Environmental regulation of carbon isotope discrimination and internal CO2 conductance in C3 leaves

Christopher Bickford

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ENVIRONMENTAL REGULATION OF CARBON ISOTOPE DISCRIMINATION AND INTERNAL CO2 CONDUCTANCE IN C3 LEAVES

BY

CHRISTOPHER PAUL BICKFORD

B.S., Botany, University of Oklahoma, 2001
M.Sc., Forestry, Northern Arizona University, 2005

DISSERTATION

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy
Biology

The University of New Mexico
Albuquerque, New Mexico

August 2009
DEDICATION

In memory of my mother, Karen Jeanne Huff Bickford, who could not be here today.
ACKNOWLEDGMENTS

Many people have aided me in this dissertation process, and I am grateful to all of them. I thank my advisors Dave Hanson and Nate McDowell for their time, patience, and advice over the years. It was a wonderful experience learning about the process of doing science from you both. I thank my family, in particular my father Paul Bickford, and friends for being there through all the ups and downs and supporting me all the way; I wouldn’t be where I am today without you. Finally, I wish to express my deepest gratitude to my wonderful wife, Karen Bagne, who has been here through this entire process.
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ABSTRACT OF DISSERTATION

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ABSTRACT

Stable carbon isotopes are powerful tools for elucidating leaf- and ecosystem-level processes, and recent technological developments provide new opportunities to assess the isotopic flux during leaf gas exchange. In these studies I used a tunable diode laser spectroscopy coupled to an infra-red gas analyzer to measure the isotopic composition of leaf gas exchange at high frequency in both field and greenhouse settings and assess environmental regulation of carbon isotope discrimination ($\Delta$) and internal conductance of CO$_2$ to sites of carboxylation ($g_i$). I measured $\Delta$ and $g_i$ across diurnal and seasonal periods in field-grown Juniperus monosperma trees and used these data to 1) assess the diurnal variation in $\Delta$ in response to environmental
drivers, 2) test predictions from existing models of $\Delta$, 3) test the linearity of the
relationship between $\Delta$ and the ratio of intercellular to ambient $\text{CO}_2$ partial
pressure ($p_i/p_a$), 4) test the hypothesis that $g_i$ varies at diurnal timescales and 5) test
the influence of $g_i$ in $\Delta$ models. Results show photosynthetic photon flux density, soil
water availability, and vapor pressure deficit were significant environmental drivers
of diurnal $\Delta$ patterns, and that existing models generally produced model
predictions of $\Delta$ within 1-3‰ of observed values. Linear models adequately
described significant relationships between observed $\Delta$ and $p_i/p_a$, but second order
models better described the relationship under some conditions. $g_i$ varied diurnally
and ranged between 0.03-2.0 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$. Accounting for this variation
improved model predictions of $\Delta$ compared with a model that omits $g_i$, and
parameterizing $g_i$ based on dynamic variables such as time of day produced the
greatest improvement in predictions. These findings demonstrate the need for model
improvements to better predict $\Delta$ under field conditions.

Greenhouse studies were conducted to address the influence of soil water
deficit (SWD) and leaf water potential ($\Psi_w$) on $g_i$. Plants with isohydric tendencies
were droughted and $g_i$ assessed using slope-based isotopic methods. Results showed
no significant difference in $\Psi_w$ or $g_i$ between droughted and control plants and
suggest $\Psi_w$ may buffer the $g_i$ response to SWD.
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Chapter 1

Introduction

Stable carbon isotope analyses have a long history in plant biology that includes differentiation of photosynthetic pathways (Smith & Epstein 1971), development of physiological theory of carbon isotope fractionation (O’Leary 1981; Farquhar et al. 1982), crop improvement (Farquhar & Richards 1984), ecological studies (Ehleringer 1993; Brooks et al. 1997), ecosystem process studies (Bowling et al. 2002; McDowell et al. 2004), and biosphere-atmosphere interactions (Yakir 2003; Randerson et al. 2006).

