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 spectrooscope 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 ($\Delta_{\text{comp}}$), 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 $\Delta_{\text{comp}}$ predictions of field-grown juniper compared with the simple model of Δ ($\Delta_{\text{simple}}$) 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 $\Delta_{\text{comp}}$ ($29\%$) and $\Delta_{\text{simple}}$ ($27\%$). 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; Le Roux et al., 2001; Hanba et al., 2003; Warren and Adams, 2006) and ecosystem respiration ($\delta^{13}$C$_{\text{resp}}$; Ogée et al., 2003; Cai et al., 2008) which has implications for interpreting water use efficiency and terrestrial carbon exchange, among other applications. $\Delta$ is a strong regulator of $\delta^{13}$C and $\delta^{13}$C$_{\text{resp}}$ (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 $\Delta$ 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.

$\Delta$ 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 $\Delta$ has been widely observed over the last three decades (Farquhar et al., 1982a, 1989; Brugnoli and Farquhar, 2000). $p_i/p_a$ is integral to two models of $\Delta$: a comprehensive model that incorporates fractionation factors associated with diffusion, carboxylation, and decarboxylation processes ($\Delta_{\text{comp}}$; Farquhar et al., 1982b); and a simplified version of $\Delta_{\text{comp}}$ that omits fractionation factors associated with decarboxylation activity and much of the diffusive pathway ($\Delta_{\text{simple}}$; Farquhar et al., 1982b). The parsimonious $\Delta_{\text{simple}}$ evolved from the same theoretical work as $\Delta_{\text{comp}}$ (Farquhar et al., 1982b) and gained wide usage primarily because of its simplicity and power in explaining observations of $\Delta$, but also because the effects of decarboxylation activity and $g_m$ were thought to be negligible in predicting $\Delta$.

Mechanistic models are used to predict $\Delta$ 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., 1982b). In addition to $p_i/p_a$, the key drivers of $\Delta_{\text{simple}}$ 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 $\sim$27‰ in $\Delta_{\text{simple}}$, which is $\sim$2‰ lower than most measurements of the Rubisco fractionation in C3 plants ($\sim$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 $\Delta$ 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 $\Delta$ 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 $\Delta$ (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 $\sim$7‰ to 13‰ (Tcherkez, 2006; Lanigan et al., 2008) and $e$ thought to be around $\sim$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 spectroscopy (TDL) coupled to infra-red gas analysers (IRGAs) was used to measure $g_m$ and $\Delta$ 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 $\Delta$ and examine the relationship between $\Delta$ and environmental and physiological drivers; and (v) assess the performance of $\Delta_{\text{comp}}$, when fitted with diurnally variable $g_m$, compared with predictions from $\Delta_{\text{simple}}$.

