig. 2). Each treatment was replicated twice in each greenhouse for a total of 24 tanks and 144 trees (3 individuals, 2 species, 6 source water treatments, 2 replicate tanks, 2 greenhouse humidities). Of these trees, random subsets of two to four plants were selected for leaf wax extractions. Periodically over the course of the growing season, stomatal conductance and transpiration rates were measured using a diffusion porometer (Li-Cor 1600; Li-Cor,
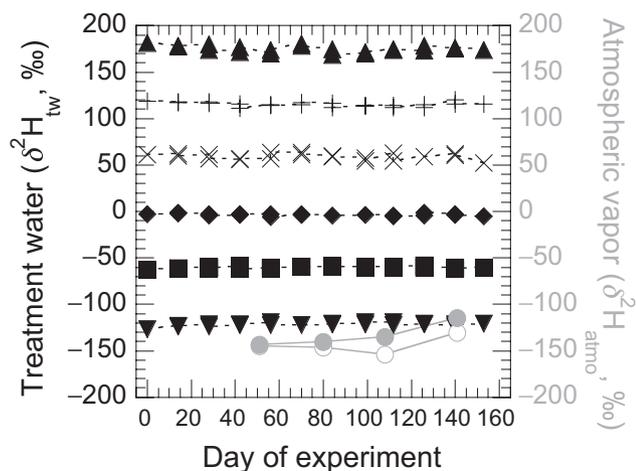
Figure 1. Isotopic composition of treatment water (black symbols) and atmospheric water vapour (grey symbols) over the course of the experiment. The experiment started on 4/8/1997. Two symbols present on any day indicate samples taken both prior to and after new water addition. Solid and open symbols for atmospheric samples represent high and low humidity treatment, respectively. Data from Roden & Ehleringer (1999a).

Lincoln, NE, USA) on leaves sampled from all water source treatments in both greenhouses.

Water sample acquisition, preparation and isotope analysis

Approximately 5 mL of water was sampled from each tank both prior to and after water additions for analysis of hydrogen isotope composition. Atmospheric water vapour was sampled from both greenhouses using a custom cryo-trap system (Helliker *et al*. 2002). Greenhouse air was pumped into a glass cold trap submerged in a dry ice/ethanol slurry (–78 °C). After 1 h, the frozen vapour was thawed and transferred to a baked 4 mL vial, sealed with Parafilm® (Bemis North America, Neenah, WI, USA) and stored in the freezer until the time of processing.

The water samples from the tanks and atmospheric vapour were reduced to H_2 as described in Roden & Ehleringer (1999a). The δ^2H values of tank water and atmospheric vapour were analysed on a Finnagan MAT Delta S isotope ratio mass spectrometer at the University of Utah with a precision of ±1‰. Isotopic compositions are reported as

$$\delta = [R_{samp}/R_{std} - 1], \tag{1}$$

where R represents the ^{2}H/^{1}H abundance ratio, and R_{samp} and R_{std} represent the sample and standard, respectively. Delta values are reported in per mil notation, which implies a factor of 1000 and are expressed relative to Vienna Standard Mean Ocean Water (VSMOW).

Leaf wax sampling, extraction, quantification and hydrogen isotope analysis

Plants were grown in 1998 and leaf samples used in this study were collected from archived materials of the entire original

plants. Archived specimens were desiccated and stored in paper bags at the University of Utah. Leaf wax lipids were extracted from approximately 0.5 g of whole leaves with 2:1 ultrapure dichloromethane/methanol by ultrasonication (15 min × 3). The resulting extract was filtered with a glass funnel plugged with ashed glass wool to remove any solid material. The filtered total lipid extract was concentrated under a stream of purified nitrogen with the aid of a heated evaporator block. Hydrocarbons were separated by column chromatography using 1 g of deactivated silica gel and 2 mL of hexane. The compounds were analysed using a Thermo Trace 2000 gas chromatography (GC) mass spectrometer equipped with a split-splitless injector at 300 °C with a fused silica, 30 m
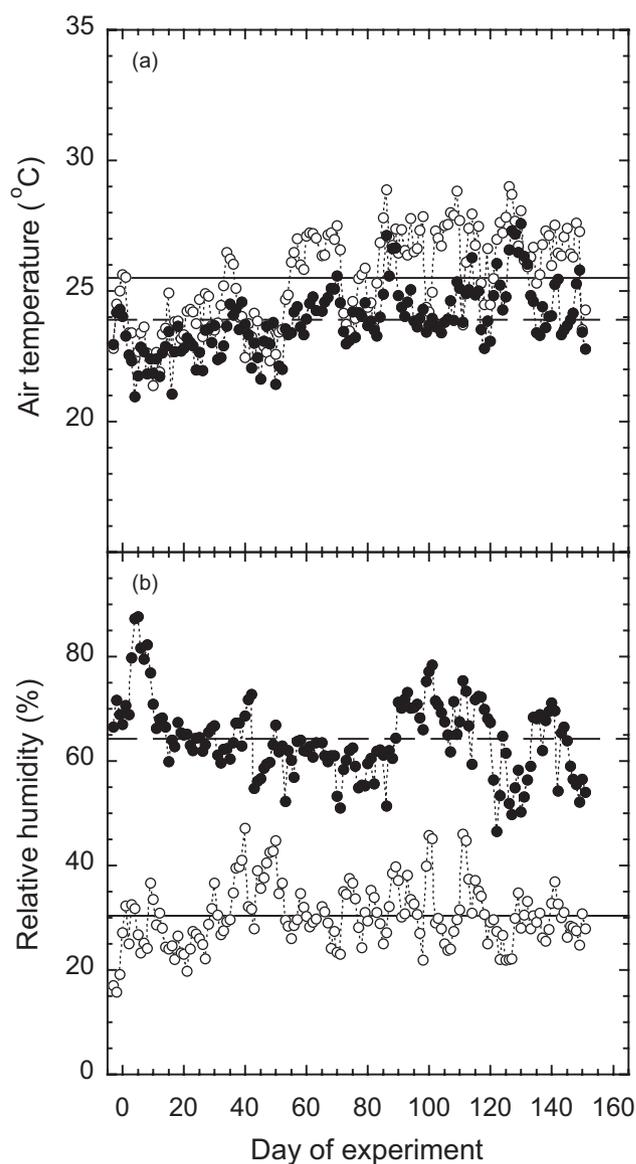

Figure 2. Greenhouse environmental parameters over the course of the experiment. Solid and open symbols represent high and low humidity treatments, respectively. Data from Roden & Ehleringer (1999a).

