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Influence of diurnal variation in mesophyll conductance on modelled ^{13}C discrimination: results from a field study

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Abstract

Mesophyll conductance to CO_2 (g_m) limits carbon assimilation and influences carbon isotope discrimination (Δ) under most environmental conditions. Current work is elucidating the environmental regulation of g_m, but the influence of g_m on model predictions of Δ remains poorly understood. In this study, field measurements of Δ and g_m were obtained using a tunable diode laser spectroscope coupled to portable photosynthesis systems. These data were used to test the importance of g_m in predicting Δ using the comprehensive Farquhar model of Δ (Δ_{comp}), where g_m was parameterized using three methods based on: (i) mean g_m; (ii) the relationship between stomatal conductance (g_s) and g_m; and (iii) the relationship between time of day (TOD) and g_m. Incorporating mean g_m, g_s-based g_m, and TOD-based g_m did not consistently improve Δ_{comp} predictions of field-grown juniper compared with the simple model of Δ (Δ_{simple}) that omits fractionation factors associated with g_m and decarboxylation. Sensitivity tests suggest that b, the fractionation due to carboxylation, was lower (25‰) than the value commonly used in Δ_{comp} (29‰) and Δ_{simple} (27‰). These results demonstrate the limits of all tested models in predicting observed juniper Δ, largely due to unexplained offsets between predicted and observed values that were not reconciled in sensitivity tests of variability in g_m, b, or e, the day respiratory fractionation.

Key words: Carbon isotope discrimination, Farquhar model, internal conductance, *Juniperus*, mesophyll conductance, stomatal conductance.

Introduction

Low mesophyll conductance of CO_2 from substomatal cavities to sites of carboxylation (g_m) can reduce the partial pressure of CO_2 (pCO_2) at the site of carboxylation, limit photosynthesis (A), and affect carbon isotope discrimination (Δ) (Farquhar *et al.*, 1989; Niinemets *et al.*, 2009). g_m varies on numerous time scales in response to environmental drivers, from rapid variation in response to changes in intercellular [CO_2] (Flexas *et al.*, 2007; Vrábl *et al.*, 2009) to shifts in response to temperature (Bernacchi *et al.*, 2002), water stress (Galmés *et al.*, 2007; Grassi *et al.*, 2009), light gradients (Piel *et al.*, 2002; Monti *et al.*, 2009), and others (for reviews, see Flexas *et al.*, 2008; Warren, 2008a). The responses of g_m to environmental drivers, however, are not universal (Tazoe *et al.*, 2009). Scaling relationships between

g_m and photosynthetic capacity have been shown (Evans and von Caemmerer, 1996; Le Roux *et al.*, 2001; Ethier *et al.*, 2006) and challenged (Warren and Adams, 2006). Similarly, a correlation between g_m and g_s has been demonstrated in several species (Loreto *et al.*, 1992; Lauteri *et al.*, 1997; Flexas *et al.*, 2002; Hanba *et al.*, 2003; Ethier *et al.*, 2006; but see Bunce, 2009), and is intriguing because of the potential for high frequency modelling of g_s and subsequent estimates of g_m. Recurrent diurnal patterns in g_m could also provide a simple method of accounting for variation in mesophyll conductance within carbon exchange models. Studies of diurnal g_m are limited (Bickford *et al.*, 2009; Grassi *et al.*, 2009) but open up the possibility of establishing a relationship between time of day and

variation in mesophyll conductance that could be used as a dynamic model parameter. Mesophyll conductance has also been recognized as an important factor influencing the $^{13}C/^{12}C$ ratio of leaf material ($\delta^{13}C_L$; Le Roux *et al.*, 2001; Hanba *et al.*, 2003; Warren and Adams, 2006) and ecosystem respiration ($\delta^{13}C_{resp}$; Ogée *et al.*, 2003; Cai *et al.*, 2008) which has implications for interpreting water use efficiency and terrestrial carbon exchange, among other applications. Δ is a strong regulator of $\delta^{13}C_L$ and $\delta^{13}C_{resp}$ (Bowling *et al.*, 2008), and therefore a better understanding of g_m in leaf-level predictions of discrimination may improve interpretation of $\delta^{13}C$ signals from multiple sources. Studies testing the role of g_m in Δ predictions are limited, but suggest (Wingate *et al.*, 2007) and demonstrate (Le Roux *et al.*, 2001; Bickford *et al.*, 2009) that the influence of g_m was important.

Δ is influenced by numerous environmental and physiological regulators and is well correlated with key physiological indicators. The ratio of intercellular to ambient pCO_2 (p_i/p_a) is a physiological parameter that succinctly describes the variability in the pCO_2 gradient driven by A and stomatal conductance (g_s), and its linear relationship with Δ has been widely observed over the last three decades (Farquhar *et al.*, 1982*a*, 1989; Brugnoli and Farquhar, 2000). p_i/p_a is integral to two models of Δ: a comprehensive model that incorporates fractionation factors associated with diffusion, carboxylation, and decarboxylation processes (Δ_{comp}; Farquhar *et al.*, 1982*b*); and a simplified version of Δ_{comp} that omits fractionation factors associated with decarboxylation activity and much of the diffusive pathway (Δ_{simple}; Farquhar *et al.*, 1982*b*). The parsimonious Δ_{simple} evolved from the same theoretical work as Δ_{comp} (Farquhar *et al.*, 1982*b*) and gained wide usage primarily because of its simplicity and power in explaining observations of Δ, but also because the effects of decarboxylation activity and g_m were thought to be negligible in predicting Δ.

Mechanistic models are used to predict Δ across a variety of temporal and spatial scales, where variation is driven by p_i/p_a interacting with key model parameters (Farquhar *et al.*, 1982*b*). In addition to p_i/p_a, the key drivers of Δ_{simple} include: (i) the carboxylation term, b, that represents net fractionation associated with phosphoenolpyruvate (PEP) carboxylase and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco); and (ii) the fractionation associated with diffusion in air and through stomata (a; 4.4‰) (Farquhar *et al.*, 1989). Theory suggests the Rubisco carboxylation fractionation may be between 25‰ and 30‰ (Tcherkez and Farquhar, 2005) and is supported by recent measurements of Rubisco fractionation near 27‰ in tobacco (*Nicotiana tabacum*; McNevin *et al.*, 2007). b is typically estimated at ~27‰ in Δ_{simple}, which is ~2‰ lower than most measurements of the Rubisco fractionation in C_3 plants (~29‰; Roeske and O'Leary, 1984) due to the influence of PEP carboxylase activity and omitted fractionation factors (Farquhar and Richards, 1984; Gessler *et al.*, 2008).

The comprehensive mechanistic Δ model incorporates the factors discussed above plus fractionation associated with CO_2 diffusion, including g_m, and decarboxylation activity.