The biophysical discrimination against the $^{13}$C$^{16}$O$_2$ isotopologue during photosynthesis ($\Delta$) is the consequence of numerous fractionation factors, most of which are relatively well understood but include important exceptions. These fractionation factors are points along the CO$_2$ photosynthetic pathway from the atmosphere to sites of carboxylation where different diffusion and carboxylation rates of the $^{12}$CO$_2$ and $^{13}$CO$_2$ isotopologues result in accumulations of $^{13}$CO$_2$ in gas, liquid, or solid samples which differ from the composition of atmospheric air (Farquhar, Ehleringer & Hubick 1989; Brugnoli & Farquhar 2000; Bowling et al. 2008). The role of many factors underlying $\Delta$ in leaf gas exchange are well understood and include the fractionations associated with CO$_2$ diffusion through the leaf boundary layer and stomata (Craig 1953), CO$_2$ entry into solution (Mook, Bommerson & Staverman 1974), diffusion through solution (O’Leary 1984), and carboxylation due to enzymatic activity (Roeske & O’Leary 1984). Early studies suggested isotopic fractionations associated with day respiration ($e$), photorespiration ($f$) and $g_i$ were minimal or non-existent (Farquhar et al. 1982), but mounting evidence strongly suggests $e$, $f$, and $g_i$ all have large roles in $\Delta$. 
Mechanistic models are used to predict $\Delta$ across a variety of temporal and spatial scales, but direct comparisons of their performance against empirical data are rare. Two related models are currently used to describe $\Delta$: a comprehensive model that incorporates fractionation factors associated with the entire diffusion pathway of CO$_2$, carboxylation and decarboxylation ($\Delta_{\text{comp}}$; Farquhar, O’Leary & Berry 1982; Farquhar & Richards 1984), and a simplified version of $\Delta_{\text{comp}}$ that omits many of the diffusion related fractionation factors and all decarboxylation components ($\Delta_{\text{simple}}$; Farquhar et al. 1982).

Variation in $\Delta_{\text{simple}}$ is driven by variation in the ratio of CO$_2$ partial pressure in the intercellular spaces and in the atmosphere ($p_i/p_a$) interacting with key model parameters (Farquhar, O’Leary & Berry 1982). These key drivers of $\Delta_{\text{simple}}$ include 1) the carboxylation term, $b$, that represents net fractionation associated with phosphoenolpyruvate (PEP) carboxylase and Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and 2) the fractionation associated with diffusion in air and through stomata ($a$; 4.4‰) (Farquhar et al. 1989). $b$ is typically estimated at ~27‰ in $\Delta_{\text{simple}}$, or ~2‰ lower than early measurements of the full Rubisco fractionation (~29‰; Roeske & O’Leary 1984), to account for omitted fractionation factors (Farquhar & Richards 1984). Recent work suggests net Rubisco fractionation may be between 25-30‰ (Tcherkez & Farquhar 2005) and $b$ may be as low as 27.4‰ in tobacco (*Nicotiana tabacum*; McNevin et al. 2007) and 26‰ in *Senecio* (Lanigan et al. 2008). $\Delta_{\text{comp}}$ incorporates the factors discussed above plus fractionation associated with CO$_2$ diffusion, including $g_i$, and decarboxylation activity. $g_i$ is dynamic and may be an important driver of $\Delta$ by reducing the diffusion rate from stomatal cavities to the chloroplast (Flexas et al. 2008), but tests of the influence of $g_i$ in predicting $\Delta$ are few (Cai et al. 2008). A major
focus of this study was to examine the diurnal patterns of $\Delta$ and $g_i$ under field conditions and use these data to test $\Delta_{\text{simple}}$ and $\Delta_{\text{comp}}$ against a high frequency dataset representative of seasonal shifts in physiological activity in a semi-arid ecosystem.