DB-5 phase column (Agilent J&W Scientific, Agilent Technologies, Santa Clara, CA, USA) with helium as the carrier at a flow of 1.5 mL min⁻¹. The GC oven temperature program utilized was 60–320 °C at 15 °C min⁻¹ with an isothermal for 30 min. Compounds were identified through comparison of elution times with *n*-alkane standards. Compound concentrations were quantified using a four-point calibration curve generated from certified reference materials of known concentration.

Compound-specific isotope analyses were performed at the University of Utah using a Thermo Trace 2000 gas chromatograph coupled to a Thermo Delta V isotope ratio mass spectrometer interfaced with a High Temperature Conversion system. The gas chromatograph injector, analytical column, carrier flow and temperature ramp conditions were identical to the above. The H_3^+ factor was determined daily prior to standard calibration and sample analysis with an average of 2.25 ± 0.11 during the 10 days of measurement. All samples were measured at least twice at two different concentrations and the values reported here represent the mean of all four analyses. Individual *n*-alkane isotope ratios were corrected to primary in-house *n*-alkane reference materials, which had previously been calibrated to the VSMOW scale and analysed daily at several concentrations [*n*-C_{20} (–54‰), *n*-C_{22} (–39‰), *n*-C_{24} (–36‰), *n*-C_{28} (–250‰) and *n*-C_{32} (–236‰)]. Accuracy for compound-specific measurements was ± 4‰ (1σ, $n = 53$) as determined from a secondary QC *n*-alkane reference material [*n*-C_{36} (–240‰)].

Leaf water model and data analysis

A general model for the evaporative enrichment of a free water surface was first developed by Craig & Gordon (1965). To model leaf water, we used an expanded Craig–Gordon model to include leaf boundary layer considerations and diffusion through stomata:

$$R_{wl} = \alpha^* \left[\alpha_k R_{wx} \left(\frac{e_i - e_s}{e_i} \right) + \alpha_{kb} R_{wx} \left(\frac{e_s - e_a}{e_i} \right) + R_a \left(\frac{e_a}{e_i} \right) \right], \qquad (2)$$

where the subscripts wl, wx and a refer to leaf water, xylem water and bulk air, respectively (Flanagan & Ehleringer 1991). The vapour pressures of the intercellular air spaces of the leaf, the leaf surface and the bulk are e_i, e_s and e_a, respectively. Leaf surface pressures were determined from the equations of Ball (1987). α^* is the liquid–vapour equilibrium fractionation factor that varies with temperature according to the equations of Majoube (1971), α_k is the kinetic fractionation associated with diffusion in air (1.0164; Cappa *et al.* 2003) and α_{kb} is the kinetic fractionation associated with diffusion through the boundary layer and is calculated by increase α_k to the ⅔ power (¹H/²H = 1.0110).

When applied to leaves, the Craig–Gordon model contains a number of assumptions that may not be valid in natural systems. These assumptions may lead to potential discrepancies between the model and bulk leaf water ²H/¹H values. In particular, assumptions of isotopic steady state, constant water volume and isotopic homogeneity may not always be valid

(Yakir 1998). Significant spatial and temporal heterogeneity in the isotope ratios of leaf waters have been observed and may relate to a non-steady state (Farquhar & Cernusak 2005; Ometto *et al.* 2005; Lai *et al.* 2006), variations in stomatal conductance (Mott 1995), water compartmentalization between the vein and mesophyll, gradients within the leaf tissues (Farquhar & Lloyd 1993) and diurnal variations associated with variations in humidity (Cernusak *et al.* 2002). Any one or a combination of these effects may produce consequences in that a simple leaf water model cannot account.

To assess the effects of ²H enrichment in leaf water on the δ^2H isotope values of leaf waxes, we determined the apparent fractionation (ε_{app}) and biosynthetic fractionation (ε_{bio}) between leaf waxes and plant source water and modelled leaf water, respectively. ε is defined as

$$\varepsilon = [R_{lipid} / R_{water} - 1], \qquad (3)$$

where R represents the ²H/¹H abundance ratio, and R_{lipid} and R_{water} are the ratios in the *n*-alkane and growth water or modelled leaf water, respectively. As with delta values, epsilon values are reported in per mil notation, which implies a factor of 1000 (Coplen 2011).

Statistics

Statistical analysis was completed using JMP® 10 Pro (SAS, Cary, NC, USA) and PRISM (GraphPad Software, Inc., La Jolla, CA, USA) for Mac OS X. Outliers were tested for using the Grubbs test at the $\alpha = 0.01$ level. Ordinary least squares (OLS) regression was used to compare the measured δ^2H values of *n*-alkanes to the measured δ^2H values of treatment waters and to the δ^2H values of modelled leaf waters. *t*-Tests were used to assess differences between humidity treatments for the given species. Regression lines were fitted to data only when the slope of the line was significantly different from 0 at $\alpha = 0.01$ level. The slopes and the *y*-intercept of the regression lines for the data sets were compared using one-way analysis of variance (ANOVA) and a Tukey's post-hoc test to identify differences at $\alpha = 0.01$.

RESULTS

Compound distributions and hydrogen isotope values of leaf wax *n*-alkanes

B. occidentalis produced *n*-C_{23}-C_{35}, with *n*-C_{31} (54 ± 38 μg g⁻¹) being the most abundant, approximately five times more abundant than the next most abundant *n*-alkanes, *n*-C_{29} (13 ± 8 μg g⁻¹) and *n*-C_{27} (10 ± 5 μg g⁻¹). *P. fremontii* produced *n*-C_{23}-C_{31} but predominantly *n*-C_{27} and *n*-C_{29}. For *n*-alkanes collected from *P. fremontii*, *n*-C_{29} (85 ± 48 μg g⁻¹) was about seven times more abundant than the next most abundant *n*-alkane, *n*-C_{27} (13 ± 8 μg g⁻¹). No other *n*-alkanes collected from *P. fremontii* were greater than 6 μg g⁻¹. Thus, throughout the remainder of the text and for analysis, we use *n*-C_{31} for *B. occidentalis* and *n*-C_{29} for *P. fremontii*.