**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 ($J_{\text{ambient}}$) which were rotated between $\sim$06:00 h and 18:00 h on each day with measurements conducted maintaining the chamber environment similar to ambient conditions; and (ii) on an adjacent mature juniper tree ($J_{\text{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 $J_{\text{ambient}}$, leaf gas exchange and $^{13}$C discrimination were measured in response to ambient conditions. For both $J_{\text{ambient}}$ and $J_{\text{manipulate}}$, temperature regulation in the chamber block was engaged when
leaf temperature ($T_L$), measured by energy balance, was $\approx 35^\circ C$. Incoming irradiance in $J_{\text{manipulate}}$ was manipulated by using a plastic shade to reduce incident light by $\sim 50\%$ once or twice per hour to regulate net photosynthetic rate ($A; \mu mol m^{-2} s^{-1}$) and assess the impact of irradiance on $F_{\text{ms}}$. 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 ($J_{\text{manipulate}}$), 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 $J_{\text{ambient}}$ and the other to measure $J_{\text{manipulate}}$. 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 [12C18O2] and [13C18O2] 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 $A$ measurements and subsequent model testing, a priori criteria were established to filter error-prone data. These filtering criteria included ensuring that the difference in [CO2] of the gas entering and exiting the leaf chamber was $> 30 \mu mol m^{-1}$, that the difference in entering and exiting $\delta^{13}C$ was $\approx 1 \%$, and that $\xi$ was $< 10$ (see below for explanation of the $\xi$ 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%/C0 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 [CO2] measurements used to calibrate each measurement cycle were (mean ± SE) 548.7 ± 0.04 μmol mol$^{-1}$ (12C16O2): 5.9 ± 0.0005 μmol mol$^{-1}$ (13C16O2); 2.2 ± 0.001 μmol mol$^{-1}$ (12C18O16O) for the high WS tank; and 347.3 ± 0.3 μmol mol$^{-1}$ (12C16O2): 3.7 ± 0.003 μmol mol$^{-1}$ (13C16O2); 1.4 ± 0.001 μmol mol$^{-1}$ (12C18O16O) for the low WS tank during 1 June, 20 June, and 19 July measurements. The [CO2] of a new high WS calibration tank used in the 23 August measurements was measured as 535.9 ± 0.3 μmol mol$^{-1}$ (12C16O2); 5.8 ± 0.003 μmol mol$^{-1}$ (13C16O2); 2.2 ± 0.001 μmol mol$^{-1}$ (12C18O16O), while the low WS tank was the same as described above. All WS calibration tanks were calibrated for 4 months against WMO-certified tanks that were filled and δ13C 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 [CO2] of the WMO-traceable tanks used in this study were, for the high tank, 539.57 μmol mol$^{-1}$ (12C16O2): 5.93 μmol mol$^{-1}$ (13C16O2): 2.21 μmol mol$^{-1}$ (12C18O16O); and for the low tank, 339.43 μmol mol$^{-1}$ (12C16O2): 3.76 μmol mol$^{-1}$ (13C16O2): 1.40 μmol mol$^{-1}$ (12C18O16O). Measurements of [CO2] 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 CO2 values observed in this study (Bickford et al., 2009).

Pre-dawn leaf water potential ($\Psi_w$) 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 Hydrophone Neutron Moisture Probes, Campbell Pacific Nuclear, Inc., Pacoche, CA, USA) at 2 week intervals between 23 May and 9 August 2007.

Model parameterization

The study tested whether variable $g_{\text{ms}}$ improved model predictions of $\Delta_{\text{obs}}$ in $J_{\text{ambient}}$ using a comprehensive model of $\Delta$ (Farquhar et al., 1982b),

$$
\Delta_{\text{comp}} = a_0 - a_p a_p - a_p a_p + b_1 + a_0 (p_1 - p_1) + b_2 \frac{g_2}{p_2} + \frac{\gamma a + f^*}{p_a}
$$
(1)

where $a_0$, $a_{wp}$, and $b_1$ represent the fractionation factors associated with CO2 diffusion through the leaf boundary layer (2.9%o), water (0.7%o), and fractionation attributed to CO2 entering solution (11.0%o). The variables $p_a$, $p_o$, $p_c$, and $p_r$ represent pCO2 (Pa) in the chamber surrounding the leaf, at the leaf surface, in the intercellular spaces, and at the sites of carboxylation, respectively. $\Gamma^*$, $R_p$, $k$, and $e$ represent the CO2 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 $p_{wp}$, $p_o$, $p_c$, and $p_r$ were calculated by incorporating atmospheric pressure in Los Alamos (~79 kPa) with mole fraction-measurements of [CO2]; $p_c$ was estimated as $p_c = p_r - A g_{\text{ms}}$ (Farquhar and Sharkey, 1982). $R_p$ 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 $k_pP_p$ for each 3 min cycle, and $\gamma$ calculated based on the expanded $T_i$ 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%o (Lanigan et al., 2008) and ~3%o respectively. $e$ has often been estimated based on the dark respiration fractionation, and previous work suggests juniper exhibits a 2-3%o 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 CO2 (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 $\Delta_{\text{comp}}$ to variation in each, and model predictions were compared with $\Delta_{\text{obs}}$. In these sensitivity tests $\Delta_{\text{comp}}$ was fitted with a $g_2 = 1.72$ μmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ ($\Delta_{\text{mean}}$) and both $\Delta_{\text{mean}}$ and $\Delta_{\text{simple}}$ were tested against all $\Delta_{\text{obs}}$ values ($n = 552$), where $\Delta_{\text{simple}}$ is:

$$
\Delta_{\text{simple}} = a + (b - a) \frac{p_1}{p_a}
$$
(2)