As previously discussed, g_m is dynamic and may influence Δ by restricting diffusion from substomatal cavities to the chloroplast. The influence of day respiration (R_d), its associated fractionation factor (e), and fractionation associated with photorespiration (f) was thought to be negligible in early studies of g_m and Δ (Evans *et al.*, 1986; von Caemmerer and Evans, 1991). Recent evidence suggests, however, that these may be non-negligible variables (Ghashghaie *et al.*, 2003; Tazoe *et al.*, 2009), with f values ranging from ~7‰ to 13‰ (Tcherkez, 2006; Lanigan *et al.*, 2008) and e thought to be around −6‰ (Ghashghaie *et al.*, 2003). R_d is difficult to measure and not well understood, but existing studies demonstrate inhibition of the respiration rate under illuminated conditions (Tcherkez *et al.*, 2005) and biochemical differences between R_d and dark respiration (R; Tcherkez *et al.*, 2008, 2009). Similarly, e is very difficult to estimate and no direct leaf-level measurements currently exist in the literature. Consequently, e is frequently estimated based on the dark respiration fractionation (e_d; Ghashghaie *et al.*, 2001; Tcherkez *et al.*, 2003; Barbour *et al.*, 2007), though the similarity, if any, of the isotope effects in R and R_d are not yet well understood (Tcherkez *et al.*, 2008).

In this study a tunable diode laser absorption spectroscope (TDL) coupled to infra-red gas analysers (IRGAs) was used to measure g_m and Δ of *Juniperus monosperma* (Engelm.) Sarg. (juniper) trees at high frequency on days representative of the growing season at a high elevation semi-arid field site in 2007. The objectives of this study were to (i) measure the diurnal variation of g_m; (ii) quantify the relationship between diurnal g_m and (a) g_s and (b) time of day (TOD); (iii) assess model sensitivity to variation in e and b; (iv) measure the diurnal variation in Δ and examine the relationship between Δ and environmental and physiological drivers; and (v) assess the performance of Δ_{comp}, when fitted with diurnally variable g_m, compared with predictions from Δ_{simple}.

Materials and methods

The study was conducted on 1 June 2007, 20 June 2007, 19 July 2007, and 23 August 2007 on Mesita del Buey near Los Alamos, NM, USA (elevation 2140 m) at a field site described in Breshears (2008) and Bickford *et al.* (2009). Precipitation at the site was 156.2 mm between May and August 2007, but was 65.5 mm in the January–April period preceding measurements.

Leaf gas exchange measurements

Two simultaneous measurements of leaf gas exchange were collected: (i) on the crowns of three mature juniper trees ($j_{ambient}$) which were rotated between ~06:00 h and 18:00 h on each day with measurements conducted maintaining the chamber environment similar to ambient conditions; and (ii) on an adjacent mature juniper tree ($j_{manipulate}$) measured continuously throughout each day but subjected to light manipulations. Measurements were occasionally interrupted by rainfall, and did not resume until foliage was dry. Among the three rotational trees comprising $j_{ambient}$, leaf gas exchange and ^{13}C discrimination were measured in response to ambient conditions. For both $j_{ambient}$ and $j_{manipulate}$, temperature regulation in the chamber block was engaged when

leaf temperature (T_L), measured by energy balance, was $\geqslant 35$ °C. Incoming irradiance in $j_{\mathrm{manipulate}}$ was manipulated by using a plastic shade to reduce incident light by ~50% once or twice per hour to regulate net photosynthetic rate (A; µmol m^{-2} s^{-1}) and assess the impact of irradiance on g_m. Shading was maintained for 15–25 min intervals within each hour across the diurnal measurement period. Natural variation in irradiance occurred during both shaded and unshaded periods, and contributed to a wide range of A and light intensity. While all light manipulations were performed on one tree ($j_{\mathrm{manipulate}}$), different groups of leaves were measured over the course of each day and across the season: two groups on 1 June, three on 20 June, two on 19 July, and three on 23 August.

Leaf gas exchange was measured by providing buffered air, via two 50.0 l volumes, to two LICOR 6400 portable photosynthesis systems (IRGAs; LI-COR Biosciences Inc., Lincoln, NE, USA); one IRGA was used to measure j_{ambient} and the other to measure $j_{\mathrm{manipulate}}$. Each IRGA was fitted with a conifer chamber (LI-COR 6400-05), and incoming and outgoing gas streams were plumbed to a TDL (TGA100A, Campbell Scientific Inc., Logan, UT, USA) for measurement of the [^{12}C^{16}O$_2$] and [^{13}C^{16}O$_2$] within each gas stream. Lines connecting each IRGA and the TDL were of different lengths, resulting in different lag times, and the 33 s and 50 s lag between the two IRGAs and the TDL were accounted for when summarizing data between the instruments. To ensure high data quality for all Δ measurements and subsequent model testing, a priori criteria were established to filter error-prone data. These filtering criteria included ensuring that the difference in [CO$_2$] of the gas entering and exiting the leaf chamber was >30 µmol mol^{-1}, that the difference in entering and exiting δ^{13}C was $\geqslant 1$ ‰, and that ξ was <10 (see below for explanation of the ξ ratio). Leaf area within the conifer chamber ranged between 29.7 cm^2 and 49.3 cm^2. Instrument precision was previously determined to be 0.06‰ over 1 h periods (Bickford et al., 2009). Three minute TDL measurement cycles were used where each calibration tank (see below) was measured for 40 s, of which the last 10 s were used to calculate the means for both isotopologues, and 25 s for each of the four measurement inlets, of which the last 15 s were used for calculating concentrations. Details of the instrument coupling and measurement cycle calibration follow procedures described in Bickford et al. (2009).

Working standard (WS) calibration tanks spanning the range of expected [CO$_2$] measurements used to calibrate each measurement cycle were (mean ±SE) 548.7±0.04 µmol mol^{-1} (^{12}C^{16}O$_2$): 5.9±0.0005 µmol mol^{-1} (^{13}C^{16}O$_2$): 2.2±0.0001 µmol mol^{-1} (^{12}C^{18}O^{16}O) for the high WS tank; and 347.3±0.3 µmol mol^{-1} (^{12}C^{16}O$_2$): 3.7±0.003 µmol mol^{-1} (^{13}C^{16}O$_2$): 1.4±0.001 µmol mol^{-1} (^{12}C^{18}O^{16}O) for the low WS tank during 1 June, 20 June, and 19 July measurements. The [CO$_2$] of a new high WS calibration tank used in the 23 August measurements was measured as 535.9±0.3 µmol mol^{-1} (^{12}C^{16}O$_2$): 5.8±0.003 µmol mol^{-1} (^{13}C^{16}O$_2$): 2.2±0.001 µmol mol^{-1} (^{12}C^{18}O^{16}O), while the low WS tank was the same as described above. All WS calibration tanks were calibrated for 4 h monthly against WMO-certified tanks that were filled and δ^{13}C calibrated at the Stable Isotope Lab of the Institute for Arctic and Alpine Research, a cooperating agency of the Climate Monitoring division of the National Oceanic and Atmospheric Administration's Earth Research Laboratory. The [CO$_2$] of the WMO-traceable tanks used in this study were, for the high tank, 539.57 µmol mol^{-1} (^{12}C^{16}O$_2$): 5.93 µmol mol^{-1} (^{13}C^{16}O$_2$): 2.21 µmol mol^{-1} (^{12}C^{18}O^{16}O); and for the low tank, 339.43 µmol mol^{-1} (^{12}C^{16}O$_2$): 3.76 µmol mol^{-1} (^{13}C^{16}O$_2$): 1.40 µmol mol^{-1} (^{12}C^{18}O^{16}O). Measurements of [CO$_2$] occasionally exceeded the lower span of the WS calibration tanks (maximum deviation: 42.6 µmol mol^{-1}), but post-hoc tests of the TDL demonstrated a linear measurement response beyond the lowest range of CO$_2$ values observed in this study (Bickford et al., 2009).

