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Research paper

A controlled test of the dual-isotope approach for the interpretation of stable carbon and oxygen isotope ratio variation in tree rings

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Seedlings of a conifer (*Pinus radiata* D. Don) and a broad leaf angiosperm (*Eucalyptus globulus* Labill.) were grown for 100 days in two growth cabinets at 45 or 65% relative humidity. The seedlings were exposed to treatments designed to modify carbon assimilation rates and capacities, stomatal conductance and transpiration to test conceptual models that attempt to clarify the interpretation of carbon isotope discrimination ($\Delta^{13}C$) by using oxygen isotope enrichment ($\Delta^{18}O$). Differences in relative humidity and within-cabinet treatments (including lower irradiance, lower nitrogen inputs, higher leaf temperature and lower moisture status than control seedlings) produced significant differences in assimilation rates, photosynthetic capacities, stomatal conductance, leaf transpiration rates and leaf evaporative enrichment. The dual-isotope approach accurately interpreted the cause of variation in wood cellulose $\Delta^{13}C$ for some of the treatments, but not for others. We also tested whether we could use $\Delta^{13}C$ variation to constrain the interpretation of $\delta^{18}O$ variation. Carbon isotope discrimination appears to be influenced by transpiration (providing information on leaf evaporative enrichment), but the results did not provide a clear way to interpret such variation. The dual-isotope approach appears to be valid conceptually, but more work is needed to make it operational under different scenarios.

Keywords: cellulose, conceptual models, $\delta^{18}O$, $\delta^{13}C$, eucalypt, pine, wood.

Introduction

Isotopic analysis of plant organic matter is used extensively in plant physiology, ecology and paleoclimatogy (Ehleringer et al. 1993, Swart et al. 1993, Griffiths 1998, Dawson et al. 2002). Because tree rings can be precisely dated, inter-annual variation in ring width and stable isotope time-series provide valuable proxy data and historical perspectives for plant biology and ecosystem function (McCarroll and Loader 2004). Carbon isotope ratios ($\delta^{13}C$) in tree-ring cellulose have been linked to plant water status, intrinsic water use efficiency, temperature and vapor pressure deficit (Leavitt 1993, Saurer et al. 1995, Stewart et al. 1995, Hemming et al. 1998, Schleser et al. 1999, Barbour et al. 2002, Leavitt et al. 2002). The stable isotopes in organic matter that are derived from water (H and O) have been utilized to infer plant water sources, precipitation amount, historic temperature and humidity variation (Gray and Thompson 1976, White et al. 1985, Edwards et al. 1986, Dawson 1993, Thorburn and Walker 1993, Feng and Epstein 1994, Miller et al. 2006).

Mechanistic models have been developed to understand fractionation events leading to isotopic variation (Farquhar et al. 1982, Francey and Farquhar 1982, Roden et al. 2000, Hemming et al. 2001, Barbour et al. 2004). Unfortunately, $\delta^{13}C$ and $\delta^{18}O$ compositions of organic matter are each a function of two primary drivers. Carbon isotope discrimination is influenced by the CO_2 concentration at the site of Rubisco, the

primary carbon-fixing enzyme of photosynthesis (C_c [chloroplast CO_2 concentration], Farquhar et al. 1989), though intercellular CO_2 concentration (C_i) is often used in models as C_c and mesophyll resistance are difficult to estimate (see Barbour et al. 2010, for discussions on mesophyll conductance). More specifically, the drop in [CO_2] from ambient to intercellular ($C_a - C_i$) equals the assimilation rate divided by the stomatal plus boundary layer conductance, whereas the further drop ($C_i - C_c$) equals the assimilation rate divided by the leaf internal transfer conductance to CO_2 (Vitousek et al. 1990). Organic matter $\delta^{13}C$ is also a function of the $\delta^{13}C$ of atmospheric CO_2, but that can be accounted for by calculating discrimination ($\Delta^{13}C$, Farquhar and Richards 1984). There are many factors that correlate with $\delta^{13}C$ variation and thus appear to affect ^{13}C fractionation such as temperature, hydraulic conductivity, soil moisture, humidity, irradiance etc. (Walcroft et al. 1996, Warren et al. 2001, McCarroll and Loader 2004), but these are likely secondary processes that are influencing the primary effects of conductance and assimilation (CO_2 supply and demand).

The oxygen in organic matter is derived from water (DeNiro and Epstein 1979). Meteoric water $\delta^{18}O$ varies with condensation temperature and Rayleigh distillation processes (Dansgaard 1964, Welker 2000). Evaporation involves both kinetic and equilibrium fractionation events that 'enrich' the remaining water in the heavy isotopes (Craig and Gordon 1965, Dongmann et al. 1974). Transpiration is, however, a two-way process isotopically with a flux into a leaf from the air as well as one out of the leaf, and so ambient humidity and its isotopic composition influence the net effect (Farquhar et al. 2007). Isotopic fractionation in organic matter differs between autotrophic and heterotrophic metabolism for hydrogen but not for oxygen. In addition, isotopic exchange with medium water at the site of organic matter synthesis influences δ^2H and $\delta^{18}O$ values. Exchange and biochemical fractionation appear to be fairly well described and constrained using mechanistic models (Sternberg and DeNiro 1983, Luo and Sternberg 1992, Roden et al. 2000, Sternberg 2009). Thus, the $\delta^{18}O$ composition of organic matter is primarily affected by variation in source water $\delta^{18}O$ and leaf evaporative enrichment. Leaf water evaporative enrichment is complicated by spatial heterogeneity (Wang and Yakir 1995), transpirational flux of unenriched water from veins to the sites of evaporation (Péclet effect, Farquhar and Lloyd 1993) and non-steady-state conditions (Farquhar and Cernusak 2005). Variation in organic matter $\delta^{18}O$ may also be influenced by other environmental factors such as precipitation amount (Miller et al. 2006), temperature (White et al. 1994, but see also Roden and Ehleringer 2000) and atmospheric water vapor $\delta^{18}O$ variation (not in equilibrium with source water, White and Gedzelman 1984, Roden et al. 2000), but their impact may be secondary influences on source or leaf water enrichment or minor in comparison with the primary effects of evaporation and source water $\delta^{18}O$ variation.

Because $\delta^{13}C$ is influenced by two independent factors, determining which factor dominates under particular conditions is problematic. Some researchers have attempted to tease apart these factors using a second isotope ($\delta^{18}O$) in what may be called a dual-isotope approach (Scheidegger et al. 2000, Grams et al. 2007). The idea is to use $\delta^{18}O$ variation to constrain the interpretation of $\Delta^{13}C$ variation. Since $\delta^{18}O$ variation is driven by evaporative processes and stomatal conductance (g_s) is a main control point for transpiration, this linkage could be exploited for interpreting $\Delta^{13}C$ variation (Scheidegger et al. 2000, Grams et al. 2007). One main assumption of these models is that source water $\delta^{18}O$ is invariant and thus the majority of organic matter $\delta^{18}O$ variation is driven by evaporative enrichment. In some instances this assumption is not valid. For example, coast redwood is known to have two primary water sources, precipitation and fog, which are isotopically distinct (Ingraham and Matthews 1990, Dawson 1998) and both water sources may be incorporated into tree-ring cellulose (Roden et al. 2009).