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

$\Delta$ and diurnal $g_{\text{ms}}$

Leaf carbon isotope discrimination ($\Delta_{\text{obs}}$) was calculated from TDL-generated data:

$$
\Delta_{\text{obs}} = \frac{\delta_c - \delta_o}{1 + \delta_o - \delta_c}
$$
(3)

where $\delta_c$ and $\delta_o$ equal the $\delta^{13}C$ of the entering and outgoing chamber gas streams, respectively, and $\xi$ equals $c_f(c_e-c_o)$ where $c_e$ and $c_o$ are the [CO2] of the gas entering and exiting the leaf chamber, respectively. $g_{\text{ms}}$ was estimated in $J_{\text{manipulate}}$ leaf gas
exchange and isotopic data using the point-based method (Evans et al., 1986),

\[ g_m = \frac{(b - b_s - a_m)A/p_a}{(D_{\text{pred}} - D_{\text{obs}}) - \Delta_{ef}} \]  

(4)

where predicted discrimination \(D_{\text{pred}}\) is \(D_{\text{simple}}\) with \(b=29\text{ppm}\). The estimate of the fractionation attributed to decarboxylation activities, \(\Delta_{ef}\), was calculated as,

\[ \Delta_{ef} = \frac{c_B + f_B}{p_a} \]  

(5)

All components of \(\Delta_{ef}\) were parameterized as described for \(\Delta_{\text{comp}}\). \(g_m\) estimates that fell below zero were excluded, and this occurred when \(D_{\text{pred}} < D_{\text{obs}}\). Measurement error in \(D_{\text{obs}}\) and \(g_m\) incorporated instrument error for both total CO2 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 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) Pa\(^{-1}\)). Secondly, a regression was fitted between TOD and \(g_m\) measured across 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 (\(\mu\)mol CO2 m\(^{-2}\) s\(^{-1}\) Pa\(^{-1}\)) to a flux density (mol CO2 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 CO2 \((g_{sc}; \text{mol CO2 m}^{-2}\text{ s}^{-1})\) was calculated as stomatal conductance to H2O \((g_{sw})\) divided by 1.6 to account for differences in diffusivity between water vapour and CO2 (Farquhar and Sharkey, 1982). The transformed \(g_m\) values were then compared with \(g_m\) data using linear regression, and the linear expression describing the relationship was used to estimate \(g_m\) (\(g_{m,\text{ls}}\)). 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 \(g_m\) estimate, presented here as \(1\ SE\), exceeded 0.10 \(g_m\) that point \(g_m\) estimate was excluded from regression analysis. Means testing was computed using the Tukey–Kramer honestly significant differences test \((P <0.05\text{ level})\). All statistical tests were performed in R (version 2.9.1; R Core Development Team, 2009).

\(\Delta_{\text{comp}}\) was parameterized in three ways for intermodel testing by calculating \(\Delta_{\text{comp}}\) using \(g_{m,\text{mean}}\) (\(\Delta_{\text{mean}}\)), \(g_{m,\text{TOD}}\) (\(\Delta_{\text{TOD}}\)), and \(g_{m,\text{ls}}\) (\(\Delta_{\text{ls}}\)). All three variations of \(\Delta_{\text{comp}}\) along with \(\Delta_{\text{simple}}\) were tested against \(\Delta_{\text{obs}}\). 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 \(\mu\)mol 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 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) Pa\(^{-1}\)) and 20 June (1.44±0.05 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) Pa\(^{-1}\)), but was higher on 19 July (3.13±0.42 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) Pa\(^{-1}\)) and 23 August (2.22±0.10 \(\mu\)mol 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 \(g_m=-3.52TOD+3.38\) 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).

**\(\Delta_{\text{obs}}\): physiological, and environmental parameters**

Mean \(\Delta_{\text{obs}}\) in \(j_{\text{ambient}}\) 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. \(\Delta_{\text{obs}}\) 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 \(\Delta_{\text{obs}}\), including \(A\) (\(P <0.0001\), \(r^2=0.13, \ F=80.7\)) and \(p_{\text{lp}}/p_{\text{pa}}\) (\(P <0.0001\), \(r^2=0.29, \ F=225.9\)).