Pre-dawn leaf water potential (Ψ_w) was measured using a Scholander-type pressure bomb (PMS Instruments Co., Corvallis, OR, USA) on six mature juniper trees near the study trees on 23 May, 27 June, 25 July, and 23 August 2007. Soil water content was measured at depths of 0.02–0.3 m using 11 neutron probes (503DR Hydrophobe Neutron Moisture Probes, Campbell Pacific Nuclear, Inc., Pacheco, CA, USA) at 2 week intervals between 23 May and 9 August 2007.

Model parameterization

The study tested whether variable g_m improved model predictions of Δ_{obs} in j_{ambient} using a comprehensive model of Δ (Δ_{comp}; Farquhar et al., 1982b),

$$\Delta_{\mathrm{comp}} = a_b \frac{p_a - p_s}{p_a} + a \frac{p_s - p_i}{p_a} + (b_s + a_w)\frac{p_i - p_c}{p_a} + b \frac{p_c}{p_a} - \frac{\frac{eR_d}{k} + f\Gamma^*}{p_a} \tag{1}$$

where a_b, a_w, and b_s represent the fractionation factors associated with CO$_2$ diffusion through the leaf boundary layer (2.9‰), water (0.7‰), and fractionation attributed to CO$_2$ entering solution (1.1‰). The variables p_a, p_s, p_i, and p_c represent pCO$_2$ (Pa) in the chamber surrounding the leaf, at the leaf surface, in the intercellular spaces, and at the sites of carboxylation, respectively. Γ^*, R_d, k, f, and e represent the CO$_2$ compensation point in the absence of day respiration (Pa), day respiration rate (µmol m^{-2} s^{-1}), carboxylation efficiency (µmol m^{-2} s^{-1} Pa^{-1}), and fractionations associated with photorespiration and day respiration (‰), respectively.

Parameters p_a, p_s, p_i, and p_c were calculated by incorporating atmospheric pressure in Los Alamos (~79 kPa) with mole fraction-measurements of [CO$_2$]; p_c was estimated as $p_c = p_i - A/g_m$ (Farquhar and Sharkey, 1982). R_d was estimated at 1.5 µmol m^{-2} s^{-1} based on reported measurements of dark respiration in juniper (Bickford et al., 2009), k was calculated as A/p_c for each 3 min cycle, and Γ^* was calculated based on the expanded T_L expression presented in Brooks and Farquhar (1985) that incorporates data from Jordan and Ogren (1984). The photorespiratory, f, and day respiratory, e, fractionations were estimated at 11.6‰ (Lanigan et al., 2008) and -3‰, respectively. e has often been estimated based on the dark respiration fractionation, and previous work suggests juniper exhibits a 2–3‰ dark respiration fractionation (Bickford et al., 2009). Recent evidence demonstrates biochemical shifts between light and dark respiration that may influence the isotopic signature of respired CO$_2$ (Tcherkez et al., 2008), but currently there are no data in the literature providing estimates of the offset between day and dark respiratory fractionation at the leaf level. Because uncertainty in e and b could contribute to model uncertainty, tests were performed to evaluate the sensitivity of Δ_{comp} to variation in each, and model predictions were compared with Δ_{obs}. In these sensitivity tests Δ_{comp} was fitted with a $g_m = 1.72$ µmol m^{-2} s^{-1} Pa^{-1} ($\Delta_{\mathrm{c.mean}}$) and both $\Delta_{\mathrm{c.mean}}$ and Δ_{simple} were tested against all Δ_{obs} values ($n=552$), where Δ_{simple} is:

$$\Delta_{\mathrm{simple}} = a + (b - a) \cdot \frac{p_i}{p_a} \tag{2}$$

and b is equal to 27‰ to account for omitted fractionation factors (Farquhar and Richards, 1984).

Δ and diurnal g_m

Leaf carbon isotope discrimination (Δ_{obs}) was calculated from TDL-generated data:

$$\Delta_{\mathrm{obs}} = \frac{\xi(\delta_o - \delta_e)}{1 + \delta_o - \xi(\delta_o - \delta_e)} \tag{3}$$

where δ_e and δ_o equal the δ^{13}C of the entering and outgoing chamber gas streams, respectively, and ξ equals $c_e/(c_e - c_o)$ where c_e and c_o are the [CO$_2$] of the gas entering and exiting the leaf chamber, respectively. g_m was estimated in $j_{\mathrm{manipulate}}$ leaf gas

exchange and isotopic data using the point-based method (Evans *et al.*, 1986),

$$g_m = \frac{(b - b_s - a_w)A/p_a}{(\Delta_{\text{pred}} - \Delta_{\text{obs}}) - \Delta_{\text{ef}}} \quad (4)$$

where predicted discrimination (Δ_{pred}) is Δ_{simple} with $b=29‰$. The estimate of the fractionation attributed to decarboxylation activities, Δ_{ef}, was calculated as,

$$\Delta_{\text{ef}} = \frac{\frac{eR_d}{k} + f\Gamma^*}{p_a} \quad (5)$$

All components of Δ_{ef} were parameterized as described for Δ_{comp}. g_m estimates that fell below zero were excluded, and this occurred when $\Delta_{\text{pred}} < \Delta_{\text{obs}}$. Measurement error in Δ_{obs} and g_m incorporated instrument error for both total CO_2 concentration and isotopic composition, and this uncertainty was propagated through analyses of g_m using a bootstrapping approach described in Bickford *et al.* (2009). Point-based estimates were used to quantify g_m in three different ways for model testing. First, a mean g_m was calculated from all g_m estimates ($g_{m.\text{mean}}$; 1.72 μmol m^{-2} s^{-1} Pa^{-1}). Secondly, a regression was fitted between TOD and g_m measured within each day. The TOD and g_m data were pooled across dates, analysed using least squares regression, and the resulting expression was used to estimate g_m ($g_{m.\text{TOD}}$). Thirdly, each g_m estimate was transformed from expression in partial pressure (μmol CO_2 m^{-2} s^{-1} Pa^{-1}) to a flux density (mol CO_2 m^{-2} s^{-1}) by multiplying g_m by the ambient pressure (~79 kPa) which increased each g_m value by 21.1%. The stomatal conductance to CO_2 (g_{sc}; mol CO_2 m^{-2} s^{-1}) was calculated as stomatal conductance to H_2O (g_{sw}) divided by 1.6 to account for differences in diffusivity between water vapour and CO_2 (Farquhar and Sharkey, 1982). The transformed g_m values were then compared with g_{sc} data using linear regression, and the linear expression describing the relationship was used to estimate g_m ($g_{m.gs}$). To ensure the analysis of the relationship between g_m and TOD or g_{sc} was robust, *a priori* criteria for g_m uncertainty were established. When the uncertainty in each point g_m estimate, presented here as 1 SE, exceeded $0.10 \times g_m$ that point g_m estimate was excluded from regression analysis. Means testing was computed using the Tukey–Kramer honestly significant differences test ($P < 0.05$ level) unless indicated otherwise. All statistical tests were performed in R (version 2.9.1; R Core Development Team, 2009).