We compiled peak areas of high-molecular weight *n*-alkanes in order to quantify variations in distributions of *n*-alkanes. Strong odd-over-even predominance in modern leaf wax *n*-alkanes is consistent with previous observations (Eglinton & Hamilton 1967; Lockhart *et al.* 1997; Piasentier *et al.* 2000). Carbon preference indices (CPIs) were used to assess the odd-over-even predominance and calculated following Marzi *et al.* (1993):

$$CPI = \frac{\begin{array}{c}(A_{23} + A_{25} + A_{27} + A_{29} + A_{31} + A_{33}) \\ + (A_{25} + A_{27} + A_{29} + A_{31} + A_{33} + A_{35})\end{array}}{2(A_{24} + A_{26} + A_{28} + A_{30} + A_{32} + A_{34})}, \quad (4)$$

where *A* represents the area of the individual *n*-alkane peak from the chromatograph trace. *B. occidentalis* and *P. fremontii* had average CPIs of 14.3 ± 6.8 and 12.9 ± 4.7, respectively (Table 1).

To assess if *B. occidentalis* and *P. fremontii* average chain lengths (ACLs) varied in response to humidity and temperature, we used the following:

$$ACL = \frac{\begin{array}{c}(A_{23}(23)) + (A_{25}(25)) + (A_{31}(27)) + (A_{29}(29)) \\ + (A_{31}(31)) + (A_{33}(33)) + (A_{35}(35))\end{array}}{(A_{23} + A_{25} + A_{27} + A_{29} + A_{31} + A_{33} + A_{35})}. \quad (5)$$

B. occidentalis had average ACLs of 30.3 ± 0.4 and 30.0 ± 0.6 for the high and low humidity treatments, respectively. *P. fremontii* had average ACLs of 28.7 ± 0.2 and 28.4 ± 0.5 for the high and low humidity treatments, respectively (Table 1). We found no systematic variation in *n*-alkanes distribution within a species between the humidity treatments for both *B. occidentalis* and *P. fremontii* ($P > 0.01$).

Growth conditions and hydrogen isotope values of environmental inputs

The mean daily greenhouse air temperature varied between 21 and 29 °C during the course of the experiment (Fig. 2). Slightly lower temperatures were observed in the high humidity greenhouse due to evaporative cooling from the humidification system with average values of 23.9 and 25.5 °C for the high and low humidity greenhouses, respectively (Fig. 2). The relative humidity within the two greenhouses varied with humidity inputs from the evaporative cooling system that drew from ambient air. The 'high humidity' treatment was approximately twice that of the 'low humidity' treatment, with average values of 64.3 and 30.4% for the high and low humidity greenhouses, respectively (Fig. 2).

Table 1. Carbon preference index (CPI) and average chain length (ACL) by humidity treatment

Species	Treatment	CPI	SD	ACL	SD
Populus fremontii	High RH	14.9	5.2	28.7	0.7
Populus fremontii	Low RH	10.9	4.7	28.5	0.5
Betula occidentalis	High RH	14.1	7.1	30.3	0.4
Betula occidentalis	Low RH	14.4	6.9	30.0	0.6

RH, relative humidity; SD, standard deviation.

Stomatal conductance and transpiration rates were measured mid-morning at three intervals during the experiment. During the course of the experiment, stomatal conductance ranged from 0.248 to 0.340 mol m^{-2} s^{-1} and from 0.162 to 0.284 mol m^{-2} s^{-1} for *B. occidentalis* and *P. fremontii*, respectively (Roden & Ehleringer 1999a). No differences in stomatal conductance between humidity and growth water treatments were detected. Measured transpiration rates for *B. occidentalis* ranged from 5.1 to 6.9 mol m^{-2} s^{-1} and from 2.7 to 3.9 mmol m^{-2} s^{-1} for low and high humidity treatments, respectively (Roden & Ehleringer 1999a). Measured transpiration rates for *P. fremontii* ranged from 3.3 to 5.7 mol m^{-2} s^{-1} and 2.0 to 3.4 mmol m^{-2} s^{-1} for low and high humidity treatments, respectively. Plants grown in the low humidity greenhouse transpired more water than in the high humidity greenhouse for both species (Roden & Ehleringer 1999a).

The δ^2H value of atmospheric vapour (δ^2H_{atmo}) in the two greenhouses was sampled at four intervals during the experiment. δ^2H_{atmo} values remained relatively stable throughout the course of this study and ranged between –115 and –155‰ (Fig. 1). While the high humidity greenhouse had more positive δ^2H_{atmo} values of atmospheric water vapour later in the experiment, no replication was performed to enable statistical analysis of these differences.

The δ^2H value of treatment growth water (δ^2H_{tw}) was maintained throughout the experiment for each treatment (Fig. 1). Treatment water was sampled both prior to and after water additions to track variations in the δ^2H value of tank water. No significant differences in the δ^2H_{tw} value were observed between treatment replicates and greenhouses. Given the lack of variation, we present the means of four δ^2H_{tw} values in Fig. 1.

As it was impractical to sample enough leaves at a sufficient frequency to capture temporal and spatial variability in leaf water of trees used in this experiment, the Craig and Gordon model was used to estimate the δ^2H value of leaf water for the different growth water and humidity treatments. As leaf waxes for broad-leaved angiosperms are produced early in the ontogeny of the leaf, the δ^2H value of modelled leaf water (δ^2H_{mlw}) was calculated for the humidity and growth water treatment at the first interval during the experiment when atmospheric water vapour was collected (Figs 1 & 2). Stomatal conductance and transpiration rates measured at a second interval were used for calculations of modelled leaf water for both the first and the second intervals, as these parameters were not measured at the first interval (Roden & Ehleringer 1999a). Leaf temperatures were assumed to reflect the measured air temperature. Modelled leaf water δ^2H_{mlw} values ranged from –59 to 62‰ and from –49 to 160‰ for the high and low humidity treatments, respectively.