![Fig. 1. Significant diurnal variation in mesophyll conductance to CO2 \((g_{sc})\) across the four measurement dates (\(P <0.0001\); \(r^2=0.37\)). Mean \(g_{sc}\) 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.](image1)

![Fig. 2. The relationship between stomatal conductance to CO2 \((g_{sc})\) and mesophyll conductance \((g_m)\) across all four measurements dates \((g_{sc}=\text{estimates}\times0.022; \ P <0.0001, \ r^2=0.27\)). Error bars represent 1 SE.](image2)
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 \( D_{obs} \) and \( T_L \) on 19 July (\( P=0.006, r^2=0.05, F=7.81 \)) but not other dates (\( P \geq 0.05 \)). Mean \( T_L \) was 31.8\( \pm \)3.43 \(^\circ\)C (mean \( \pm \)SD) across all dates. There were also weak but significant relationships between \( D_{obs} \) and vapour pressure deficit (VPD) on each day except 23 August (\( P<0.0001, r^2=0.05 \)). 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 \( D_{obs} \) 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. \( \Psi_w \) measured in nearby juniper trees (\( n=6 \)) was highest early in the season at \(-0.62\pm0.06 \) MPa (23 May) and then declined to \(-2.1\pm0.2 \) MPa (27 June) and \(-3.4\pm0.33 \) MPa (25 July) before increasing to \(-2.75\pm0.34 \) MPa (23 August). The relationship between \( \Psi_w \) and \( D_{obs} \) was not significant (\( P=0.15, r^2=0.75 \)).

Model performance

\( \Delta_{comp} \) did not consistently outperform \( \Delta_{simple} \), and the reductions in \( \Delta_{comp} \) model bias observed over most of the study varied little with different parameterizations of \( g_m \). \( \Delta_{simple} \) 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 \( \Delta_{comp} \) showed comparable RMSE, and the differences in error were within 0.05\% of one another. Model bias was significantly greater than zero in predictions of \( \Delta_{obs} \) 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 \( \Delta \) by at least 1\%\( \), and that the limited improvements in predictions of \( \Delta \) by incorporating \( g_m \) were small compared with the bias between \( \Delta_{obs} \) and \( \Delta_{comp} \), 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,
spaces and the atmosphere around the leaf (relationships with $D$ across months these parameters exhibited significant linear
Across tested $e$ values the use of lower $b$ values in $\Delta_{\text{mean}}$
consistently reduced model bias and error. Error changed minimally when $\Delta_{\text{mean}}$ was parameterized with $e=-3^{\circ}/C_0$ and $b=29^{\circ}/C_0$ and $g_m$ was decreased to 0.172 $\mu$mol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ (bias=1.66, RMSE=2.46) or increased to 17.2 $\mu$mol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ (bias=3.76, RMSE=2.43) compared with a $g_m=1.72$ $\mu$mol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ (bias=3.57, RMSE=2.42), though model bias did decline 54% at the lowest $g_m$ value ($P<0.0001$). $\Delta_{\text{simple}}$ showed an 85% reduction in model bias and a 4.7% reduction in error when fit with $b=22^{\circ}/C_0$ instead of $b=27^{\circ}/C_0$ (Table 3). Excluding 19 July, all variations of $\Delta_{\text{comp}}$ and $\Delta_{\text{simple}}$ overestimated $\Delta_{\text{obs}}$ by 3–7$^{\circ}/C_0$ as determined by model bias, though accounting for the variance, as in the RMSE term, reduced total error to between 1.3$^{\circ}/C_0$ and 2.4$^{\circ}/C_0$ on individual days. Using RMSE as the metric, the best fit to $\Delta_{\text{obs}}$ using $\Delta_{\text{mean}}$ was with $e=-3^{\circ}/C_0$ and $b=25^{\circ}/C_0$ (RMSE=2.25), but that fit was still poorer than predictions from $\Delta_{\text{simple}}$ where $b=22^{\circ}/C_0$ (RMSE=2.19).