Δ_{comp} was parameterized in three ways for intermodel testing by calculating Δ_{comp} using $g_{m.\text{mean}}$ ($\Delta_{c.\text{mean}}$), $g_{m.\text{TOD}}$ ($\Delta_{c.\text{TOD}}$), and $g_{m.gs}$ ($\Delta_{c.gs}$). All three variations of Δ_{comp} along with Δ_{simple} were tested against Δ_{obs}. Model performance was evaluated using model bias and the root mean squared error (RMSE) as test statistics. Both were calculated from residuals where all models conformed to a slope of 1 and intercept of 0 (i.e. residuals=model prediction$-\Delta_{\text{obs}}$). The mean of these residuals represents model bias, while the standard deviation of the residuals represents the RMSE (Bickford *et al.*, 2009).

Results

Diurnal g_m

g_m ranged between 0.4 and 4.6 μmol m^{-2} s^{-1} Pa^{-1} in $j_{\text{manipulate}}$ across the four measurement days and generally declined across the morning to late day period (Fig. 1). Mean g_m was not different between 1 June (mean ±SE=1.69±0.09 μmol m^{-2} s^{-1} Pa^{-1}) and 20 June (1.44±0.05 μmol m^{-2} s^{-1} Pa^{-1}), but was higher on 19 July (3.13±0.42 μmol m^{-2} s^{-1} Pa^{-1}) and 23 August (2.22±0.10 μmol m^{-2} s^{-1} Pa^{-1}; $P < 0.05$). There was a significant relationship between g_{sc}

and g_m ($r^2=0.27$; $P < 0.0001$; Fig. 2) and TOD and g_m ($P < 0.0001$). The linear expression $g_m = -3.52\text{TOD}+3.38$ described the TOD–g_m relationship ($r^2=0.37$, $F=154.6$). The relationship between photosynthetic photon flux density (PPFD) and g_m was weak, but significant ($r^2=0.05$, $P=0.0004$; Fig. 3).

Δ_{obs}, physiological, and environmental parameters

Mean Δ_{obs} in j_{ambient} was 13.5±0.1‰ on 1 June, 15.9±0.2‰ on 20 June, 17.0±0.2‰ on 19 July, and 14.7±0.1‰ on 23 August. Δ_{obs} was significantly different between all dates ($P > 0.05$; Fig. 4). When pooled across months, some physiological parameters exhibited significant but weak linear relationships with Δ_{obs}, including A ($P < 0.0001$, $r^2=0.13$, $F=80.7$) and p_i/p_a ($P < 0.0001$, $r^2=0.29$, $F=225.9$),
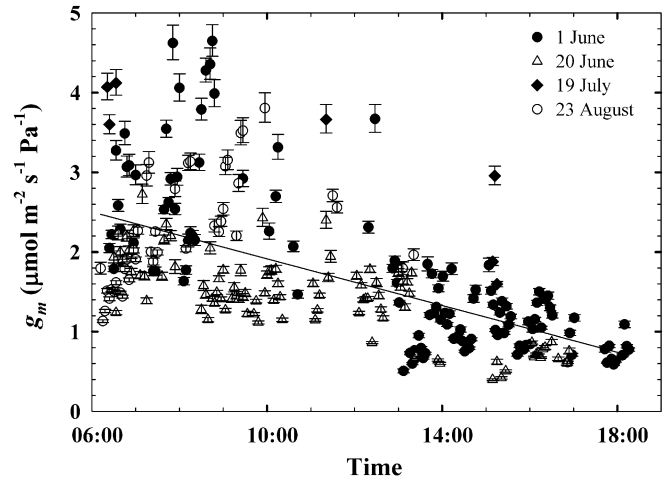

Fig. 1. Significant diurnal variation in mesophyll conductance to CO_2 (g_m) across the four measurement dates ($P < 0.0001$; $r^2=0.37$). Mean g_m was not different between 1 June and 20 June, but was higher on 19 July and 23 August ($P < 0.05$; Tukey's HSD). Error bars represent 1 SE.
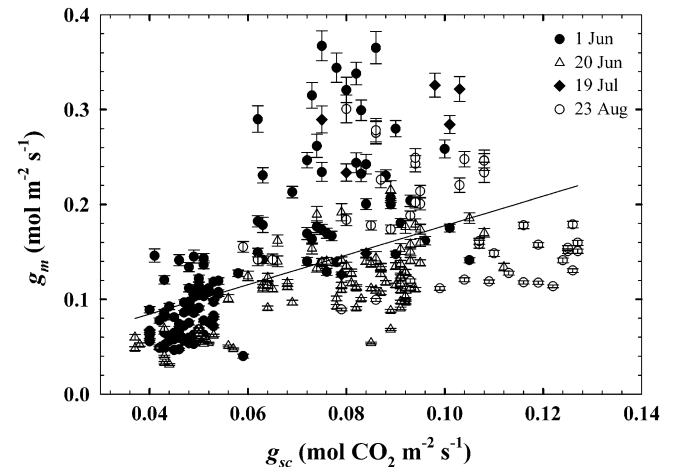

Fig. 2. The relationship between stomatal conductance to CO_2 (g_{sc}) and mesophyll conductance (g_m) across all four measurements dates ($g_m=1.55g_{sc}+0.022$; $P < 0.0001$, $r^2=0.27$). Error bars represent 1 SE.
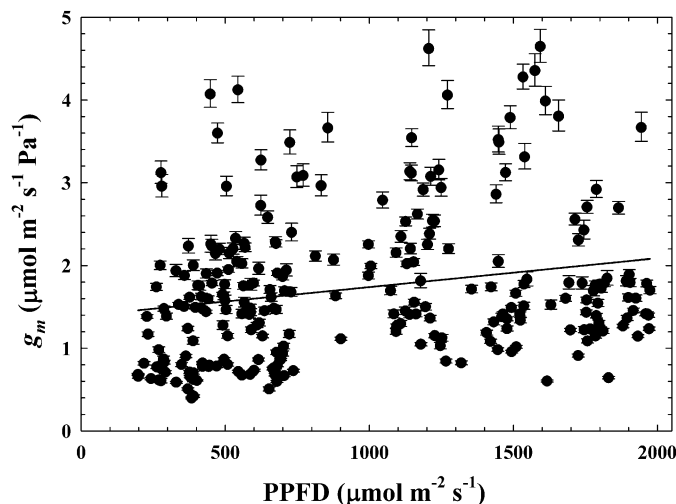
Fig. 3. The relationship between photosynthetic photon flux density (PPFD) and g_m (r^2=0.05, P=0.0004). Error bars represent 1 SE.

but not g_{sw} (P=0.24, r^2=0.0006, F=1.3; Fig. 5). A was higher on 23 August compared with 20 June, but was not significantly different among other dates (P >0.05; Table 1); g_{sw} was similar on 1 June and 19 July, but was different on all other days (P ≤0.05; Table 1).