Hydrogen isotope values of leaf wax *n*-alkanes

The δ^2H values of *B. occidentalis* and *P. fremontii n*-alkanes ($\delta^2H_{n\text{-alkane}}$) varied linearly with growth water δ^2H values (Fig. 3). The δ^2H values of *B. occidentalis* and *P. fremontii n*-alkanes ranged between –225 and –1‰ (Supporting Information Table S2). Multiple plants grown under each
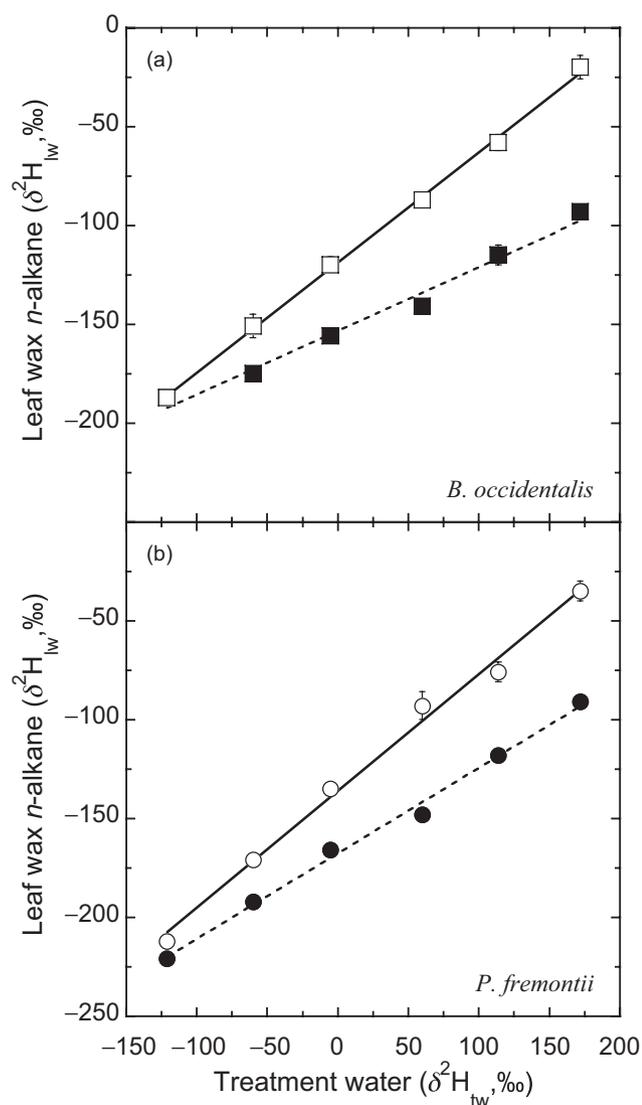
Figure 3. Isotope ratios of treatment water against leaf wax *n*-alkane for *Betula occidentalis* (a) and *Populus fremontii* (b). Solid and open symbols represent high and low humidity treatments, respectively.

treatment water and humidity combination were measured several times to allow for population statistics to be calculated. Regression analysis produced linear relationships between δ^2H values of *B. occidentalis* *n*-alkanes and δ^2H values of growth waters of $\delta^2H_{n\text{-alkane}} = 0.32\ \delta^2H_{tw} - 153$ and $\delta^2H_{n\text{-alkane}} = 0.56\ \delta^2H_{tw} - 119$ for the high and low humidity treatments, respectively (Fig. 3a). Similarly, we found regressions between *P. fremontii* $\delta^2H_{n\text{-alkane}}$ and δ^2H_{tw} values of $\delta^2H_{n\text{-alkane}} = 0.44\ \delta^2H_{tw} - 167$ and $\delta^2H_{n\text{-alkane}} = 0.59\ \delta^2H_{tw} - 136$ for the high and low humidity treatments, respectively (Fig. 3b). All regressions between $\delta^2H_{n\text{-alkane}}$ and δ^2H_{tw} values had $r^2 \geq 0.98$ and $P < 0.0001$. Plants grown under low humidity conditions produced *n*-alkanes with more positive δ^2H values than plants exposed to higher humidity. These differences in slope and intercept were statistically significant ($P < 0.01$) between both humidity treatments for both species and sug-

gested that humidity, and thus evapotranspiration, has a strong effect on $\delta^2H_{n\text{-alkane}}$ values.

Calculated ε_{app} values between *B. occidentalis* and *P. fremontii* *n*-alkanes and treatment water δ^2H values were linearly related to treatment water δ^2H values (Table 2). The ε_{app} values of *B. occidentalis* *n*-alkanes ranged between −228 and −67‰, with an average of −145 ± 49‰ (Table 2). The ε_{app} values of *P. fremontii* *n*-alkanes ranged between −230 and −103‰, with an average of −157 ± 36‰ (Table 2).

Regression analysis produced linear relationships between δ^2H values of *B. occidentalis* *n*-alkanes and modelled leaf water δ^2H values of $\delta^2H_{n\text{-alkane}} = 0.77\ \delta^2H_{mlw} - 147$ and $\delta^2H_{n\text{-alkane}} = 0.78\ \delta^2H_{mlw} - 148$ for the high and low humidity treatments, respectively. Similarly, we found regressions between *P. fremontii* $\delta^2H_{n\text{-alkane}}$ and δ^2H_{mlw} values of $\delta^2H_{n\text{-alkane}} = 1.05\ \delta^2H_{mlw} - 158$ and $\delta^2H_{n\text{-alkane}} = 0.81\ \delta^2H_{mlw} - 166$ for the high and low humidity treatments, respectively. All regressions between $\delta^2H_{n\text{-alkane}}$ and δ^2H_{mlw} values had $r^2 \geq 0.98$ and $P < 0.0001$. The combined relationships for high and low humidity treatments for *B. occidentalis* and *P. fremontii* were $\delta^2H_{n\text{-alkane}} = 0.78\ \delta^2H_{mlw} - 147$ and $\delta^2H_{n\text{-alkane}} = 0.82\ \delta^2H_{mlw} - 162$, respectively (Fig. 4). Both regressions had $r^2 \geq 0.98$ and $P < 0.0001$. We found that the slopes for the combined regression between leaf wax and modelled leaf water δ^2H values were not statistically different between the two species, while the intercepts were different. Thus, we are not justified to combine both *B. occidentalis* and *P. fremontii* relationships into a single regression.