In addition, these conceptual models use A_{max} (photosynthetic capacity) for the 'demand' side of the *A*:*g* relationship. There are a number of ways to quantify this parameter and it can be confusing as to whether model outputs are describing changes in environmentally or physiologically constrained maximum photosynthetic rates. Scheidegger et al. (2000) define A_{max} as 'the average maximum net photosynthesis at ambient CO_2 concentration and under optimal environmental conditions.' Sub-optimal environmental conditions (e.g., low light or temperatures) can reduce *A* without changes in physiological capacity (at saturating irradiance and optimal temperatures). But the *A* one obtained at the same g_s was reduced and in that important sense capacity was reduced. Environmental conditions (e.g., reduced soil nitrogen or chronic low light) can also modify the investment a leaf makes in photosynthetic systems leading to reductions in *A* because they are constrained by reduced physiological capacity. We here define A_{max} as *A* at saturating light intensity and at 1000 μmol mol^{-1} [CO_2] to measure physiological capacity, and in this study we measured both *A* and A_{max} in an attempt to clarify model interpretation.

Scheidegger et al. (2000) studied three herbaceous plants under field conditions where environmental parameters were uncontrolled and potentially variable. In addition, Keitel et al. (2006) and Sullivan and Welker (2007) tested these conceptual models under field conditions, but used tree species. Grams et al. (2007) grew seedlings of two tree species in controlled environments while modifying physiological traits through different CO_2 and ozone exposures. All these studies measured the isotopic composition of leaf organic matter rather than tree-ring cellulose. Another objective of this study was to test, under controlled experimental conditions, whether

the dual-isotope approach can be utilized for the interpretation of isotopic variation in tree-ring organic matter.

We were also interested in whether the approach could be modified to help interpret $\delta^{18}O$ variation. As with $\Delta^{13}C$, $\delta^{18}O$ is primarily influenced by two independent environmental factors: source water $\delta^{18}O$ and humidity (vapor pressure deficit or more precisely the difference between ambient and intercellular vapor pressures). Because of the linkage between stomatal conductance, transpiration and carbon isotope discrimination, it may be possible to use $\Delta^{13}C$ variation to constrain the interpretation of $\delta^{18}O$ variation. This would allow us to tease out the effects of source water $\delta^{18}O$ variation from humidity and evaporative enrichment. This dual-isotope approach would also require a simplifying assumption, that of constant photosynthetic capacity and/or rates. Thus, variation in $\Delta^{13}C$ would be primarily a function of stomatal conductance and that driven by evaporative conditions. This would allow a determination of the importance of different water sources (e.g., fog versus precipitation for redwood trees).

This experiment was designed to provide a number of treatments that would alter conductance, transpiration, photosynthetic rates or A_{max}. Source water $\delta^{18}O$ remained constant throughout. The main treatment difference was humidity. We used a needle leaf gymnosperm (*Pinus radiata* D. Don) and a broad leaf angiosperm (*Eucalyptus globulus* Labill.) to test diverse leaf morphologies and stem anatomies from ecotypes commonly used in isotope dendroclimatology. These species have also been used in studies on leaf water enrichment (Cernusak et al. 2005, M. G. Barbour, personal communication) providing information for each species on leaf effective path lengths (L_m) for use in the Péclet model (Farquhar and Lloyd 1993, Barbour et al. 2000). The concept that $\delta^{18}O$ variation in organic matter is primarily a function of variation in stomatal conductance needs further testing as this is a key assumption of these conceptual models. Our objective was to test how treatments that alter conductance, transpiration and photosynthetic rates influence both $\delta^{18}O$ and $\Delta^{13}C$ and whether a dual-isotope approach can clarify interpretations of isotopic variation in organic matter and tree rings in particular.

Materials and methods

Materials and growing conditions

Seeds from a needle leaf gymnosperm (*Pinus radiata*) and a broad leaf angiosperm (*Eucalyptus globulus* Labill. subsp. *bicostata* J. B. Kirkp), obtained from the Australian Tree Seed Centre (CSIRO Forestry), were germinated in flats containing standard 4:3:3:1 soil mixture of mushroom compost:moist river sand:sphagnum peatmoss (TE-EM Canadian):perlite (P500) in a controlled temperature greenhouse. As soon as seedlings were large enough to transplant, two of each species were planted in 5 l PVC pipe pots (150 mm diameter, 50 total) containing vermiculite, a non-nutritive medium. To reduce soil evaporation, all pots were sealed with closed cell foam with four slits to allow the seedlings to protrude and a small resealable port-hole that allowed water and nutrient inputs. The pots were watered to field capacity with a nutrient solution (1/4 strength Hoagland's) and weighed weekly with a balance (Mettler Toledo, Columbus OH, USA) and placed in one of two growth cabinets (Thermoline Plant Growth Cabinet, Wetherill Park, Australia).

Air and leaf temperatures were measured every 2 weeks in each cabinet with a thermocouple (Model 7001CH; Jenco Electronics Grand Prairie, TX, USA; note: growth cabinet air temperature was monitored continuously for environmental control but not recorded). Air temperature averaged 21 °C throughout the 100-day experiment. The primary treatment difference between growth cabinets was humidity. Growth chamber relative humidity (RH) was controlled and tracked within the cabinets (using wet bulb/dry bulb technology). Relative humidity was also measured and recorded four times (for 4–5 days each time) during the experiment using a more sensitive humidity sensor (Tinytag Ultra TGU 1500; Gemini Data Loggers Ltd, Chichester, UK) than that used by the cabinet humidity control system. Although humidity control produced large variance in RH (Figure 1c inset) the cabinets consistently produced a 20% difference in daytime RH. Light levels were measured every 2 weeks with a Li-Cor 190s quantum sensor (Li-Cor, Lincoln, NE, USA). The cabinets maintained values at 1000 (±100, SD) μmol m^{-2} s^{-1} (photosynthetically active radiation (PAR)) at seedling height.

Seedlings were grown in the cabinets for 100 days and exposed to five treatments for the entire period of growth. One treatment was the control with standard conditions for each cabinet (shorthand designation is C). The low-nitrogen treatment (N) was designed to reduce photosynthetic rates and A_{max} but not necessarily stomatal conductance. All treatments except for N (and the drought treatment, see below) received 1/4 strength Hoagland's solution weekly (see Epstein 1972 for details of macro- and micro-nutrient concentrations). The low-nitrogen treatment was generated by applying a Hoagland's solution modified to contain half the nitrogen inputs while maintaining the other mineral nutrients at similar levels (1/4 strength). The low-light treatment (L) was designed to reduce photosynthetic rates but not A_{max}. These plants were grown in the same growth cabinets but were under a framework covered in shade cloth that reduced light levels to 50% of that received by other plants. The increased temperature treatment (T) was designed to increase transpiration rates but not substantially influence stomatal conductance, photosynthetic rates or A_{max}. These plants were grown in the same cabinets, but exposed to lamps (FATI [free air temperature increase] described by Loveys et al. 2006) that emit directionally isolated