There were weak but significant relationships between Δ_{obs} and T_L on 19 July (P=0.006, r^2=0.05, F=7.81) but not other dates (P ≥0.05). Mean T_L was 31.8±3.43 °C (mean ±SD) across all dates. There were also weak but significant relationships between Δ_{obs} and vapour pressure deficit (VPD) on each day except 23 August (P ≤0.03), and when VPD data were pooled across months (P <0.0001, r^2=0.05). VPD was significantly higher on 1 June and lower on 23 August compared with other days (P ≤0.05), but was similar on the remaining days (P >0.05; Table 1). Finally, there was a weak but significant linear relationship between Δ_{obs} and PPFD across all dates (P <0.0001, r^2=0.16). Soil water content at 200 mm over the study period ranged from a high of 19.2% on 23 May to a low of 12.0% on 25 July, before recovering to 13.9% on 9 August. Ψ_w measured in nearby juniper trees (n=6) was highest early in the season at −0.62±0.06 MPa (23 May) and then declined to −2.1±0.2 MPa (27 June) and −3.4±0.33 MPa (25 July) before increasing to −2.75±0.34 MPa (23 August). The relationship between Ψ_w and Δ_{obs} was not significant (P=0.15, r^2=0.75).

Model performance

Δ_{comp} did not consistently outperform Δ_{simple}, and the reductions in Δ_{comp} model bias observed over most of the study varied little with different parameterizations of g_m. Δ_{simple} exhibited lower RMSE on 1 June and 23 August, and across the pooled measurements dates (Table 2, Fig. 6), but also exhibited higher model bias on most dates (P <0.0001, paired t-test). All three variations of Δ_{comp} showed comparable RMSE, and the differences in error
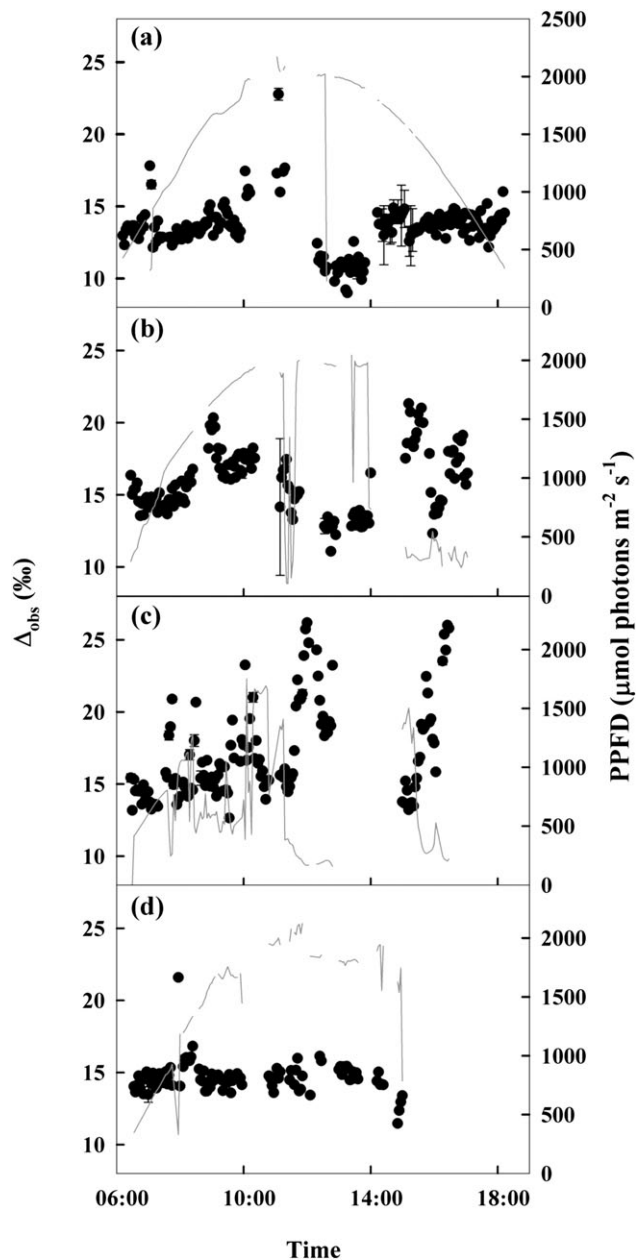

Fig. 4. Diurnal variation in carbon isotope discrimination (Δ; filled circles) and photosynthetic photon flux density (PPFD; grey line) on 1 June (A), 20 June (B), 19 July (C), and 23 August 2007 (D). The abrupt shifts in Δ mid-day on 1 June can be attributed to variation among trees, but variation seen on other dates results from plant environmental response. Error bars represent 1 SE.

were within 0.05‰ of one another. Model bias was significantly greater than zero in predictions of Δ_{obs} from all four models on all dates (P <0.0001 for all, paired t-test). A primary conclusion from Table 2 is that all models overpredicted Δ by at least 1‰, and that the limited improvements in predictions of Δ by incorporating g_m were small compared with the bias between Δ_{obs} and Δ_{comp}, which averaged 3.6‰ across the study.