ε_{bio} values between *n*-alkanes and modelled leaf water δ^2H values were not significantly correlated with treatment water δ^2H values. ε_{bio} of *B. occidentalis* *n*-alkanes ranged between −173 and −128‰, with an overall mean of −150 ± 9‰ and an average of −147 ± 8‰ and −152 ± 10‰ for the high and low humidity treatments (Table 2). ε_{bio} of *P. fremontii* *n*-alkanes ranged between −183 and −139‰, with an overall mean of −164 ± 10‰ and an average of −158 ± 10‰ and −167 ± 9‰ for the high and low humidity treatments (Table 2). While the ranges of ε_{bio} for each humidity treatment overlapped and appear very similar, we found statistical differences in ε_{bio} between the two humidity treatments from either species [$t(78) = 2.3607$, $P = 0.0207$ and $t(69) = 4.1296$, $P = 0.0001$ for *B. occidentalis* and *P. fremontii*, respectively]. In addition, calculated ε_{bio} values were statistically different between the two species [$t(149) = 8.6778$, $P < 0.0001$].

DISCUSSION

Effects of humidity treatment on the compound distributions of *n*-alkanes

We found that *B. occidentalis* and *P. fremontii* *n*-alkane compositions were dominated by $n\text{-}C_{31}$ and $n\text{-}C_{29}$, respectively. These data are consistent with previous studies of *Betula* (Sachse *et al*. 2006; Pedentchouk *et al*. 2008) and *Populus* (Kahmen *et al*. 2011, 2013b; Tipple *et al*. 2013) species *n*-alkane distributions. Compound distributions of *B. occidentalis* and *P. fremontii* had remarkably consistent leaf wax compositions with less than 5% variations in ACL for

Table 2. Hydrogen isotope values of waters and leaf waxes

Species	Treatment	δ^2H_{tw}	δ^2H_{mlw}	δ^2H_{lw}	Pooled SD	ε_{app}	Pooled SD	ε_{bio}	Pooled SD	n
Populus fremontii	High RH	−121	−59	−221	2	−114	2	−172	2	5
Populus fremontii	High RH	−60	−34	−192	2	−140	3	−163	3	4
Populus fremontii	High RH	−5	−11	−166	3	−162	3	−156	3	5
Populus fremontii	High RH	60	16	−148	1	−196	1	−161	1	4
Populus fremontii	High RH	114	38	−118	3	−208	2	−150	3	4
Populus fremontii	High RH	172	62	−91	2	−224	2	−144	2	5
Populus fremontii	Low RH	−121	−49	−212	0	−104	0	−171	0	5
Populus fremontii	Low RH	−60	−6	−171	1	−118	1	−166	1	4
Populus fremontii	Low RH	−5	34	−135	2	−131	2	−163	2	9
Populus fremontii	Low RH	60	80	−93	7	−145	6	−160	6	6
Populus fremontii	Low RH	114	119	−76	5	−171	5	−174	5	7
Populus fremontii	Low RH	172	160	−35	5	−177	4	−168	4	13
Betula occidentalis	High RH	−121	−59	−187	1	−75	2	−136	1	5
Betula occidentalis	High RH	−60	−34	−175	1	−122	1	−145	1	4
Betula occidentalis	High RH	−5	−11	−156	2	−152	2	−146	2	5
Betula occidentalis	High RH	60	16	−141	1	−190	1	−155	1	8
Betula occidentalis	High RH	114	38	−115	5	−206	4	−148	5	8
Betula occidentalis	High RH	172	62	−93	2	−227	2	−147	2	5
Betula occidentalis	Low RH	−121	−49	−187	3	−76	3	−145	3	6
Betula occidentalis	Low RH	−60	−6	−151	6	−96	7	−146	6	8
Betula occidentalis	Low RH	−5	34	−120	4	−115	4	−148	4	5
Betula occidentalis	Low RH	60	80	−87	4	−139	4	−155	4	5
Betula occidentalis	Low RH	114	119	−58	4	−155	4	−158	4	10
Betula occidentalis	Low RH	172	160	−20	6	−164	5	−155	5	11

RH, relative humidity; SD, standard deviation.

a given species (Table 1). Due to the functionality of leaf waxes to control water balance, the compound distribution of leaf wax has been suggested to be dependent upon environmental parameters (Shephard & Griffiths 2006). Several recent studies on modern plants have demonstrated strong linkages between chain length distribution and temperature, where higher growth temperatures correlated with the production of a greater proportion of longer *n*-alkane chain lengths (Sachse *et al*. 2006; Tipple & Pagani 2013). Leaf wax-derived *n*-alkanes extracted from dust samples have shown increased ACL with increased aridity, suggesting humidity may have an affect on chain length distributions (Rommerskirchen *et al*. 2003, 2006; Schefuss *et al*. 2003; Hughen *et al*. 2004; Vogts *et al*. 2009, 2012). We found that chain length distributions were not different among the high and low humidity treatments for a given species. As these plants were grown hydroponically and were not water limited, these data indicate that humidity alone did not affect *n*-alkane distribution of plants used in this study. These results suggest that additional factors (i.e. temperature and/or a water stress) may be the primary drivers of chain length variability within an individual species.

Variations in the δ^2H values of *n*-alkanes within species

We found that inter-plant variability of the δ^2H values of *n*-alkanes was minimal, with the majority of plants grown under the same condition producing similar δ^2H values of

n-alkanes. However, we observed substantial differences (±10–19‰) in the δ^2H values of *n*-alkanes of several individual *P. fremontii* and *B. occidentalis* plants. While these individuals appear to be different from the population, they were not identified as outliers (Supporting Information Table S1). This result was unexpected as we hypothesized that identical plants grown under indistinguishable climate and growth conditions should have very similar δ^2H values of leaf waxes.