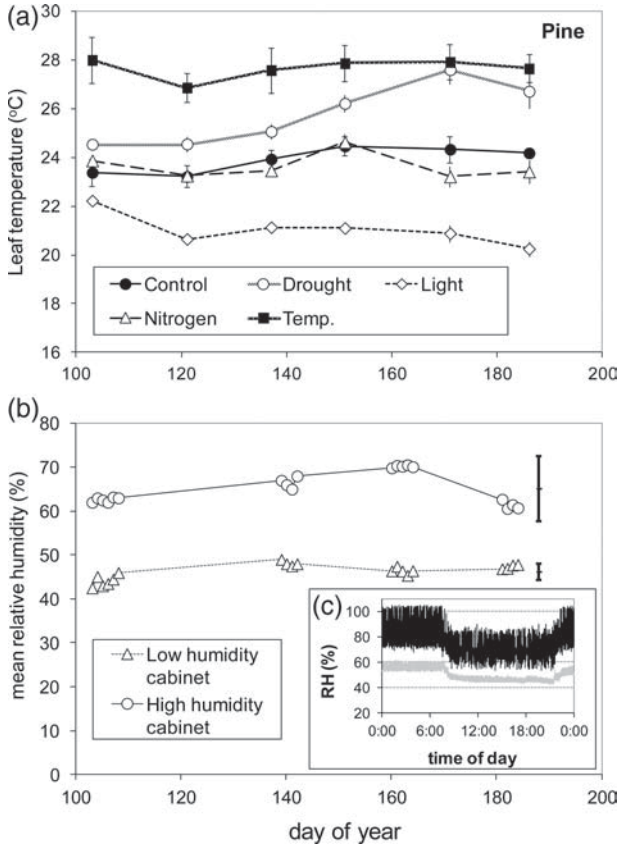

Figure 1. (a) Time course of leaf temperatures for *Pinus radiata* seedlings grown under five experimental conditions (values are mean ± SE pooled for both growth cabinets). (b) Mean daytime relative humidity for each growth cabinet for specific days over the course of the experiment. Bars indicate the standard deviation of mean values for all time periods. (c, inset) Diurnal trace of relative humidity for both cabinets showing humidity control and variability.

infrared radiation exposing the plants to a heat source without any visible wavelengths to influence photosynthesis. The drought treatment (D) was designed to influence stomatal conductance, transpiration and photosynthetic rates. These plants were initially watered after transplanting but not subsequently. All pots were weighed weekly, the amount of water needed to return the pots to field capacity (considering the plant to be a negligible component to pot weight) was tracked and water added according to usage. For the drought treatment, pots were allowed to dry out and seedling water potentials (Ψ_w) were monitored every 2 weeks with a pressure chamber (Soil Moisture Equipment, Santa Barbara, CA, USA). When drought stress appeared severe due to the combination of low Ψ_w and signs of wilting, the drought pots were watered with the amount of water transpired since the last weighing. This kept the drought plants alive but did not substantially reduce the effects of the water stress treatment. The drought treatment produced stunted plants which created problems for obtaining enough material for isotope analysis and so samples had to be pooled. All treatments were grown together in two cabinets (at different humidities, five pots per treatment per cabinet) but only the C, D and N treatments were periodically re-randomized in the cabinets. The T and L treatments had to be isolated from the others as the shade cloth and FATI lamps were positionally distinct.

Physiological measurements

Photosynthetic gas exchange was measured every 2 weeks on two seedlings grown in each treatment and in each growth cabinet using a Li6400 system (LiCor, Lincoln NE, USA). Each seedling was measured under ambient conditions (PAR = 1000 or 500 μmol m^{-2} s^{-1} for low light treatment, $[CO_2]$ = 375 ppm, RH = 50 or 80% [depending on cabinet], air temperature = 26 °C) and conditions designed to estimate A_{max} (PAR = 2000 μmol m^{-2} s^{-1}, $[CO_2]$ = 1000 ppm, RH and temperature same as ambient). Photosynthetic capacity can be defined in a number of ways (e.g., saturating CO_2 at prevailing light intensities). Here we are using saturating irradiance and elevated CO_2 in an attempt to provide conditions that test limitations on metabolism imposed by each treatment and to match the common understanding of what A_{max} means. Photosynthetic rates (A), stomatal conductance (g_s), transpirations rates (E) and intercellular CO_2 concentrations (C_i) were recorded every 2 weeks for the duration of the experiment.

Isotope sampling

Tap water used for irrigation was sampled every 2 weeks in glass vials and sealed for $\delta^{18}O$ analysis. Chamber water vapor was also sampled every 2 weeks for $\delta^{18}O$ analysis using a pump to draw chamber air through a glass trap submerged in a mixture of dry ice and ethanol. Samples for analysis of atmospheric CO_2 $\delta^{13}C$ were collected by drawing chamber air into evacuated cylinders and isolating the CO_2 into sealed glass tubes using a cryogenic cut-off line. Stems and leaves were sampled every 2 weeks on two seedlings per treatment and water was extracted for $\delta^{18}O$ analysis. We used a cryogenic vacuum water extraction technique developed by Dr Chin Wong (Wong personal communication, see also Ehleringer et al. 2000).

Organic matter sampling and cellulose extraction

At the end of the experiment, the remaining stems (those not destructively sampled previously) were dried and the outermost portions of wood were sampled under a 40× dissecting microscope using a scalpel. The outermost portion was sampled to avoid potential influences of carbohydrate sources not directly affected by the imposed treatments (seed source and initial conditions). Samples from the drought treatment were pooled as the seedlings were too small to produce enough material for individual sampling. The samples were ground to a fine powder in a ball mill and a subset was set aside for whole

wood analysis. Those destined for cellulose extraction were placed into Soxhlet thimbles with silica wool on each end to prevent loss of sample. The procedure is described in detail by Loader et al. (1997) and Barbour et al. (2002). Briefly, lipids and resins are extracted using chloroform and ethanol solvents in a Soxhlet apparatus. Lignin was extracted by bleaching in acidified sodium chlorite at 70 °C. Hemicellulose was removed in 10% NaOH followed by rinsing in distilled water.

Isotope analysis

For $\delta^{13}C$ analysis, ~2 mg of dried cellulose (or whole wood) was loaded into tin capsules, combusted in a Carlo-Erba elemental analyzer (Carlo-Erba, Milan, Italy) and introduced as CO_2 into a mass spectrometer (Micromass Isochrom; VG Isotech, Middlewick, UK). For $\delta^{18}O$ analysis, ~1.5 mg of dried cellulose (or whole wood) was loaded into silver capsules, pyrolysed in a Carlo-Erba elemental analyzer and introduced as CO into a different Micromass Isochrom mass spectrometer using the methods described by Farquhar et al. (1997). A beet sucrose standard was used for both analyses ($\delta^{13}C = -24.6‰$ PDB and $\delta^{18}O = 30.8‰$ VSMOW, SD = 0.2‰).

Statistical analysis

Data were analyzed by means of a general linear model analysis of variance using the Minitab 15 statistical program. A Tukey's multiple comparisons test was used to determine significant differences between treatments. Unless otherwise noted, $P < 0.05$ was used to reject null hypotheses.

Results

Air temperature in both cabinets was 21 °C, except for under the FATI lamps where it was on average 3 °C warmer. Throughout the experiment, leaf temperatures were similar between seedlings grown in the C and N treatments, and consistently higher and lower for seedlings in the T and L treatments, respectively (Figure 1a). Temperature reductions for leaves in the L treatment were presumably due to reduced radiative energy inputs. Leaf temperatures for plants exposed to the D treatment were initially similar to control seedlings then increased over time, presumably due to reduced conductance and latent heat loss. Eucalypt and pine leaf temperatures were similar. Although cabinet control of humidity includes significant variation (Figure 1c), mean RH was consistently ~20% greater in the high-humidity cabinet (Figure 1b).