Isotopic fractionation associated with the decarboxylation activities of day respiration and photorespiration ($\Delta_{ef}$) has a demonstrable effect on $\Delta$. Direct measurements of $e$ are not currently possible, and consequently the isotopic fractionation associated with dark respiration ($e_d$) is often used as a surrogate estimator for $e$, though recent evidence demonstrates day and dark respiration involve different biochemical pathways (Tcherkez et al. 2008). Several environmental variables have been shown to influence $e_d$, including drought (Duranceau et al. 1999; Ghashghaie et al. 2001; Ghashghaie et al. 2003), temperature (Tcherkez et al. 2003) and irradiance (Barbour et al. 2007). Studies have demonstrated a large range of $f$ values (~3-8‰), but the role of environmental regulation is largely confined to temperature effects on photorespiration (Brooks & Farquhar 1985; Gillon & Griffiths 1997). Recently, Tcherkez (2006) reported the primary regulating enzyme of photorespiration, glycine decarboxylase, has a large role in $f$ and proposed that $f$ is approximately +10‰, a finding reinforced in recent empirical work showing $f$ equal to 11.6‰ in Senecio (Lanigan et al. 2008). Both $e$ and $f$ are parameterized within the comprehensive model of carbon isotope discrimination ($\Delta_{\text{comp}}$; Farquhar et al. 1982; Farquhar & Richards 1984) in conjunction with their respective respiratory fluxes, day respiration ($R_d$) and photorespiration ($O$). Within $\Delta_{\text{comp}}$ the interaction between $e$, $R_d$, $f$, and $O$ is summarized in a decarboxylation term ($\Delta_{ef}$) that is subtracted from the carboxylation component. The interactive contribution of $e$ and $R_d$ has a negative sign, but results in positive forcing on predicted $\Delta$. Concurrent $f$ and $O$
activities have a positive isotopic signature, and consequently have a negative forcing effect in $\Delta_{\text{comp}}$. To some extent these negative and positive influences nullify one another in the cumulative respiratory isotopic signature, but if one process dominates under a set of environmental conditions then positive or negative forcing of the overall $\Delta$ signature may occur. One research foci of this study was to examine the patterns of $\Delta_{ef}$ under field conditions and in a controlled experimental setting to assess diurnal variation in the field and in response to water stress.

Temperature and water stress have been shown to impact diffusion of CO$_2$ across cell walls and through cellular membranes to sites of carboxylation ($g_i$). Temperature has been shown to regulate $g_i$ within the biologically significant range of 10° to 40°C in tobacco (Bernacchi et al. 2002), a finding supported in more recent work using different species (Warren & Dryer 2006; Yamori et al. 2006). Water stress reduces $g_i$, as demonstrated experimentally in *Pseudotsuga* seedlings (Warren, Livingston & Turpin 2004) and *Olea* (Diaz-Espejo, Nicolas & Fernandez 2007) and in a comprehensive field study using *Quercus* and *Fraxinus* (Grassi & Magnani 2005). However, the physiological signal linking leaf water deficit and shifts in $g_i$ remains elusive. The strong regulatory effect of lamina water balance on leaf processes such as stomatal conductance (Buckley 2005) warrants exploration of leaf water potential ($\psi_w$) as a regulator of $g_i$. Reports of strong linkages between aquaporin function and $g_i$ (Flexas et al. 2006; Miyazawa et al. 2008; Uehlein et al. 2008) provide a possible mechanism for explain rapid variation in $g_i$ in response to a multitude of environmental factors, as has been demonstrated in response to CO$_2$ concentration (Flexas et al. 2007). In this study I explore if linkages exist between
and Ψ_w by assessing g_i in droughted isohydric plants, where leaves maintain relatively constant diurnal Ψ_w in response to soil water deficit.

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Chapter 2

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High frequency field measurements of diurnal carbon isotope discrimination and internal conductance in a semi-arid species, *Juniperus monosperma*

CHRISTOPHER P. BICKFORD¹, NATE G. MCDOWELL², ERIK B. ERHARDT³, DAVID T. HANSON¹

¹University of New Mexico, Department of Biology, MSC03-2020, Albuquerque, NM 87131; ²Earth and Environmental Sciences Division, MS-J495, Los Alamos National Laboratory, Los Alamos, NM 87544 USA; ³University of New Mexico, Department of Mathematics and Statistics, MSC03-2150, Albuquerque, NM 87131

Running title: Diurnal Δ and $g_i$
Abstract

We present field observations of carbon isotope discrimination ($\Delta$) and internal conductance of CO$_2$ ($g_i$) collected using tunable diode laser spectroscopy ($TDL$). $\Delta$ ranged from 12.0‰–27.4‰ over diurnal periods with daily means of 16.3 ± 0.2‰ during drought to 19.0 ± 0.5‰ during monsoon conditions. We observed a large range in $g_i$, from 0.04–8.53 $\mu$mol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ among measured leaves, but most $g_i$ estimates were less than 4.0 $\mu$mol m$^{-2}$ s$^{-1}$ Pa$^{-1}$. We tested the comprehensive Farquhar, O’Leary & Berry (1982) model of $\Delta$ ($\Delta_{comp}$), a simplified form of $\Delta_{comp}$ ($\Delta_{simple}$), and recently suggested amendments ($\Delta_{revised}$; Wingate et al. 2007). Sensitivity analyses demonstrated that varying $g_i$ had a substantial effect on $\Delta_{comp}$, resulting in mean differences between observed $\Delta$ ($\Delta_{obs}$) and $\Delta_{comp}$ ranging from 0.04‰ to 9.6‰. First order regressions adequately described the relationship between $\Delta$ and the ratio of substomatal to atmospheric CO$_2$ partial pressure ($p_i/p_a$) on all three days, but second order models better described the relationship in July and August. The three tested models each predicted $\Delta_{obs}$ best on different days. In June $\Delta_{simple}$ outperformed $\Delta_{comp}$ and $\Delta_{revised}$, but incorporating $g_i$ and all non-photosynthetic fractionations improved model predictions in July and August.