There are several possible explanations for these observations. Here, we found that the largest inconsistency in δ^2H values occurred in the plants treated with the most ^{2}H-enriched source waters (Table 2). One possibility is that there may be additional uncertainty associated with the most positive δ^2H values of *n*-alkanes, given that these measured values fall near or slightly outside the reference material brackets. However, the variability within the individual plants subjected to the most ^{2}H-enriched source waters was not different than the other treatments, suggesting that the measured isotope values are reliable (Supporting Information Table S1). An alternative prospect is that plants subjected to the lower humidity conditions may be more variable as several studies have observed in field-grown plants experiencing low humidity conditions indicate more variability in the δ^2H values of leaf waxes (Hou *et al*. 2007a; Feakins & Sessions 2010a). However, in this study, we found that plants grown under the low humidity treatment did not have significantly more variable δ^2H values of *n*-alkanes than those from plant grown in the higher humidity greenhouse ($P = 0.0787$),
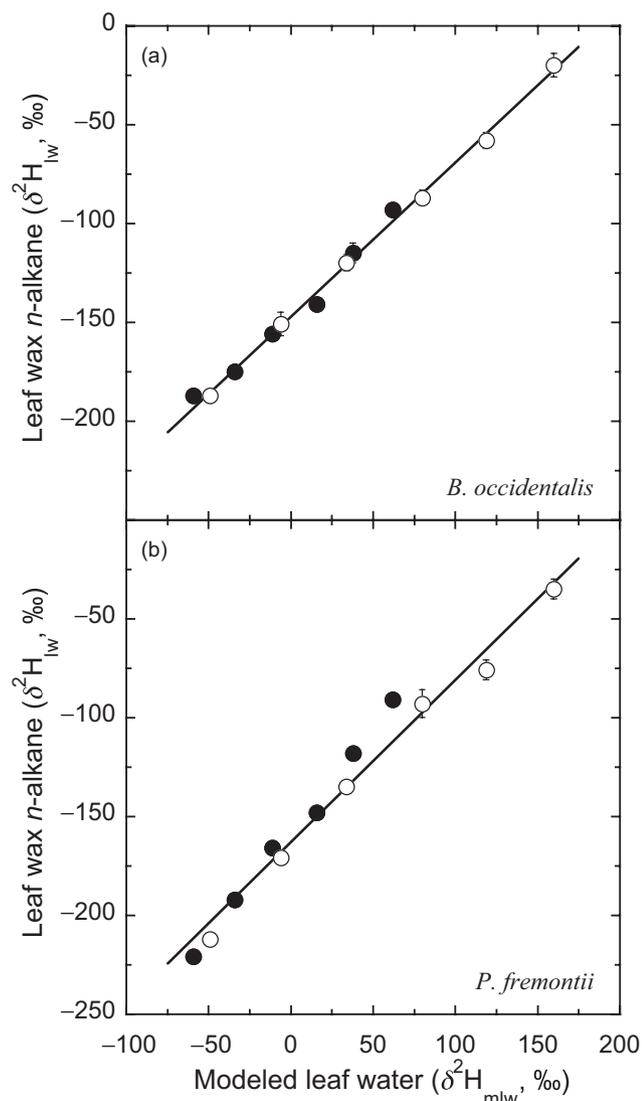
Figure 4. Modelled leaf water against measured *n*-alkane δ^2H values. Solid and open symbols represent high and low humidity treatments, respectively.

indicating in this case humidity had no affect on the inter-plant variability.

Another potential reason for this observation is that some tree saplings were in the beginning stages of leaf development prior to the start of the experiment (see the Materials and Methods section). In this case, the δ^2H values of leaf wax *n*-alkanes from these plants might reflect an earlier period and were not fully growth under the controlled conditions. It has been established that the δ^2H values of leaf waxes reflect the plant-water environment in the earliest ontogeny of the leaf (Kahmen *et al.* 2011; Tipple *et al.* 2013). Further, it has been shown for a similar *Populus* species as the one used in this experiment that leaf wax lipids are largely created during the bud-swelling phase of leaf development (Tipple *et al.* 2013).

Finally, it is also possible that differences in δ^2H values of *n*-alkanes may reflect variations in the isotope ratio or

sources of NADPH used in their biosynthesis. Differences in δ^2H values of lipids from different compound classes were derived from the precursor molecule and variations in the source of NADPH used in the biosynthesis of lipids (Schmidt *et al.* 2003; Zhang *et al.* 2009) and this model has been applied to explain variations between distinct compound classes and photosynthetic pathways (McInerney *et al.* 2011; Kahmen *et al.* 2013b). Recently, Kahmen *et al.* (2013a,b) suggested that variable contributions of unique NADPH sources could explain differences in the δ^2H values of *n*-alkanes of different plant families. As we focused upon the same leaf wax compounds within a single species grown under identical growth conditions and source waters, variations in the δ^2H values of *n*-alkanes may also reflect differences in partitioning of distinct NADPH sources to the hydrogen in *n*-alkanes during the early developmental stages.

Therefore, at this point, we can only speculate regarding the reasons for the isotopic differences of plants grown under identical conditions; however, subtle differences in phenology may have altered the timing of leaf wax synthesis and NADPH sources may have affected the δ^2H values of leaf wax *n*-alkanes from plants grown side by side.

Leaf transpiration and atmospheric exchange affect leaf wax hydrogen isotope values

An understanding of the hydrological and ecological information recorded in the $^2H/^1H$ ratios of leaf waxes and how these materials relate to other plant tissues remains ambiguous as the environmental and biological processes that shape the δ^2H values of leaf wax *n*-alkanes have not been sufficiently resolved. Nonetheless, the most recent research suggests clear connections between the leaf water environment and the isotope ratios of leaf waxes (Kahmen *et al.* 2011, 2013a,b; Tipple *et al.* 2013).