Seedlings exposed to drought had substantially lower Ψ_w than control seedlings (Figure 2a). Eucalypt seedlings were more stressed than pine seedlings. The rise in Ψ_w after day 170 was the result of minimal watering to avoid lethal levels of stress. Over the course of the experiment, stomatal conductance (g_s) remained reasonably stable for seedlings in most treatments with the exception of D where it steadily declined

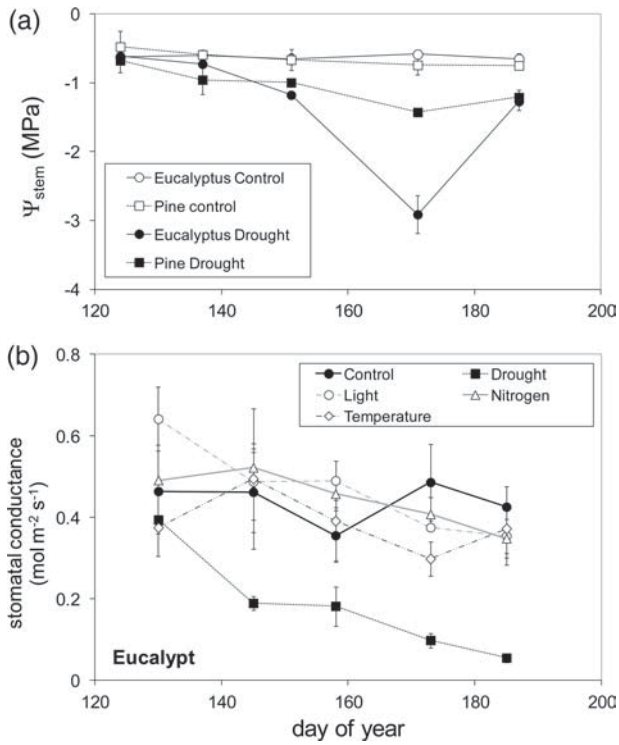

Figure 2. (a) Time course of mean mid-day water potentials of pine and eucalypt stems that were either exposed to drought (water withheld) or well-watered (control). Values are pooled means (±SD) from plants in both growth cabinets as there was no significant difference in Ψ_{stem} between humidity treatments. (b) Stomatal conductance for eucalypt seedlings grown under five experimental conditions (values are mean ± SE, pooled data) over the course of the experiment.

with the onset of drought conditions (Figure 2b). Although it was difficult to maintain a particular level of drought stress by withholding water, seedlings in the D treatment clearly had a reduced water status over most of the experimental period.

The treatments created significant differences ($P < 0.05$) in carbon assimilation rates (Table 1, Figure 3a). Since the analysis-of-variance test found no effect of humidity on assimilation rates, the data were pooled in Figure 3 (see Table 1 for results separated by humidity). Pine and eucalypt seedlings had similar photosynthetic rates. All treatments produced some reductions in seedling assimilation rates as compared with the control. These results are derived from the last 60 days of the experiment because the treatments likely exerted their greatest influence on gas exchange parameters during that period. These results were obtained under ambient conditions (C_a = ~400 ppm, PAR = 1000 μmol m^{-2} s^{-1} [except for treatment L, which was measured at 500 μmol m^{-2} s^{-1}], temperature ~26 °C, RH ~50–80%). Photosynthetic capacity (A_{max}) of each seedling was also measured (PAR = 2000 μmol m^{-2} s^{-1}, C_a ~1000 ppm, temperature and RH similar to ambient). A_{max} for seedlings grown in the L treatment was not different from control seedlings (Figure 3b) implying that ambient reductions in carbon assimilation (Figure 3a) are solely due to reductions

Table 1. Mean gas exchange results for the last 60 days of the experiment for eucalypt and pine seedlings grown in different vapor pressure deficits. Significant differences ($P < 0.05$) between the high- and low-humidity cabinets within each treatment are indicated by an asterisk. Significant differences between treatments ($P < 0.05$, Tukey's multiple comparison test) are indicated by differences between the letters below each treatment value (only for comparisons within each column, not between species).

Treatment	Humidity	Eucalypt			Pine		
		A (μmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	E (mmol m^{-2} s^{-1})	A (μmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	E (mmol m^{-2} s^{-1})
Control (C)	Low	13.0	0.407	5.05	11.5	0.238	4.59
	High	12.0	0.438	*3.51	*13.5	*0.325	*3.71
		a	a	a	a	a	a
Drought (D)	Low	4.36	0.131	2.28	4.6	0.06	1.27
	High	3.41	0.136	1.61	*2.0	0.02	*0.26
		b	b	b	b	b	b
Light (L)	Low	10.1	0.439	5.32	9.3	0.225	4.37
	High	10.2	0.447	*3.53	8.7	0.199	*2.34
		a	a	a	c	c	ac
Nitrogen (N)	Low	8.47	0.419	5.72	9.5	0.246	4.67
	High	8.83	0.390	*3.70	8.2	0.220	*2.79
		c	a	a	c	ac	ac
Temperature (T)	Low	10.1	0.277	4.43	9.3	0.151	3.13
	High	*13.5	0.408	*3.43	8.3	0.189	2.62
		a	a	a	c	c	c

in irradiance. The N and D treatments produced seedlings with reduced A_{max} and thus reduced ambient photosynthetic rates. Pine seedlings generally had lower A_{max} than the eucalypt seedlings but photosynthetic rates under ambient conditions were similar (Figure 3a and b).

Surprisingly, analysis of variance found no effect of humidity on stomatal conductance (g_s) although a T-test did highlight a significant difference in control pine seedlings (Table 1). Eucalypt seedlings in the T treatment also showed a decline in g_s with humidity (although not statistically significant due to measurement variability, Table 1) likely in an effort to reduce water loss at higher leaf temperatures. In general, similar g_s values were observed for eucalypt seedlings growing in all treatments with the exception of D and possibly T (Figure 3c, Table 1). Pine seedlings had lower stomatal conductance than eucalypt seedlings (per projected leaf area). These results indicate that reductions in ambient carbon assimilation for seedlings growing in the N treatment were due to reduced A_{max} not g_s, while for those in the L treatment it was neither A_{max} nor g_s but simply reductions in light interception, and for those exposed to drought both A_{max} and g_s were affected. Seedlings exposed to elevated temperatures maintained assimilation rates similar to control seedlings because they maintained a high A_{max} while g_s reductions were minimal. Environmentally imposed reduction in carbon assimilation is a reduction in the 'capacity' to do photosynthesis which modifies the A:g relationship even if physiological capacity (A_{max}) remains high. Therefore, the use of the term A_{max} in dual-isotope models is confusing and should be avoided.

Instantaneous daytime transpiration rates (E) were estimated from chamber humidity, air temperature, leaf temperatures, measured stomatal conductance and an estimate of boundary layer conductance. Not surprisingly, chamber humidity had a large effect on E for both pine (data not shown) and eucalypt seedlings as did g_s (especially for treatment D, Figure 4a). Treatments that modified leaf temperature (L and T) also influenced transpiration while the N treatment did not appear to impact on water loss as compared with the control. E was also measured periodically with a gas exchange system, and the results generally agree with the patterns from estimated E (Table 1). Estimates of E were considered more valuable as they were calculated from growth environmental conditions that were not always identical to conditions in the gas exchange cuvette. For example, leaf temperatures that can affect E differed in the growth cabinets but not the cuvette environment. These treatments produced variable conditions that produced variation in carbon gain, conductance and transpiration which could have modified leaf water evaporative enrichment (in ^{18}O) and the isotopic composition of organic matter.

The atmosphere in both growth cabinets had a $\delta^{13}C$ value of ~−9.3‰. Mean tap water $\delta^{18}O$ was −6 ‰. Mean cabinet water vapor $\delta^{18}O$ was −17.3‰. Leaf water evaporative enrichment (in ^{18}O) was greater in the low-humidity cabinet (Figure 4b, Table 2). Leaf water $\delta^{18}O$ values were higher for seedlings growing in the T and D treatments and slightly lower in the L treatment as compared with control seedlings. It appears that reduced nitrogen inputs did not affect evaporative enrichment for leaves of either species (Figure 4b, Table 2). Since young seedlings have small stems and relatively porous periderms, stem water $\delta^{18}O$ was consistently enriched above tap water $\delta^{18}O$ (most commonly between 0.5 and 1‰).