**Table 1.** Mean diurnal net photosynthetic rate ($A$; $\mu$mol 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 ($\%_{\text{obs}}$) best performing model in each month and across the study $\Delta_{\text{simple}}$ predictions showed the lowest RMSE across the study, but exhibited higher model bias ($\%_{\text{obs}}$) across the whole study compared with all three parameterizations of $\Delta_{\text{comp}}$ ($P<0.0001$).

| Model | 1 June | 20 June | 19 July | 23 August | Whole study |
|-------|--------|---------|---------|-----------|-------------|
|       | Bias | RMSE | Bias | RMSE | Bias | RMSE | Bias | RMSE | Bias | RMSE |
| $\Delta_{\text{c.mean}}$ | 3.20 | 1.65 | 3.56 | 1.35 | 1.45 | 1.62 | 7.17 | 1.55 | 3.57 | 2.42 |
| $\Delta_{\text{TOD}}$ | 3.18 | 1.61 | 3.55 | 1.36 | 1.45 | 1.62 | 7.20 | 1.52 | 3.56 | 2.42 |
| $\Delta_{\text{gip}}$ | 3.05 | 1.66 | 3.45 | 1.36 | 1.32 | 1.63 | 7.06 | 1.57 | 3.44 | 2.43 |
| $\Delta_{\text{simple}}$ | 2.78 | 1.33 | 3.73 | 1.43 | 1.59 | 1.96 | 6.97 | 1.32 | 3.80 | 2.30 |

**Diurnal $g_{sw}$**

Two diurnal $g_{sw}$ trends were evident across the study. On 1 June, $g_{sw}$ increased in the early morning period to relatively high values ($\sim2-3$ $\mu$mol m$^{-2}$ s$^{-1}$ Pa$^{-1}$) and then declined to lower values for the remainder of the day ($\sim1$ $\mu$mol m$^{-2}$ s$^{-1}$ Pa$^{-1}$), a pattern repeated on 20 June and
the 23 August morning and mid-day periods. On 19 July, \( g_m \) was highest during the earliest measurements (\( \sim 4 \mu mol \ m^{-2} \ s^{-1} \ Pa^{-1} \)) and remained relatively high through the afternoon period (\( \sim 1.5–3 \mu mol \ m^{-2} \ s^{-1} \ Pa^{-1} \)). 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 \( \Psi_w \) 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 CO2-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 CO2 transfer to the sites of carboxylation or, as discussed below, substantially affecting \( \Delta \). These findings agree with data in other species demonstrating that \( g_m \) was higher than

![Graph](image-url)

**Fig. 6.** Model tests of observed discrimination (\( \Delta_{obs} \)) on 1 June (A), 20 June (B), 19 July (C), and 23 August 2007 (D). Four models were tested against \( \Delta_{obs} \) including the simple model of discrimination (\( \Delta_{simple} \); filled circles), the comprehensive model of discrimination using a mean mesophyll conductance (\( g_m \)) of \( 1.72 \mu mol \ m^{-2} \ s^{-1} \ Pa^{-1} \) (\( \Delta_{c.mean} \); open triangles), the comprehensive model of discrimination using a \( g_m \) estimated from the regression between diurnal \( g_m \) and time of day (TOD) (\( \Delta_{c.TOD} \); filled squares), and the comprehensive model of discrimination using a \( g_m \) estimated from the regression describing the relationship between stomatal conductance of CO2 and \( g_m \) (\( \Delta_{c.gs} \); open circles). \( \Delta_{predicted} \) represents discrimination predictions of any of the four models. On two dates \( \Delta_{c.mean} \) or \( \Delta_{c.TOD} \) performed best, but on other dates and across the whole study \( \Delta_{simple} \) exhibited the lowest model error. These results support the use of \( \Delta_{simple} \) to predict leaf-level diurnal carbon discrimination of field-grown juniper.

**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 \( 1.72 \mu mol \ m^{-2} \ s^{-1} \ Pa^{-1} \) (\( \Delta_{c.mean} \); Equation 1), and \( b \) was adjusted in the simplified version of carbon discrimination (\( \Delta_{simple} \); Equation 2)

| \( e \) (%) | \( b \) (%) | Bias (%) | RMSE (%) | \( e \) (%) | \( b \) (%) | Bias (%) | RMSE (%) |
|------------|------------|----------|----------|------------|------------|----------|----------|
| -1         | 29         | 3.04     | 2.42     | 27         | 3.80       | 2.30     |
| 0          | 27         | 1.77     | 2.34     | 25         | 1.03       | 2.74     |
| -3         | 29         | 3.57     | 2.42     | 27         | 2.30       | 2.32     |
| -6         | 29         | 4.36     | 2.46     | 27         | 3.09       | 2.35     |
| 15         | 1.82       | 2.25     |          | 22         | 0.59       | 2.19     |
$g_{sc}$ (Loreto et al., 1992; Galmés et al., 2006), but differ from studies showing lower $g_{sw}$ 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, 2008b). The diurnal decline in $g_{m}$ observed across all study dates did not consistently improve $\Delta$ 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).