Studies of modern leaf wax have explored environmental correlations, most often focus upon plants growing along climatic gradients or controlled conditions to assess the relationship between the δ^2H isotope values of leaf waxes and environmental parameters (Sessions *et al.* 1999; Chikaraishi & Naraoka 2003, 2006; Bi *et al.* 2005; Krull *et al.* 2006; Liu *et al.* 2006; Sachse *et al.* 2006, 2009; Smith & Freeman 2006; Hou *et al.* 2007b; Liu & Yang 2008; Pedentchouk *et al.* 2008; Feakins & Sessions 2010a; Kahmen *et al.* 2013a; Tipple & Pagani 2013). These studies indicate that the apparent or observed hydrogen isotope fractionation (ε_{app}) between leaf wax and precipitation ranged from −34 to −204‰ varying with environmental conditions (see Sachse *et al.* 2012).

It is expected that ε_{app} varies as a function of climate as plants do not directly use precipitation in the biosynthesis of leaf wax; however, net biosynthetic fractionation (ε_{bio}) between leaf wax compounds and the pool of water used during biosynthesis may be complicated due to potential differences in biosynthetic pathway (Sessions *et al.* 1999; Diefendorf *et al.* 2011), metabolic pathways (Zhang *et al.* 2009; Kahmen *et al.* 2013b) or number of carbon atoms in the chains (Zhou *et al.* 2010). Nonetheless, the biosynthetic water pool represents some combination of source waters available

to the plant and leaf water, both of which can be significantly ^{2}H-enriched due to evaporation. Thus, the ^{2}H/^{1}H ratio of leaf waxes largely records the ^{2}H/^{1}H ratio of the plant's biosynthetic water pool (Feakins & Sessions 2010a; Kahmen *et al.* 2013b). However, the δ^2H isotope values of biosynthetic waters have been difficult to directly obtain. In order to assess how the biosynthetic water pool and leaf wax were affected by humidity, several recent studies have sought to measure the δ^2H isotope values of leaf and stem waters along with leaf wax *n*-alkanes of field- and greenhouse-grown plants (Feakins & Sessions 2010a; Kahmen *et al.* 2013b). These experiments allow for the isolation of confounding factors such as variations in source water, temperature and light intensity. However, establishing the δ^2H isotope values of the biosynthetic water pool within a plant remains a challenge. While these recent studies both argue that leaf waxes record the plant-water environment through leaf water ^{2}H enrichment, they do not agree on the absolute values and stability of ε_{bio} for plant leaf wax synthesis.

To separate the potentially confounding variables of environment and biology, we grew two temperate angiosperm tree species in six different and distinct source waters with hydrogen isotope values ranging from −120 to +180‰ and under two humidity conditions for an entire growing season. While these source waters do not represent a natural system, the wide range of source water δ^2H values allowed for the effects of transpiration to be clearly illustrated. We found clear ^{2}H enrichment in leaf waxes for trees grown under low humidity conditions (Fig. 3). The δ^2H values of leaf wax *n*-alkanes for the low humidity treatment in both species were more enriched in ^{2}H than the higher humidity treatment (Fig. 3). Higher δ^2H values indicate increased preferential transpirational loss of ^{1}H to the atmosphere from the leaf water exposed to less humid conditions. These data are consistent with recent controlled growth experiments on broad-leaved angiosperms grown with identical δ^2H values of source water (Kahmen *et al.* 2013b). The clear difference in slope between the two humidity treatments indicates increased ^{2}H enrichment in leaf waxes of plants exposed to lower humidity atmospheres (Fig. 3). Thus, these data show that evaporative ^{2}H enrichment in leaf water is reflected in the δ^2H values of leaf waxes of broad leaf species. These findings suggest that transpiration can alter the δ^2H values of leaf waxes and that the δ^2H value of leaf water is the dominant factor in shaping leaf wax isotope signatures.

We noted that the apparent fractionation values (ε_{app}) between source waters and leaf wax *n*-alkanes for the low humidity treatment in both species were more positive than the higher humidity treatment (Table 2). More positive ε_{app} values indicate increased ^{2}H enrichment of leaf water under less humid conditions. These data are in accordance with recent controlled growth and natural experiments on broad-leaved plant species under different humidity conditions (Feakins & Sessions 2010a; Kahmen *et al.* 2013b). These data demonstrate that the δ^2H values and ε_{app} values for leaf waxes of broad-leaved angiosperms are altered by both the δ^2H values of atmospheric water and humidity. Together, these

findings suggest that the δ^2H value of leaf water is the dominant factor in shaping leaf wax isotope signatures.

The ^{2}H/^{1}H ratio of leaf waxes is derived from leaf water at the time of synthesis

Our findings indicate that δ^2H values of *n*-alkanes are ^{2}H-enriched due to the effects of leaf evapotranspiration. To elucidate the relationships between leaf water and leaf wax *n*-alkanes, we modelled the δ^2H value of leaf water using the modified Craig and Gordon model. We found extremely strong correlations between the measured δ^2H values of leaf waxes and modelled δ^2H values of leaf waters for both humidity conditions in both species (Fig. 4). These findings provide strong evidence that the δ^2H values of *n*-alkanes record the environmental variables that altered leaf water ^{2}H/^{1}H ratios in these broad-leaved species.

The hydrogen isotope ratios of plant materials are expected to behave as a single component fractionation model as all the hydrogen within plants derives from environmental waters (Schmidt *et al.* 2003). For a single component fractionation model, the slope (α) and intercept (ε) of the regression should provide the single constant fractionation as $\varepsilon = \alpha - 1$, which represents all biosynthetic processes (Sessions & Hayes 2005). We expect these factors to be constant between both species in this study as the biosynthetic pathways are identical. Furthermore, this factor should be represented by the single biosynthetic fractionation (ε_{bio}) that represents all fractionation steps that occurred during the biosynthesis of leaf waxes (Sessions & Hayes 2005). We found that the fractionation factors calculated from the slopes (α_{slope}) indicate a fractionation factor near 0.8 (0.78 and 0.82 for *B. occidentalis* and *P. fremontii*, respectively) for the fractionation between lipid and leaf water (Fig. 4). The fractionation factors calculated from the intercept ($\alpha_{intercept}$) equal 0.84 and 0.85 for *B. occidentalis* and *P. fremontii*, respectively. In the single component model, the fractionations should be equivalent. Here, we found that the fractionations derived from *B. occidentalis* data were not correspondent, while *P. fremontii* data indicated better agreement. These data suggest that the estimates of fractionation from the *P. fremontii* data are the closest to representing a single process with a ε_{bio} between −163 and −181‰. However, the fractionations derived from *B. occidentalis* were not equivalent, implying that the system may be more complex than previously assumed.