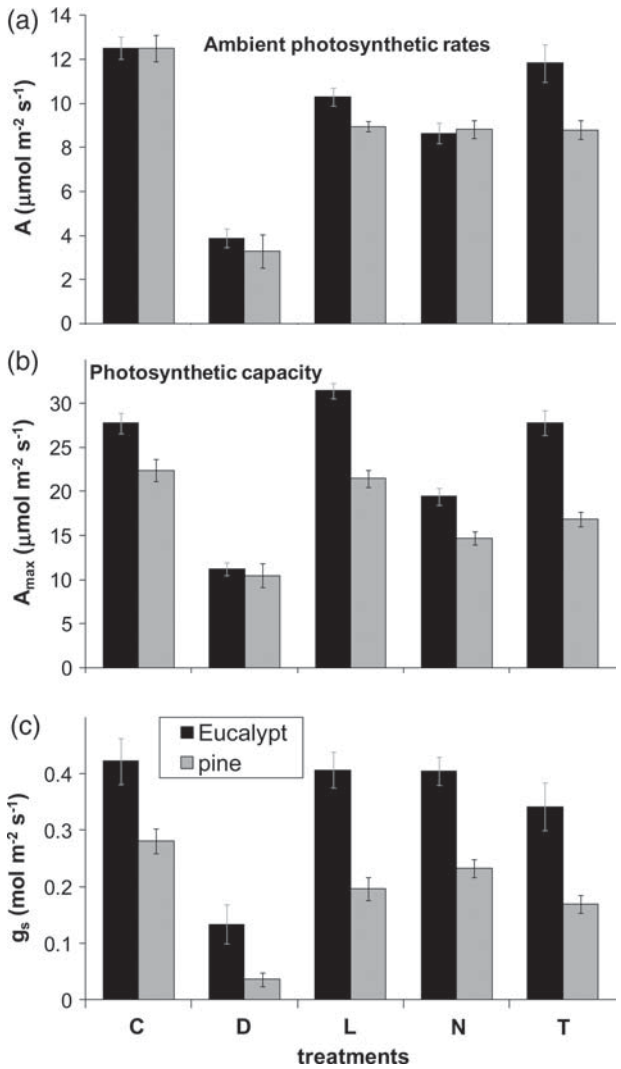

Figure 3. Mean gas exchange parameters for pine and eucalypt seedlings grown under five experimental conditions (±SE). (a) Photosynthesis under standard conditions (as close to ambient as possible). (b) Photosynthetic rates under saturating irradiance and elevated CO_2 concentrations. (c) Stomatal conductance under ambient conditions. Treatments were control (C), drought (D), reduced light (L), reduced nitrogen (N) and elevated temperature (T). Values are pooled means from plants in both growth cabinets as there were few significant differences in gas exchange measurements between humidity treatments.

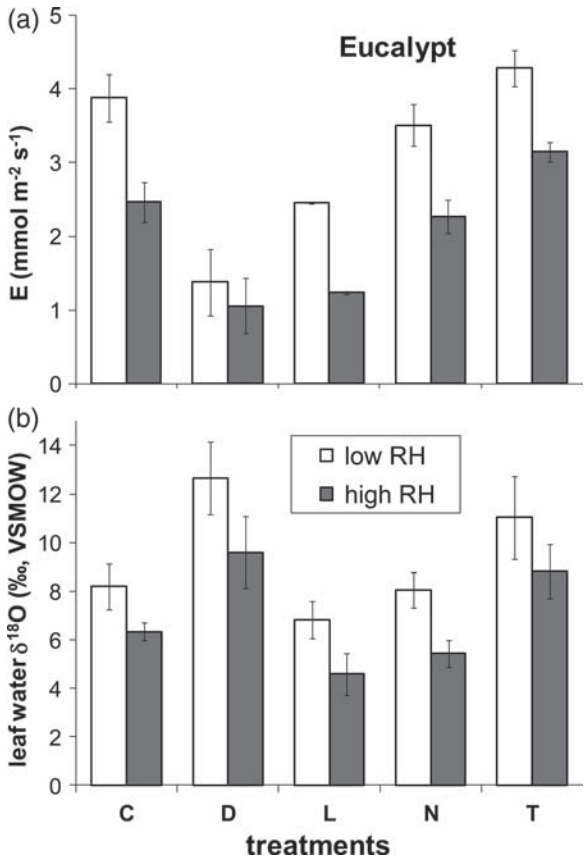

Figure 4. (a) Instantaneous daytime transpiration rates estimated from growth cabinet environmental and gas exchange information for eucalypt seedlings grown under five experimental conditions (values are mean ± SE estimates from periods coincident with gas exchange measurements). (b) Mean (±SE) oxygen isotope composition of bulk leaf water for eucalypt seedlings grown under five experimental conditions and different humidity. See Figure 3 legend or text for treatment codes.

The drought treatment had the greatest stem water enrichment (as much as 3‰); however, some of that may be associated with soil evaporative enrichment (or the back diffusion of enriched leaf water for seedlings with low transpirational flow rates). Because the pots were sealed with foam, most treatments had minimal soil evaporation. However, the drying soil in pots of the D treatment might have produced greater enrichment in soil water $\delta^{18}O$ than other treatments. If leaf water $\Delta^{18}O$ was calculated using stem water as source then differences in enrichment between D and C plants were reduced (data not shown).

Although most of the stem was processed to α-cellulose, some was saved to determine the viability of using whole wood for tree-ring analysis (see Barbour et al. 2001, Battipaglia et al. 2008). Our results indicate that whole wood is a viable alternative to cellulose for $\delta^{13}C$ analysis (Figure 5a); however, the relationship for $\delta^{18}O$ was not so tight and was inconsistent between species (Figure 5b).

Carbon isotope discrimination was greatest for the L treatment and least for the control (Figure 6a). Although $\Delta^{13}C$ was consistently higher for plants grown in high humidity, the differences were not statistically significant (Table 2). Pine produced similar result as eucalypt, though the D treatment produced seedlings with lower values of $\Delta^{13}C$ than control seedlings. Both L and N treatments reduced seedling A and/or A_{max} compared with the control with minimal differences in stomatal conductance (Figure 3). The D treatment reduced both seedling assimilation and conductance and the combination resulted in similar levels of discrimination to control seedlings. Mean eucalypt $\Delta^{13}C$ was higher than pine values by over 0.7‰

Table 2. Mean leaf water $\delta^{18}O$ and stem cellulose $\delta^{18}O$ and $\delta^{13}C$ for eucalypt and pine seedlings grown in different vapor pressure deficits. Significant differences ($P < 0.05$) between the high- and low-humidity cabinets within each treatment are indicated by an asterisk. Significant differences between treatments ($P < 0.05$, Tukey's multiple comparison test) are indicated by differences between the letters below each treatment value (only for comparisons within each column, not between species). Seedlings exposed to drought (D) were stunted and replicate plants were pooled into one sample for analysis; thus ‡ indicates that statistical comparisons were not possible without measurement replication.