Sensitivity tests showed reduced model bias and RMSE in $\Delta_{c.mean}$ when e and b were set to moderate and low values,
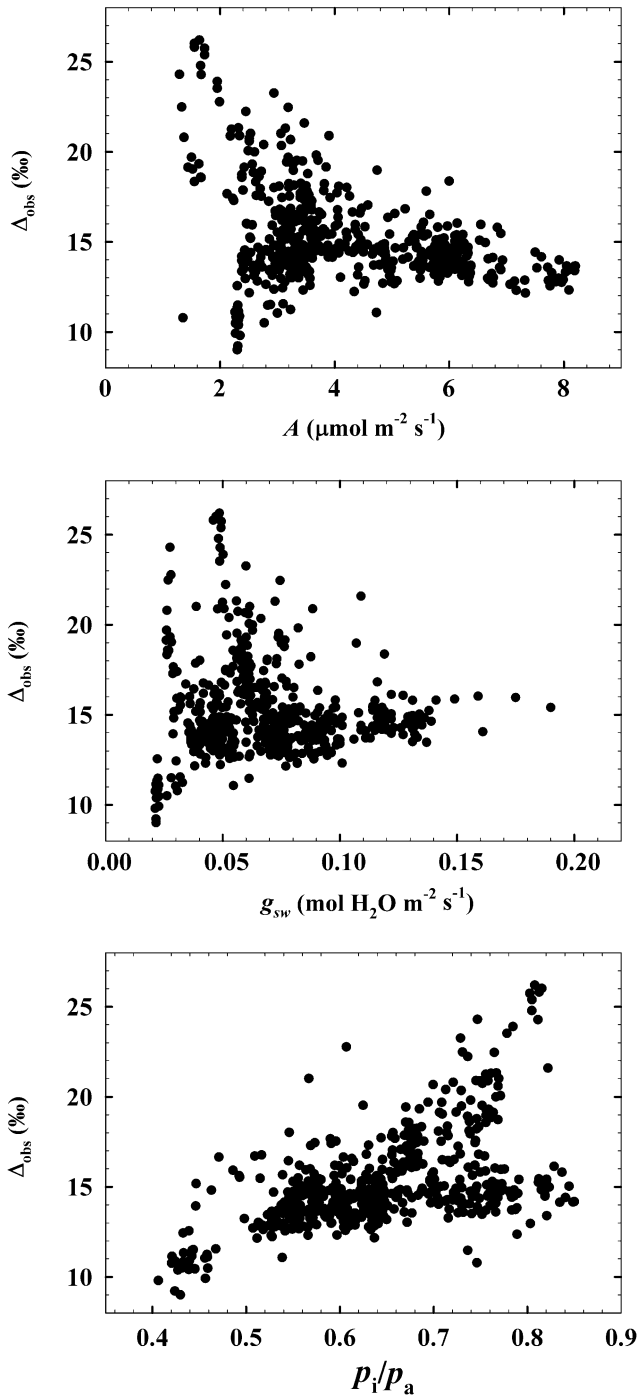
Fig. 5. The relationship between observed discrimination (Δ_{obs}) and net photosynthetic rate (*A*), stomatal conductance to H$_2$O (g_{sw}), and the ratio of partial pressure of CO$_2$ in intercellular spaces and the atmosphere around the leaf (p_i/p_a). When pooled across months these parameters exhibited significant linear relationships with Δ_{obs}, including *A* (P <0.0001, r^2=0.13) and p_i/p_a (P <0.0001, r^2=0.29), but not g_{sw} (P=0.24, r^2=0.0006).

Table 1. Mean diurnal net photosynthetic rate (*A*; μmol m^{-2} s^{-1}), stomatal conductance to H$_2$O (g_{sw}; mol m^{-2} s^{-1}), and vapour pressure deficit (VPD; kPa), each reported with 1 SE, and number of observations each day

Different letters denote significant differences between dates (P ≤0.05; Tukey's honestly significant differences test).

	A	SE	g_{sw}	SE	VPD	SE	Observations
1 June	4.34 a,b	0.15	0.06 a	0.002	2.86 a	0.04	182
20 June	3.97 a	0.09	0.07 b	0.001	2.17 b	0.04	138
19 July	4.07 a,b	0.13	0.06 a	0.002	2.31 b	0.06	134
23 August	4.54 b	0.12	0.11 c	0.003	1.22 c	0.03	98

Table 2. Summary of model prediction tests of observed discrimination, where the values in bold highlight the lowest RMSE (‰) best performing model in each month and across the study

Δ_{simple} predictions showed the lowest RMSE across the study, but exhibited higher model bias (‰) across the whole study compared with all three parameterizations of Δ_{comp} (P <0.0001).

Model	1 June		20 June		19 July		23 August		Whole study	
	Bias	RMSE	Bias	RMSE	Bias	RMSE	Bias	RMSE	Bias	RMSE
$\Delta_{c.mean}$	3.20	1.65	3.56	**1.35**	1.45	**1.62**	7.17	1.55	3.57	2.42
$\Delta_{c.TOD}$	3.18	1.61	3.55	1.36	1.45	**1.62**	7.20	1.52	3.56	2.42
$\Delta_{c.gs}$	3.05	1.66	3.45	1.36	1.32	1.63	7.06	1.57	3.44	2.43
Δ_{simple}	3.78	**1.33**	3.73	1.43	1.59	1.96	6.97	**1.32**	3.80	**2.30**

consistently reduced model bias and error. Error changed minimally when $\Delta_{c.mean}$ was parameterized with $e=-3$‰ and $b=29$‰, and g_m was decreased to 0.172 μmol m^{-2} s^{-1} Pa^{-1} (bias=1.66, RMSE=2.46) or increased to 17.2 μmol m^{-2} s^{-1} Pa^{-1} (bias=3.76, RMSE=2.43) compared with a g_m=1.72 μmol m^{-2} s^{-1} Pa^{-1} (bias=3.57, RMSE=2.42), though model bias did decline 54% at the lowest g_m value (P <0.0001). Δ_{simple} showed an 85% reduction in model bias and a 4.7% reduction in error when fit with $b=22$‰ instead of $b=27$‰ (Table 3). Excluding 19 July, all variations of Δ_{comp} and Δ_{simple} overestimated Δ_{obs} by 3–7‰, as determined by model bias, though accounting for the variance, as in the RMSE term, reduced total error to between 1.3‰ and 2.4‰ on individual days. Using RMSE as the metric, the best fit to Δ_{obs} using $\Delta_{c.mean}$ was with $e=-3$‰ and $b=25$‰ (RMSE=2.25), but that fit was still poorer than predictions from Δ_{simple} where $b=22$‰ (RMSE=2.19).

Discussion

Diurnal g_m

Two diurnal g_m trends were evident across the study. On 1 June, g_m increased in the early morning period to relatively high values (~2–3 μmol m^{-2} s^{-1} Pa^{-1}) and then declined to lower values for the remainder of the day (~1 μmol m^{-2} s^{-1} Pa^{-1}), a pattern repeated on 20 June and