Recently, Kahmen *et al.* (2013b) reported ε_{bio} values −160‰ for *Populus balsamifera*, a related species to the *P. fremontii* used in this study. The plants grown by Kahmen *et al.* (2013b) were limited to a single source water value, while here we used a wide range of source water isotope ratios, and the fractionations we observed for *P. fremontii* are consistent with the prior findings (Fig. 4). This may indicate that species closely related to the *Populus* genera may have similar ε_{bio} values. However, variations in ε_{bio} of several dicot species have been observed and have been suggested to indicate that species may have relatively unique ε_{bio} values (Kahmen *et al.* 2013b).

These observed variations might be due to unique species-specific ε_{bio} values; however, the experimental design employed here makes it difficult to distinguish potentially small variations in ε_{bio} due to the large range in the δ^2H values of source water. An additional possibility is that differences in ε_{bio} values may also be due to inaccurate model parameterizations or tuning in the calculation of δ^2H values of leaf water. While our findings suggest that *B. occidentalis* and *P. fremontii* may indeed have slightly different ε_{bio} values, it has been shown that broad-leaved angiosperm leaf waxes are produced early in the growing season as leaves flush and reflect the leaf water dynamic during that interval (Kahmen *et al.* 2011; Tipple *et al.* 2013). Furthermore, it has been established that the δ^2H values of *n*-alkanes are not significantly altered after the period of leaf wax development (Tipple *et al.* 2013). Thus, it is important to understand when leaf waxes are formed for a particular species and the atmospheric water and environmental conditions of the distinct period of leaf wax development. A limitation of this current study is that the first atmospheric water vapour $^2H/^1H$ measurement was made nearly 2 months after the start of the experiment and was used to develop the leaf water model. Given that atmospheric water δ^2H values in the western United States can vary significantly in the early growing season (Tipple *et al.* 2013) and considerable diurnal variations in the δ^2H values of leaf water can occur (Cernusak *et al.* 2002), if waxes were synthesized prior to the first measurement of atmospheric water vapour or if *B. occidentalis* and *P. fremontii* biosynthesize leaf waxes at different times during the day or ontogeny, then this model may not capture the leaf water at the time of lipid synthesis. As an example of the sensitivity of the model to variations in leaf temperature and the δ^2H value of atmospheric water vapour, a change of 1 °C and 40‰ in atmospheric water vapour brings modelled leaf water δ^2H values for *B. occidentalis* in line with those of *P. fremontii*, with a slope of 0.83 and an intercept of −163‰. This example illustrates the sensitivity of δ^2H values of leaf waxes to variations in the plant-water environment at the time of wax synthesis and suggests that subtle variations in the δ^2H value of atmospheric water vapour may be reflected in lipid isotope signatures. Furthermore, the δ^2H value of atmospheric water vapour is often assumed to be in isotopic equilibrium with a plants' source water for Craig and Gordon modelling applications (McInerney *et al.* 2011) and these data suggest that if the δ^2H values of atmospheric water vapour are not correctly parameterized for the time of lipid synthesis, errors in relationships between leaf water and leaf wax isotope ratios may occur.

It is expected that lipid biosynthetic pathway are universal between similar plant groups (Schmidt *et al.* 2003); however, many empirical studies of greenhouse-grown and field-grown plants have borne out the limited stability of these fractionations (Yang *et al.* 2009; Zhou *et al.* 2011; Sachse *et al.* 2012; Kahmen *et al.* 2013a). However, the use of δ^2H values of leaf waxes to understand plant-water dynamics requires relatively stable ε_{app} and ε_{bio} values to assess leaf, source and meteoric waters in modern and ancient settings. Here, we have shown that the isotope ratio of leaf water is the dominant control on leaf wax isotope ratios and that constraining the environmental conditions during the finite interval of lipid synthesis may be important to interpret δ^2H values of leaf waxes.

CONCLUSIONS

We grew *P. fremontii* and *B. occidentalis* saplings in an experimental hydroponic system with six different waters and two different humidities. We analysed hydrogen isotope ratios of high-molecular weight *n*-alkanes and observed highly correlated, linear relationships between the δ^2H values of *n*-alkanes and growth waters. The slope between leaf wax *n*-alkane and growth water δ^2H values for the two humidity conditions differed, suggesting that transpiration and the δ^2H value of atmospheric water vapour affected both the δ^2H values of leaf water and *n*-alkanes. A modified Craig and Gordon model was used to estimate the δ^2H values of leaf water for each humidity and source water combination. Leaf wax *n*-alkanes and modelled leaf water $^2H/^1H$ ratios for both species and humidity conditions were linearly related and combined regressions of δ^2H values of *n*-alkanes and modelled leaf water had slopes near 0.8. Our data suggest that the hydrogen isotope values of leaf waxes are a proxy for the δ^2H values of leaf water at the time of wax synthesis and that the δ^2H values of leaf waxes record variations in humidity and the δ^2H values of atmospheric water vapour. Thus, if humidity and the δ^2H values of atmospheric water vapour could be assumed or modelled for past conditions, then the δ^2H values of leaf waxes can be used to assess changes in the δ^2H values of source water.

In addition, we found that fractionations derived from the slope and intercept from *P. fremontii* was similar, while those from *B. occidentalis* were different, suggesting that the assumption regarding the relationship between leaf water and wax were more complex. The reason for this relationship remains unclear and indicates the possibility of species-specific biosynthetic fractionations or that different species may reflect slightly different periods of the growing season. A sensitivity analysis of the leaf water model demonstrated that subtle changes in the input parameters to the model could produce similar intercepts for *B. occidentalis*. We suggest that future work in both natural and controlled systems should focus on the interval of leaf wax synthesis to clarify if individual species has stable or variable ε_{bio} values.

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SUPPORTING INFORMATION

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

Table S1. Hydrogen isotope values of individual plants.
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