Keywords: mesophyll conductance, carbon isotopes, Juniperus monosperma, Farquhar model, decarboxylation activity, $p_i/p_a$, transfer conductance
Introduction

Stable carbon isotope analyses have a long history in plant biology that includes differentiation of photosynthetic pathways (Smith & Epstein 1971), development of physiological theory of carbon isotope fractionation (O’Leary 1981; Farquhar et al. 1982), crop improvement (Farquhar & Richards 1984), ecological studies (Ehleringer 1993; Brooks et al. 1997), ecosystem process studies (Bowling et al. 2002, McDowell et al. 2004), and biosphere-atmosphere interactions (Yakir 2003; Randerson et al. 2006).

The biological and physical discrimination against the $^{13}\text{C}^{16}\text{O}_2$ isotopologue during diffusion and carboxylation is a strong regulator of the isotopic signature of ecosystem exchange with the atmosphere as it largely determines the $^{13}\text{C}$ composition of the substrate pool which supplies respiratory activity (Barbour et al. 2005; Knohl et al. 2005; Bowling, Pataki & Randerson 2008). The transfer of this signature throughout the ecosystem provides a useful signal to partition components of ecosystem carbon exchange and aid in carbon cycle modeling (Ciais et al. 1995; Tu & Dawson 2005; McDowell et al. 2008a).

A substantial body of literature describing a linear relationship between leaf carbon isotope discrimination ($\Delta$) and the ratio of internal to atmospheric CO$_2$ partial pressure ($p_i/p_a$) has accumulated in the last three decades (Farquhar et al. 1982b; Brugnoli et al. 1988; Farquhar, Ehleringer & Hubick 1989; Ehleringer, Phillips & Comstock 1992; Brugnoli & Farquhar 2000). The $p_i/p_a$ ratio is useful because it succinctly describes the dominant physical and biochemical constraints to photosynthesis. Similarly, the linear relationship between $\Delta$ and $p_i/p_a$ observed in previous studies emphasizes the importance of stomatal conductance and biochemistry in $\Delta$. The full
model of $\Delta$ developed by Farquhar et al. (1982) also accounts for other factors such as internal conductance of CO$_2$ from stomatal cavities to sites of carboxylation ($g_i$) and apparent isotopic fractionations associated with the decarboxylation processes of day respiration and photorespiration ($\Delta_{cf}$), as well as other diffusion related fractionations. Recent evidence suggests that $g_i$ and $\Delta_{cf}$ are sensitive to environmental factors that vary diurnally (Bernacchi et al. 2002; Ghashghaie et al. 2003; Warren, Livingston & Turpin 2004), but their role in the variation in $\Delta$ observed in a field setting remains poorly understood.

Temperature and water stress have been shown to impact $g_i$. Bernacchi et al. (2002) found temperature regulated $g_i$ within the biologically significant range of 10° to 40°C in tobacco, a finding supported in work presented by Yamori et al. (2006) and Warren & Dreyer (2006) using different species. Water stress also reduces $g_i$, as demonstrated experimentally in Pseudotsuga seedlings (Warren et al. 2004) and Olea (Diaz-Espejo, Antonio & Fernandez 2007) and in a comprehensive field study using Quercus and Fraxinus (Grassi & Magnani 2005). Recently, a strong linkage between aquaporin function and $g_i$ was established (Flexas et al. 2006; Uehlein et al. 2008), providing a possible mechanism for rapid variation in $g_i$ in response to a multitude of environmental factors, as has been demonstrated in response to CO$_2$ concentration (Flexas et al. 2007). While seasonal changes in $g_i$ have been documented in a field setting (Grassi & Magnani 2005; Diaz-Espejo et al. 2007) diurnal variation in $g_i$ has not yet been reported.