respectively (compare Tables 2 and 3). Model bias increased 31% as e shifted from more positive (-1‰) to more negative (-6‰) values when b was set at 29‰. Model error, however, showed the lowest values when e was -3‰. Across tested e values the use of lower b values in $\Delta_{c.mean}$
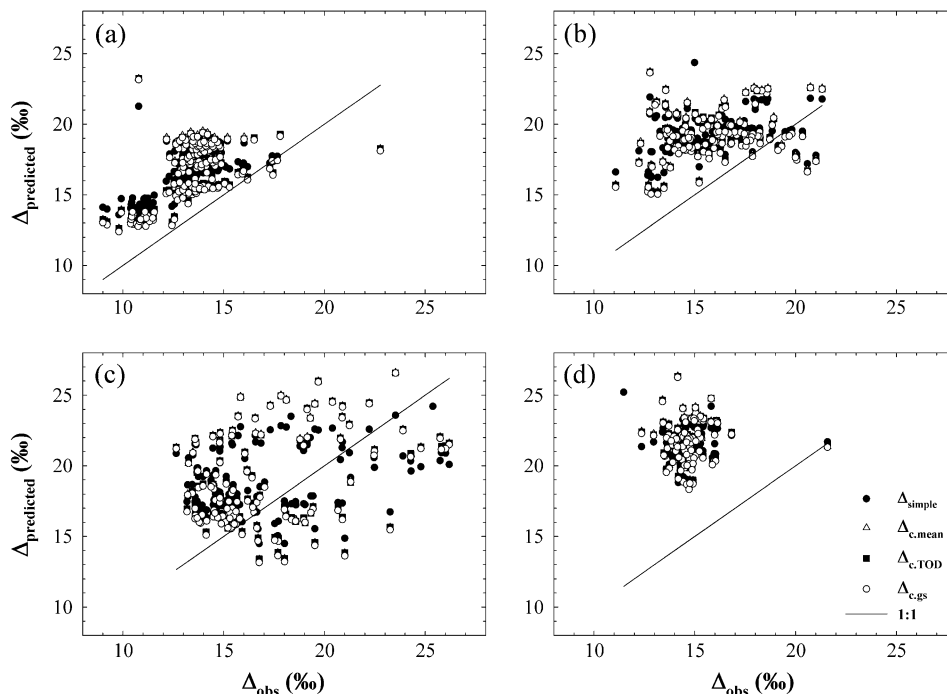
Fig. 6. Model tests of observed discrimination (Δ$_{obs}$) on 1 June (A), 20 June (B), 19 July (C), and 23 August 2007 (D). Four models were tested against Δ$_{obs}$ including the simple model of discrimination (Δ$_{simple}$; filled circles), the comprehensive model of discrimination using a mean mesophyll conductance (g_m) of 1.72 μmol m^{-2} s^{-1} Pa^{-1} (Δ$_{c.mean}$; open triangles), the comprehensive model of discrimination using a g_m estimated from the regression between diurnal g_m and time of day (TOD) (Δ$_{c.TOD}$; filled squares), and the comprehensive model of discrimination using a g_m estimated from the regression describing the relationship between stomatal conductance of CO_2 and g_m (Δ$_{c.gs}$; open circles). Δ$_{predicted}$ represents discrimination predictions of any of the four models. On two dates Δ$_{c.mean}$ or Δ$_{c.TOD}$ performed best, but on other dates and across the whole study Δ$_{simple}$ exhibited the lowest model error. These results support the use of Δ$_{simple}$ to predict leaf-level diurnal carbon discrimination of field-grown juniper.

the 23 August morning and mid-day periods. On 19 July, g_m was highest during the earliest measurements (~4 μmol m^{-2} s^{-1} Pa^{-1}) and remained relatively high through the afternoon period (~1.5–3 μmol m^{-2} s^{-1} Pa^{-1}). These trends in diurnal g_m probably represent a composite response to changes in plant microclimate and other regulators. Leaf water status and temperature are known to affect mesophyll conductance, with drought decreasing (Warren *et al.*, 2004; Flexas *et al.*, 2004) and higher temperature increasing g_m (Bernacchi *et al.*, 2002; but see Warren and Dreyer, 2006). The diurnal decline in g_m observed in this study is consistent with previous work showing reduced g_m under water-stressed conditions, though the range of pre-dawn Ψ$_w$ seen during this study would be characterized as moderate water stress in juniper (Linton *et al.*, 1998; McDowell *et al.*, 2008b). Increases in T_L across each day may have buffered any drought effect and prevented greater reduction of g_m, but such complex interactions cannot be determined with the current data set. Finally, cooporins, the CO_2-transporting protein channels, may have played a strong role in regulating diurnal shifts in g_m, but their regulation and interactions are still not well understood (Uehlein *et al.*, 2008; Heinen *et al.*, 2009).

Significant relationships existed between g_m and g_{sc}, g_m and TOD, and g_m and PPFD. The g_{sc}–g_m data show that g_m was higher than g_{sc}, and thus was not substantially limiting

Table 3. Results from sensitivity tests where the parameters representing the day respiration fractionation (e; ‰) and fractionation during carboxylation (b) were adjusted in the comprehensive model of carbon discrimination where g$_m$ was held constant at 1.72 μmol m^{-2} s^{-1} Pa^{-1} (Δ$_{c.mean}$; Equation 1), and b was adjusted in the simplified version of carbon discrimination (Δ$_{simple}$; Equation 2)

All other variables are as described in Model parameterization.

	Δ$_{c.mean}$			Δ$_{simple}$		
e (‰)	*b* (‰)	Bias (‰)	RMSE (‰)	*b* (‰)	Bias (‰)	RMSE (‰)
−1	29	3.04	2.42			
	27	1.77	2.34	27	3.80	2.30
	25	0.50	2.27			
−3	29	3.57	2.42			
	27	2.30	2.32	24	1.87	2.22
	25	1.03	2.24			
−6	29	4.36	2.46			
	27	3.09	2.35	22	0.59	2.19
	15	1.82	2.25			

CO_2 transfer to the sites of carboxylation or, as discussed below, substantially affecting Δ. These findings agree with data in other species demonstrating that g_m was higher than

g_{sc} (Loreto *et al.*, 1992; Galmés *et al.*, 2006), but differ from studies showing lower g_m compared with g_{sc} (Hanba *et al.*, 2003). These comparisons could be confounded if point-based calculations consistently overestimated juniper g_m, but estimates from this study are similar to point-based g_m values observed in a previous study of juniper (Bickford *et al.*, 2009). g_{sc}–g_m data in this study deviate from a 1:1 relationship, possibly due to different regulatory processes between stomatal and mesophyll conductance to CO_2 (but see Mott, 2009). Consensus is lacking, as others have observed nearly 1:1 g_{sc}–g_m relationships (Lauteri *et al.*, 1997), no significant relationship between g_{sc} and g_m (Bunce, 2009), and substantial variability in the g_{sc}–g_m relationship between species (Warren, 2008*b*). The diurnal decline in g_m observed across all study dates did not consistently improve Δ predictions, but using TOD as a relatively simple method to capture recurrent diurnal environmental patterns (i.e. declining leaf water status and parabolic temperature shifts) that affect mesophyll conductance and other photosynthetic processes may be productive in other systems. The weak relationship between g_m and PPFD shows that variation in light had little impact on juniper g_m, a finding that generally agrees with a study showing no effect of light on g_m in wheat (Tazoe *et al.*, 2009) but contrasts with those showing stronger effects of light on g_m (Loreto *et al.*, 2009; Monti *et al.*, 2009).

Δ, environmental, and physiological parameters

Diurnal patterns across the study were consistent with previous studies showing environmental regulation of Δ_{obs}. As previously observed in model and empirical studies, VPD and PPFD acted as environmental drivers of Δ (Baldocchi and Bowling, 2003; Chen and Chen, 2007; McDowell *et al.*, 2008*a*; Bickford *et al.*, 2009), probably through their strong influence on A and g_{sc}. Leaf water status was also a likely co-regulator of discrimination. Δ was inversely related to Ψ_w, increasing when Ψ_w decreased from 1 June to 19 July, and decreasing when Ψ_w again increased in August. Δ was comparable with previous observations in juniper during the same months in 2006, but was lower on 23 August (Bickford *et al.*, 2009), probably due to substantially more negative pre-dawn Ψ_w in August 2007 (−2.75 MPa) compared with August 2006 (−0.58 MPa; McDowell *et al.*, 2008*b*). The non-significant relationship between Ψ_w and mean Δ_{obs} was probably due to low sample size ($n=4$).