Treatment	Humidity	Eucalypt			Pine		
		Leaf water $\delta^{18}O$ (‰)	Cellulose $\delta^{18}O$ (‰)	Cellulose $\delta^{13}C$ (‰)	Leaf water $\delta^{18}O$ (‰)	Cellulose $\delta^{18}O$ (‰)	Cellulose $\delta^{13}C$ (‰)
Control (C)	Low	8.2	26.8	–25.2	8.0	28.7	–25.1
	High	6.3	*25.0	–25.5	*5.0	*26.4	–25.4
		a	a	a	a	a	ab
Drought (D)	Low	12.7	27.4	–26.0	14.2	30.3	–23.5
	High	*9.6	‡25.5	‡–25.8	*12.5	‡27.3	‡–23.8
		b	‡	‡	b	‡	‡
Light (L)	Low	6.8	27.0	–26.8	5.5	27.7	–26.6
	High	*4.6	*25.2	–27.4	*4.1	26.1	–26.4
		a	a	b	a	a	a
Nitrogen (N)	Low	8.1	26.3	–26.2	8.9	28.0	–25.3
	High	*5.4	*24.5	–26.5	*4.8	*26.3	–26.4
		a	a	bc	a	a	ab
Temperature (T)	Low	11.0	27.5	–25.5	12.4	31.6	–23.8
	High	*8.8	*25.9	–25.9	*11.2	*28.4	–24.9
		b	b	ac	b	b	b

indicating that the similar assimilation rates and reduced g_s (Figure 3) of pine created lower intracellular [CO_2] leading to less discrimination. Gas exchange estimates confirmed that C_i of pine needles, under ambient conditions, was on average 25 ppm lower than that for eucalypt leaves.

Humidity differences clearly have significant effects on $\delta^{18}O$ variation (Figure 6b, Table 2). Expressing oxygen isotope enrichment ($\Delta^{18}O$, Figure 6c) relative to stem water changed the overall trends with regard to the D treatment only as its stem water was significantly enriched over tap water (irrigation source). Large differences in transpiration (Figure 4a, compare C with L) did not appear to greatly influence the $\Delta^{18}O$ of stem cellulose (Figure 6c).

Modeling and discussion

The dual-isotope conceptual model uses a plot of $\delta^{18}O$ against $\delta^{13}C$ and the direction of change between conditions is considered diagnostic for interpreting $\delta^{13}C$ signals (Scheidegger et al. 2000, Grams et al. 2007). The arrows in Figure 7 point from the low- to high-humidity data point for each treatment. According to these conceptual models, the direction of the arrow indicates that $\delta^{13}C$ changes are due to an increase in conductance with little or no change in assimilation (Figure 7b, inset). With the exception of control pine seedlings (Table 1), no significant differences between humidity treatments in stomatal conductance were observed. If one assumes the arrows in Figure 7a are horizontal rather than sloped (the mean standard error bars in Figure 7a describe measurement variability and indicate how much variation in arrow angle is possible) then the conceptual model predicts that $\delta^{13}C$ changes are due to increases in A and g_s, which were also not consistently observed with the exception of the elevated temperature treatment (Figure 7c, please note the scaling of A and g_s in a graph to accurately portray arrow orientations is not spelled out by either Scheidegger et al. 2000, or Grams et al. 2007). Pine seedlings showed similar carbon and oxygen isotope shifts with humidity (data not shown) as eucalypts. Similarly, the observed A:g relationships were not predicted by the conceptual models for pine. Clearly, differences in vapor pressure deficit can modify leaf evaporative enrichment and modify cellulose $\delta^{18}O$ without necessarily influencing stomatal conductance and the A:g relationship. A prerequisite in these conceptual models is that source water and atmospheric vapor $\delta^{18}O$ were similar between treatments. Our experimental design satisfied these conditions. Another requirement may be similar vapor pressure deficits which are possible in common garden experiments but could be problematic for historical studies (tree rings) or comparisons between distant sites.

This data set was also used to test the conceptual models for treatment differences other than humidity. Figure 8 shows the same data as in Figure 7 only the arrows are not extended between humidity treatments but from the control to the N, T, L or D treatments (within the same humidity treatment). According to both Scheidegger et al. (2000) and Grams et al. (2007), the slope of the arrow for the elevated temperature treatment in the $\delta^{18}O$ versus $\delta^{13}C$ plot (Figure 8a) implies that both assimilation and conductance declined with increasing

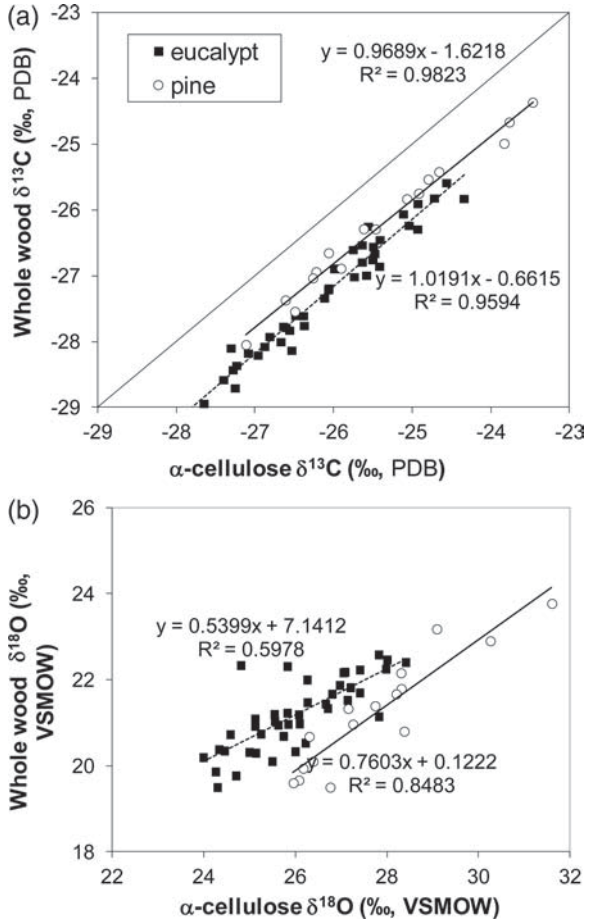

Figure 5. Comparison between whole wood and α-cellulose material extracted from the same samples of pine and eucalypt stems grown under different vapor pressure deficits and environmental conditions designed to vary carbon (a) and oxygen (b) isotopic compositions. The diagonal line in panel a is the 1 : 1 relationship.

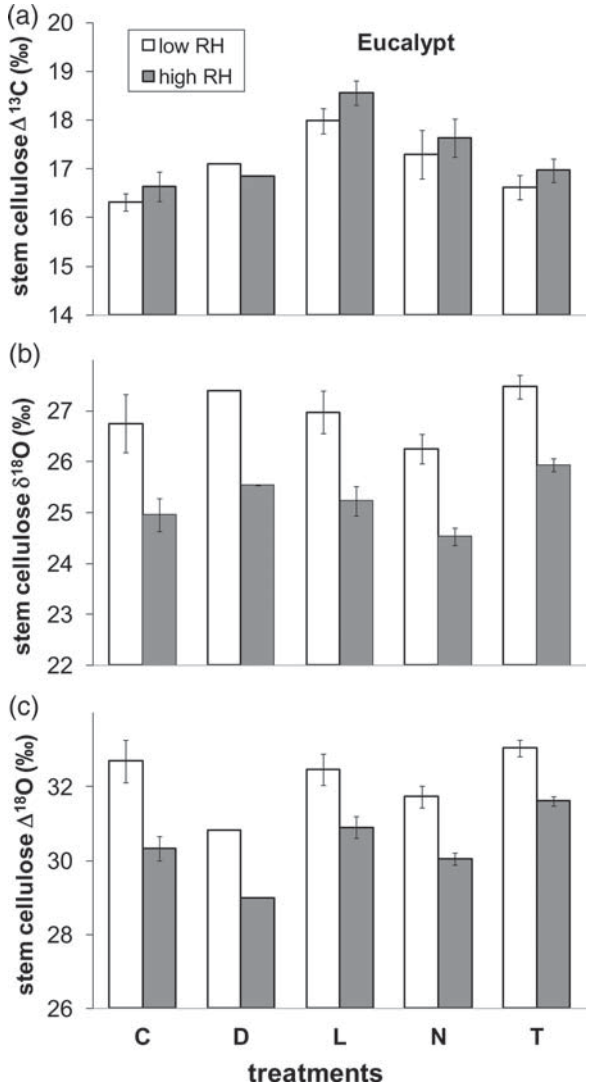

Figure 6. Carbon isotope discrimination (a), oxygen isotope composition (b) and oxygen isotope enrichment above source water (c) of α-cellulose extracted from eucalypt stems grown under five experimental conditions and different vapor pressure deficits (values are mean ± SE). See Figure 3 legend or text for treatment codes.