The influence of environmental factors on $\Delta_{cf}$ is less well known. Temperature and light have been shown to influence day respiration and photorespiration, both of
which affect CO$_2$ evolution within a leaf (Brooks & Farquhar 1985; Kozaki & Takeba 1996; Atkin et al. 2000; Atkin et al. 2005). The apparent fractionation associated with day respiration ($e$) and photorespiration ($f$) are each the result of biochemical reactions that may be subject to environmental control (Ghashghaie et al. 2003). A consistent enrichment of 6‰ in the dark respired $^{13}$C/$^{12}$C ratio ($\delta^{13}$C$_{\text{resp}}$) of CO$_2$ compared to sucrose of droughted and control Phaseolus leaves has been observed (Duranceau et al. 1999). Such respiratory enrichment has been shown to depend on species and on plant water status (Ghashghaie et al. 2001), temperature (Tcherkez et al. 2003), and light exposure (Barbour et al. 2007a). Estimates of $e$ have largely been inferred from studies of dark respiration, but recent evidence suggests these dark respiration fractionations may not be representative of day respiratory fractionation (Tcherkez et al. 2008). Field observations of the diurnal patterns of the cumulative fractionation associated with respiratory and photorespiratory processes, estimated here in $\Delta_{ef}$, may allow us to better understand the influence of environmental factors on this component of $\Delta$.

In recent years advances in optical systems utilizing tunable diode laser spectroscopy (TDL) have simplified high frequency measurements of the abundance of individual isotopologues $^{13}$C$^{16}$O$_2$, $^{12}$C$^{16}$O$_2$, and $^{12}$C$^{18}$O$^{16}$O in ecosystem studies (Bowling et al. 2003; Griffis et al. 2004; McDowell et al. 2008a) and leaf-scale studies in greenhouse settings (Barbour et al. 2007a,b). Similar TDL leaf-scale measurements can now be attempted in a field setting. The objectives of this study were to 1) examine the temporal variation in $\Delta$, $\delta^{13}$C$_{\text{resp}}$, $g_i$, and $\Delta_{ef}$ under ambient field conditions, 2) test the hypothesis that $g_i$ varies across the day, 3) test the hypothesis that $\Delta$ varies linearly in response to shifts in $p_i/p_a$ under field conditions, 4) test the influence of $g_i$ in a
comprehensive leaf model of $\Delta$, and 5) test the predictive capabilities of three models: the comprehensive Farquhar et al. (1982) model of $\Delta$ ($\Delta_{\text{comp}}$), a recently suggested amendment to $\Delta_{\text{comp}}$ ($\Delta_{\text{revised}}$; Wingate et al. 2007) and the simplified form of the comprehensive model ($\Delta_{\text{simple}}$). We used a combined TDL-infra-red gas analyzer (IRGA) system to obtain high frequency field measurements of leaf gas exchange synchronized with online isotopic measurements, similar to those used in previous greenhouse studies (Barbour et al. 2007a). Previous work has demonstrated substantial diurnal variation in leaf discrimination in diverse field settings including tropical forest (Harwood et al. 1998) and mesic conifer forest (Wingate et al. 2007). We report ~ 20 $\Delta$ measurements/hour over diurnal periods during both dry and wet seasons from a semi-arid woodland.

**Methods**

The field site was located on Mesita Del Buey in Los Alamos, New Mexico USA (latitude 35° 50' N, longitude 106° 16’W; elevation 2140 m) in a piñon-juniper woodland (*Pinus edulis* Engelm. and *Juniperus monosperma* Engelm. Sarg., respectively) dominated primarily by juniper and understory grasses and forbs (Breshears 2008; McDowell et al. 2008b). This semi-arid region typically has a bi-modal precipitation regime, with substantial winter snowfall (October–April), followed by a dry period (May–June), and monsoonal precipitation from July through early September (Breshears 2008). Precipitation at our site in 2006 totaled 119 mm in winter and 224 mm in summer. Soils on the site are Typic Haplustalfs and Typic Ustochrepts (Davenport, Wilcox & Breshears 1996).
Leaf gas exchange measurements