Variation in the physiological parameters A and p_i/p_a, but not g_{sw}, was correlated with variability in Δ_{obs}. Consistent with theory, Δ_{obs} was generally higher when A was low and p_i/p_a was high (Fig. 4). Conversely, Δ_{obs} tended to be lower when A was high and p_i/p_a was low. The diffuse pattern between Δ_{obs} and p_i/p_a seen at higher p_i/p_a (>0.7) is attributed to variation among measured trees (data not shown). A large range of Δ_{obs} was seen at low g_{sw}, consistent with previous work showing relatively high Δ when g_{sw} and A are low (Bickford *et al.*, 2009), and probably contributed to the non-significant relationship

between the two factors. This was unexpected because g_{sw} regulates CO_2 transport into the leaf, but the poor relationship may support an even stronger role for carboxylase activity in regulating Δ in juniper. Finally, the isotope effect associated with diffusion through airspaces and dissolution of CO_2 to HCO_3^- to equilibrium is accounted for in Δ_{comp}, but the diffusion or facilitated passage of CO_2 or bicarbonate across the cell wall and organelle membranes is still being elucidated (Uehlein *et al.*, 2008) and may create further fractionation events that influence the Δ that is measured, though these data do not demonstrate a strong g_m effect on juniper Δ.

Model performance

Parameterizing g_m based on its relationship to g_s and TOD did not consistently improve model predictions over Δ_{simple}, nor did the use of a mean g_m in Δ_{comp}. Incorporating g_m via Δ_{comp} did reduce model bias when set to low values, but had a negligible effect on the error term whether set to low or high values. Thus, much unexplained variance remains in predictions of juniper Δ in the field, as is evident in the large unresolved model bias between predicted and observed Δ inherent in all models tested across the four dates. From a whole-study perspective, the results demonstrate no improvement in model error when using Δ_{comp} compared with Δ_{simple}, supporting the use of the parsimonious simple model to predict juniper Δ over the diurnal periods and across the seasonal gradient in this study. It is possible, however, that utilizing the g_m–TOD or g_m–g_{sc} relationship to parameterize Δ_{comp} may result in significant reductions in model error in other plant systems. These findings contrast with previous work showing improved model fit when utilizing a mean g_m in Δ_{comp} across diurnal and seasonal time scales (Bickford *et al.*, 2009), though Δ_{simple} did outperform Δ_{comp} on one date in that limited study. These results also contrast with a recent study showing improved model predictions of respired $\delta^{13}C$ values when g_m was linked to variation in g_{sw} compared with using a static g_m in model predictions (Cai *et al.*, 2008). These discrepancies demonstrate the need for more studies in diverse systems. The substantial unexplained variance observed in the model bias, and subsequently in the error term, across all months warrants further examination. Model bias was relatively high on most days (Fig. 5), particularly 23 August, and in the pooled data (Table 2), showing that all models consistently overestimated Δ_{obs}. The most likely reason for this is model parameterization error (discussed below in the sensitivity analysis).

Sensitivity tests showed that variation in e and b improved model performance. Implementing an e value of −3‰ generally minimized error compared with values of −1‰ or −6‰, but did not show a similar reduction in model bias. Step-change reductions in b from the value used in this study (29‰), however, resulted in consistently lower model bias and error. Two factors could explain these findings: (i) that the fractionation associated with b was lower and/or more variable than that reported until recently; or (ii) that

R_d was higher and/or more variable than estimated in this study. The simultaneous reduction in model bias and error observed in this study when reduced b values were implemented demonstrates the strong regulatory role of b in model performance, but without assays of PEP and Rubisco activity and Rubisco discrimination no conclusions about the isotope effect or variability in b over diurnal periods can be made. Importantly, this does not suggest that the result of the sensitivity tests demonstrates that b is lower than shown in theoretical (Tcherkez and Farquhar, 2005) or empirical studies (Roeske and O'Leary, 1984; McNevin *et al.*, 2007). A lower b, however, could be explained by relatively high PEP carboxylation activity proportional to Rubisco activity (Farquhar and Richards, 1984; Lanigan *et al.*, 2008), a lower intrinsic isotope effect of the carboxylases comprising b (Raven and Farquhar, 1990; Brugnoli and Farquhar, 2000), or temperature effects on carboxylase activity, as mean T_L was >30 °C. PEP carboxylation is typically associated with C_4 photosynthesis and results in low discrimination against ^{13}C when hydration of CO_2 to HCO_3^- by carbonic anhydrase is in equilibrium (approximately $-5.7‰$; Farquhar *et al.*, 1989), but the extent of PEP carboxylase activity in C_3 photosynthesis is not well understood.

Alternatively, the influence of respiratory activity may have been higher than was estimated in this study. Estimates were based on previous work showing a high dark respiration rate, which were used as a surrogate estimator of R_d, and a 2–3‰ dark respiration fractionation in juniper (Bickford *et al.*, 2009). Error may have been introduced if R_d was subject to diurnal variation that was not accounted for, or if a substantial offset exists between e and the dark respiration fractionation. Recent evidence shows the day and dark respiratory biochemical pathways are not the same, and may result in different isotopic fractionation (Tcherkez *et al.*, 2008); however, the magnitude of the difference at the leaf level is not yet understood.

Δ_{simple} also showed sensitivity to variation in b, and sensitivity tests support greater variability in b among C_3 plants than is currently assumed. Previous studies using Δ_{simple} have shown b values <27‰ resulting in the best fit of observed Δ (Brugnoli and Farquhar, 2000), and this is usually attributed to the reduced b value accounting for omitted fractionation factors. Δ_{comp} and Δ_{simple} were tested with the same Δ_{obs} data set, however, and improvements were found in both models when lower b values were used. The results of the sensitivity tests are slightly confounded by the use of Δ_{pred} and Δ_{ef}, of which e and b are components, in the calculations of g_m. In this application, however, the impact on the sensitivity tests is minimal since the exercise was designed to illustrate the impact of varying b and e at given a constant g_m. That said, the results would be strengthened by estimates of g_m from an independent method such as chlorophyll fluorescence, which relies on assumptions different from those of the isotopic method (Pons *et al.*, 2009). Previous work has shown similar g_m estimates (Loreto *et al.*, 1992) and small differences in g_m estimates from the two methods (Vrábl *et al.*, 2009), and

chlorophyll fluorescence-based estimates may have provided useful data on the variability in g_m observed in this study. Overall, the results of the model tests and sensitivity analysis show non-negligible model bias and error in predicting juniper leaf Δ which was not reconciled by incorporating variability in g_m or other parameters.

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