temperature (Figure 8b). However, for plants exposed to higher temperatures, only those grown in low humidity showed reductions in A and g_s. (Figure 8c, Table 1). The reduced nitrogen treatment produced a relationship that implies (if the arrow has some slope [not vertical], Figure 8a) no change in A, but an increase in g_s (Figure 8b), which was not observed. If the slope was more vertical (mean SE bars indicate possible variation in the data and thus slope), then this implies a reduction in A with little change in g_s, which was observed (Figures 3 and 8c). The reduced light treatment produced a vertical arrow (Figure 8d) that implies a reduction in A with little change in g_s (Figure 8e), which was observed (Figure 8f, Table 1). Here again, it is not A_{max} that was affected but ambient photosynthetic rates due to reduced light inputs (Figure 3). Another possible assumption for these models might be consistent light energy inputs. Finally, the drought treatment (Figure 8d) produced a relationship that implies a reduction in both A and g_s (Figure 8d), and this indeed was observed (Figure 8f). Results in Figure 8 are for eucalypt seedlings only as they produced the most consistent responses and the best fit between model predictions and measured physiological responses. Model performance for pine was less satisfactory, producing reasonable predictions for only a few treatments (possibly T and low humidity L, data not shown but refer to Tables 1 and 2). Thus, these conceptual models work fairly well for some scenarios but not all.

Other studies have found that these conceptual models adequately constrained the interpretations of $\Delta^{13}C$ variation in leaf organic matter under field conditions with herbaceous plants (Scheidegger et al. 2000) and tree species (Keitel et al. 2006, Sullivan and Welker 2007, Moreno-Gutiérrez et al. 2011), or under controlled environmental conditions and ozone stress (Grams et al. 2007). The differences between these studies and our results may relate to material measured

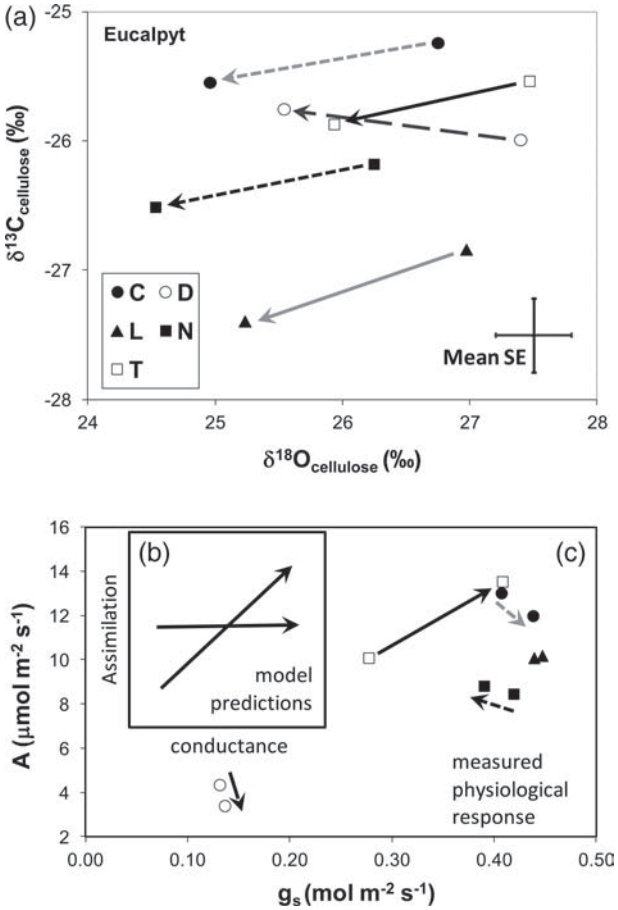

Figure 7. (a) Data points are the mean carbon and oxygen isotopic composition for eucalypt seedlings grown under five experimental conditions and different vapor pressure deficits (see Figure 3 legend or text for treatment codes). The arrows are pointing from the low- to the high-humidity measurements within each experimental treatment. (b) The predicted causes of differences in $\delta^{13}C$ between humidity treatments (arrow direction is also from low to high humidity) in terms of stomatal conductance and photosynthetic rate as the primary drivers in the conceptual models of Scheidegger et al. (2000) and Grams et al. (2007). (c) The measured photosynthetic and stomatal conductance response for eucalypt seedlings grown under five experimental conditions and different vapor pressure deficits.

(wood rather than leaves), controlled versus uncontrolled environments and the treatments applied to generate physiological differences. Although some studies have found these conceptual models useful in interpreting results using tree rings rather than leaf materials (Brooks and Coulombe 2009, Brooks and Mitchell 2011), the uncoupling of $\delta^{13}C$ and $\delta^{18}O$ signals in tree-ring cellulose from leaf-level processes (see Offermann et al. 2011) may have obscured model interpretation in our study.

An assumption of these models is that evaporative conditions that affect $\delta^{18}O$ values will translate to C_c/C_a changes and ^{13}C discrimination through modifications of stomatal conductance. Our results demonstrate that humidity impacts on transpiration as well as leaf water and cellulose ^{18}O enrichment (Figures 4 and 6, Table 2). However, making the linkage to stomatal conductance is problematic as few significant differences in g_s were observed for seedlings grown in different humidity treatments (Table 1). This may simply reflect the primary effect of vapor pressure deficit on both transpiration and leaf water ^{18}O enrichment with stomata playing only a minor role. In addition, stomatal responses to humidity are not consistent (or linear) for all species (Lambers et al. 1998). A direct correlation between $\delta^{18}O$ in tree rings and stomatal conductance, independent of vapor pressure deficit (as seen for leaves, Barbour and Farquhar 2000, Barbour et al. 2000), may have been obscured in our results by the overriding effects of humidity on evaporative enrichment. In this case, it may be questionable to use $\delta^{18}O$ variation as a proxy for changes in g_s in these models unless similar ambient vapor pressures can be assumed (Grams et al. 2007). Alternatively, over the course of the experiment, there may well have been an integrated humidity effect on stomatal conductance captured by ^{18}O enrichment that we were unable to discover using instantaneous gas exchange measurements.

As a twist to the conceptual models discussed above, one of our goals was to determine whether $\delta^{13}C$ variation can be used to constrain our interpretation of $\delta^{18}O$ variation under conditions where source water $\delta^{18}O$ values could not be considered invariant. If $\delta^{13}C$ variation is influenced by leaf evaporative processes then we may be able to differentiate between humidity and source water effects on $\delta^{18}O$ variation in organic matter. As with the Scheidegger et al. (2000) conceptual model a simplifying assumption is required, that is relatively constant photosynthetic rates and/or capacities. This was clearly not the case for the D, L or N treatments (Figure 3). However, with few exceptions there were no significant within-treatment differences in A or A_{max} between different humidity conditions. Within a treatment, higher humidity produced seedlings with higher $\Delta^{13}C$ values (Figure 6a). With the exception of the drought treatment, increasing humidity caused a reduction in E and an increase in carbon isotope discrimination. If growth conditions limit variation in photosynthetic capacity or rate then $\Delta^{13}C$ variation could possibly be used to describe (at least qualitatively) evaporative conditions. If nutrient inputs to the system are consistent and light levels are relatively invariant from year to year (no over-topping or tree fall gaps created), then possibly A_{max} could also be invariant.