We measured diurnal (06:00–19:00 MST) leaf gas exchange from the bottom third of the canopy on two juniper trees on 12 June 2006, two different juniper trees on 11 July 2006, and a single juniper on 14 August 2006. We coupled a TDL (TGA100A, Campbell Scientific Inc.) to a portable photosynthesis system (LI-COR 6400, LI-COR Biosciences) fitted with a conifer chamber (LI-COR 6400-05) to quantify the concentration of CO$_2$ and its isotopologues $^{13}$C$^{16}$O$_2$ and $^{12}$C$^{16}$O$_2$ in gas entering and exiting the leaf chamber, herein referred to as the reference and sample gas streams (i.e. Barbour et al. 2007a). We supplied atmospheric air via a 50 L buffer volume to the LIxCor 6400, which recorded the CO$_2$ and water vapor concentration of the reference and sample gas every 10 seconds. These same gas streams were dried to a constant low humidity and plumbed directly into the TDL using ultra-low porosity tubing (Synflex type 1300 ¼” diameter, Saint Gobrain Performance Plastics) wherein the TDL measured the CO$_2$ isotopologues $^{13}$C$^{16}$O$^{16}$O and $^{12}$C$^{16}$O$^{16}$O at a rate of 500Hz. These 500Hz data were then averaged down to 10Hz, and all means were calculated from the 10Hz data. Our 3 minute TDL measurement cycle consisted of two reference tanks and the reference and sample gas streams, each measured for 45 s, from which we calculated means of isotopologue concentrations over the last 15 s of each inlet cycle. We combined these TDL data with IRGA generated data after incorporating the 33 second lag between the two instruments.

We used a LI-COR conifer chamber to maximize leaf area and allow natural light interception on the scale-like juniper foliage, regulating the chamber flow rate between 250 and 500 µmol s$^{-1}$ to maintain a sufficient CO$_2$ drawdown and control chamber
humidity. We attempted to maintain CO$_2$ drawdown $\geq$ 40 µmol CO$_2$ mol$^{-1}$ air within the leaf chamber. Under moderate conditions chamber temperature was unregulated, but under conditions of high ambient air temperature ($> 35^\circ$C) and solar radiation the IRGA block temperature control was engaged to control leaf temperature below 35°C, as measured by energy balance. On 12 June, we collected data from six leaf areas diurnally and from two leaf areas at night. On 11 July, we collected data from five leaf areas diurnally and two leaf areas during dark measurements. In both June and July each leaf area was measured for 30 minutes to an hour and individual leaves were typically measured more than once each day. Finally, on 14 August we collected all data from one leaf area diurnally during a seven hour period, and one leaf area during dark measurements. The isotopic signature of nocturnal respiration ($\delta^{13}$C$_{\text{resp}}$) was measured immediately following daylight measurements and beginning when ambient photosynthetic photon flux density (PPFD) fell below 30 µmol photons m$^{-2}$ s$^{-1}$ and foliage exhibited net CO$_2$ efflux. To achieve a true dark measurement, we applied a heavy shade cloth over the leaf chamber to reduce PPFD to zero and waited for stable chamber conditions (e.g. leaf temperature, respiration rate), which occurred within 5 minutes after the shade cloth was applied. We also determined the carboxylation capacity of these juniper trees on 22 June and 23 July 2007 using assimilation ($A$) responses to changes in sub-stomatal CO$_2$ concentration ($A/p_i$). We collected these data using a LI-COR 6400 fitted with a chamber light source (LI-COR 6400-02B). We measured predawn and mid-day xylem water potential ($\psi_w$) on five to ten nearby juniper trees on each measurement day using a Scholander-type pressure bomb (PMS Instruments Co., Corvallis, OR, USA; McDowell et al. 2008b).
The working standard (WS) calibration tanks used during our diurnal measurements were calibrated against World Meteorological Organization (WMO) certified standard tanks (541.67 µmol CO$_2$ mol$^{-1}$ air, δ$^{13}$C = −16.16‰ and 350.34 µmol CO$_2$ mol$^{-1}$ air, δ$^{13}$C = −8.39‰) within 24 hours of each measurement campaign. The inter-tank calibration between WMO and working standard tanks typically required 2 hours to complete. Molar mixing ratios of $^{12}$CO$_2$: $^{13}$CO$_2$ in the WS tanks used in the June campaign were 354.04 ± 0.27:3.82 ± 0.003 µmol CO$_2$ mol$^{-1}$ air (mean ± standard error; n = 11 inter-tank calibrations) and 563.85 ± 0.27:6.09 ± 0.003 µmol CO$_2$ mol$^{-1}$ air (n = 11). Molar mixing ratios of $^{12}$CO$_2$: $^{13}$CO$_2$ in the WS tanks used in the July and August campaigns were 340.46 ± 0.29:3.67 ± 0.003 µmol CO$_2$ mol$^{-1}$ air (n = 10) and 518.71 ± 0.08:5.60 ± 0.001 µmol CO$_2$ mol$^{-1}$ air (n = 6). The WMO certified tanks were filled and δ$^{13}$C calibrated at the Stable Isotope Lab (SIL) 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. Measurement variation in the δ$^{13}$C of a known tank in the TDL measurement mode we used exhibited a standard deviation of 0.06‰ across an hour and 0.20‰ across the day. To account for diurnal instrument drift the TDL measured the high and low WS tanks during each three minute cycle and we calculated the deviation between the measured values and the known values to determine a gain and offset for each isotopologue in each tank being measured (Bowling et al. 2003). These gain and offset values were then applied to all data. The TDL measures the absolute concentration of each isotopologue, so the range of $^{12}$CO$_2$ and $^{13}$CO$_2$ in each WS tank should span the measurement range. During the three measurement days our measurements occasionally exceeded the lower end of the total...
[CO₂] in our WS tanks (maximum deviation: 45.7 µmol/mol). To test that the calibration was valid below the lower tank, we used a WMO traceable standard tank (total [CO₂] = 142.86 µmol/mol, δ¹³C = −7.96‰) and an additional unknown tank that had a target total [CO₂] of 250 µmol/mol. We measured these two tanks and two WS tanks (344.88 µmol/mol, −8.16‰ and 548.16 µmol/mol, −16.42‰) in series. We calculated the total [CO₂] and isotope ratio of the unknown tank by calculating the gain and offset values in two ways: 1) using the span between the 142.86 µmol/mol tank and the 344.86 µmol/mol tank and 2) using the span between the 344.86 µmol/mol tank and the 548.16 µmol/mol tank measurements. The unknown tank was calculated to have a total [CO₂] of 247.44 µmol/mol and a δ¹³C of −20.45‰ using the lower calibration span (#1) and a total [CO₂] of 247.43 µmol/mol and a δ¹³C of −20.45‰ using the higher calibration span (#2), a net difference of 0.01 µmol/mol and 0.00‰. We also determined the [CO₂] and δ¹³C of the 142.86 µmol/mol WMO tank using gain and offset values calculated using the higher calibration span (#2). The result was a total [CO₂] of 142.66 µmol/mol and a δ¹³C of −7.88‰, a net difference of 0.20 µmol/mol and 0.08‰ from SIL certified values. Based on this assessment, we conclude our TDL has a linear response that extends beyond the lowest CO₂ range we measured in this study.