$\Delta$, environmental, and physiological parameters

Diurnal patterns across the study were consistent with previous studies showing environmental regulation of $\Delta_{obs}$. As previously observed in model and empirical studies, VPD and PPFD acted as environmental drivers of $\Delta$ (Baldocchi and Bowling, 2003; Chen and Chen, 2007; McDowell et al., 2008a; 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. $\Delta$ was inversely related to $\Psi_{sw}$, increasing when $\Psi_{sw}$ decreased from 1 June to 19 July, and decreasing when $\Psi_{sw}$ again increased in August. $\Delta$ 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 $\Psi_{sw}$ in August 2007 ($-2.75$ MPa) compared with August 2006 ($-0.58$ MPa; McDowell et al., 2008b). The non-significant relationship between $\Psi_{sw}$ and mean $\Delta_{obs}$ was probably due to low sample size ($n=4$).

Variation in the physiological parameters $A$ and $p/p_a$, but not $g_{sw}$, was correlated with variability in $\Delta_{obs}$. Consistent with theory, $\Delta_{obs}$ was generally higher when $A$ was low and $p/p_a$ was high (Fig. 4). Conversely, $\Delta_{obs}$ tended to be lower when $A$ was high and $p/p_a$ was low. The diffuse pattern between $\Delta_{obs}$ and $p/p_a$ seen at higher $p/p_a$ (>0.7) is attributed to variation among measured trees (data not shown). A large range of $\Delta_{obs}$ was seen at low $g_{sw}$, consistent with previous work showing relatively high $\Delta$ 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 $\Delta$ in juniper. Finally, the isotope effect associated with diffusion through airspaces and dissolution of CO$_2$ to HCO$_3$ is accounted for in $\Delta_{comp}$, 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 $\Delta$ that is measured, though these data do not demonstrate a strong $g_{m}$ effect on juniper $\Delta$.

Model performance

Parameterizing $g_{m}$ based on its relationship to $g_{sw}$ and TOD did not consistently improve model predictions over $\Delta_{simple}$, nor did the use of a mean $g_{m}$ in $\Delta_{comp}$. Incorporating $g_{m}$ via $\Delta_{comp}$ 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 $\Delta$ in the field, as is evident in the large unresolved model bias between predicted and observed $\Delta$ inherent in all models tested across the four dates. From a whole-study perspective, the results demonstrate no improvement in model error when using $\Delta_{comp}$ compared with $\Delta_{simple}$, supporting the use of the parsimonious simple model to predict juniper $\Delta$ over the diurnal periods and across the seasonal gradient in this study. It is possible, however, that utilizing the $g_{m}$–TOD or $g_{m}$–$g_{sw}$ relationship to parameterize $\Delta_{comp}$ 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 $\Delta_{comp}$ across diurnal and seasonal time scales (Bickford et al., 2009), though $\Delta_{simple}$ did outperform $\Delta_{comp}$ 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 $g_{sw}$ 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 $\Delta_{obs}$. 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
observed in this study when reduced $b$ 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; Langan 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 $>30^\circ C$. PEP carboxylation is typically associated with $C_4$ photosynthesis and results in low discrimination against $^{13}\text{C}$ when hydration of $\text{CO}_2$ to $\text{HCO}_3^-$ by carbonic anhydrase is in equilibrium (approximately $-5.7\%_\text{oo}$; 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 $b$ 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.

$\Delta_{\text{simpl}}$ 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 $\Delta_{\text{simpl}}$ have shown $b$ values $<27\%_\text{oo}$ resulting in the best fit of observed $\Delta$ (Brugnoli and Farquhar, 2000), and this is usually attributed to the reduced $b$ value accounting for omitted fractionation factors. $\Delta_{\text{comp}}$ and $\Delta_{\text{simpl}}$ were tested with the same $\Delta_{\text{ps}}$ 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 $\Delta_{\text{pred}}$ and $\Delta_{\text{efi}}$ 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 (Vrâbl 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 $\Delta$ which was not reconciled by incorporating variability in $g_m$ or other parameters.

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