These ideas are very tentative and extreme caution must be exercised when interpreting these kinds of data. Although increased discrimination with humidity was reasonably consistent, few differences were statistically significant. In addition, the large differences between the L and C treatments indicate that photosynthesis may have such large effects on discrimination that even minor year-to-year variation in light or nutrient inputs could make our simplifying assumption problematic. It is

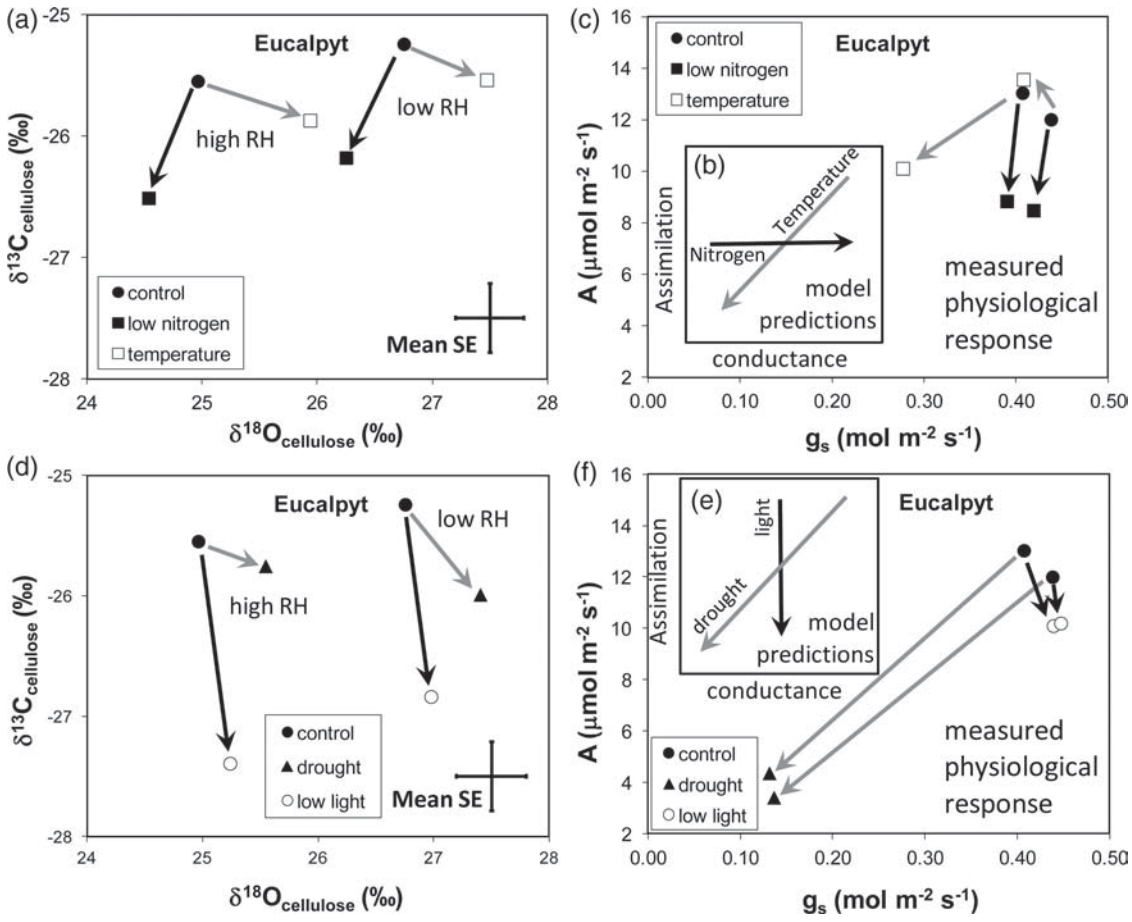

Figure 8. Data points are the mean carbon and oxygen isotopic composition for eucalypt seedlings grown under five experimental conditions and different vapor pressure deficits (a and d). All arrows are pointing from the control value to each of the other treatment values for seedlings grown in the same growth cabinet (identical humidity as indicated). Inset panels b and e show the predicted cause of differences in $\delta^{13}C$ between experimental treatments (arrow direction is also from the control to treatment) in terms of stomatal conductance and photosynthetic rates as the primary drivers in the conceptual models of Scheidegger et al. (2000) and Grams et al. (2007). Panels c and f present the mean photosynthetic and stomatal conductance measurements for the same seedlings as in panels a and d, respectively.

also difficult to understand how $\Delta^{13}C$ can be affected by humidity without a concomitant change in stomatal conductance (few significant differences in g_s between growth cabinets) and its impact on C_c/C_a. If trees were exposed to significant water stress then interpretation becomes even more difficult as both A and g_s were severely affected for seedlings grown in the D treatment. These results imply that using $\Delta^{13}C$ to constrain interpretation of $\delta^{18}O$ variation may not be impossible, but it is certainly not straightforward.

The use of seedlings was an important experimental issue in this study as juvenile growth is known to produce wood with altered $\delta^{13}C$ values (Duquesnay et al. 1998, McCarroll and Pawellek 2001) potentially influencing model performance. However, the 'juvenile effect' has mostly been studied in terms of $\delta^{13}C$ in association with recycling of respired CO_2 (Schleser and Jayasekera 1985, Cernusak et al. 2001) or changes in hydraulic conductivity and sapwood ratios with tree height (Monserud and Marshall 2001, McDowell et al. 2002). Juvenile effects in terms of $\delta^{18}O$ are equivocal (McCarroll and Loader 2004) although they could be expressed as different ratios of lignin to cellulose (Battipaglia et al. 2008). Nevertheless, as all seedlings were of the same age and α-cellulose was extracted, it is unlikely that juvenile effects would have greatly influenced our results. We assumed that the benefits of an experimental system that controlled important environmental variables (as compared with field-based designs) would outweigh the difficulties of using seedlings.

Conclusions

Interpreting $\delta^{18}O$ variation in whole-wood tree-ring chronologies may be problematic as there was no consistent relationship for different species. We recommend using α-cellulose for $\delta^{18}O$ analysis. A clean interpretation of dual isotope signals has not emerged from these results. Measuring $\delta^{18}O$ together with $\delta^{13}C$ is still recommended as together they provide more information regarding environmental and physiological conditions than one isotope alone. Humidity does

appear to have some minor effect on $\Delta^{13}C$ values. If an assumption that A remains stable over time can be justified for a tree species then it may be possible to use $\Delta^{13}C$ to provide information about evaporative conditions. However, there are many interacting factors that influence both C and O isotope ratios and environmental inputs can vary widely making quantitative interpretations problematic. This study also highlights some difficulties using the dual-isotope approach to constrain the interpretation of $\Delta^{13}C$ variation (Scheidegger et al. 2000, Grams et al. 2007). The simplifying assumption of constant source water $\delta^{18}O$ may not hold in all cases and even when it does (as in this study) the conceptual models did not correctly predict changes in A, A_{max} and g_s for all treatments. This can be especially problematic if vapor pressure deficits vary between test conditions. The pivotal impact of humidity on evaporative enrichment may overshadow effects of g_s on $\delta^{18}O$ variation making it less useful as a proxy for conductance.

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Conflict of interest

None declared.

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