The IRGA was calibrated the morning of each measurement day, and the reference and sample gas analyzers of the IRGA were frequently matched to the same gas stream, while disconnected from the TDL inlet tubes. After reconnecting the TDL inlet tubes with the IRGA, the system was leak tested by gently blowing around the chamber, all connections, and the pressure equilibrating vent tube located on the sample line to the TDL. The TDL was also used to measure the reference and sample gas streams with an
empty leaf chamber and differences were lower than instrument precision (data not shown).

\[ \Delta \text{ & } \delta^{13}C_{\text{resp}} \text{ calculations} \]

We calculated \( \Delta_{\text{obs}} \) in the chamber following Evans et al. (1986):

\[
\Delta_{\text{obs}} = \frac{\xi(\delta_o - \delta_e)}{1 + \delta_o - \xi(\delta_o - \delta_e)} \tag{1}
\]

where \( \xi = c_e/(c_e-c_o) \) is the ratio of the reference CO\(_2\) concentration entering the chamber \(c_e\) relative to the sample CO\(_2\) concentration outgoing from the chamber \(c_o\), and \( \delta_e \) and \( \delta_o \) are the \( \delta^{13}C \) of the reference and sample gas, respectively. All variables incorporated in \( \Delta_{\text{obs}} \) and \( \delta^{13}C_{\text{resp}} \) (below) are derived from TDL measurements of \([^{12}\text{CO}_2]\) and \([^{13}\text{CO}_2]\), removing inter-instrument variability. Mixing ratios of total \([\text{CO}_2]\) were calculated following Barbour et al. (2007a). Because the TDL measures the concentration of each isotopologue \( \delta_o \) and \( \delta_e \) are calculated from the ratio of the molar abundance of each isotopologue and then presented in ratio to the Vienna Pee Dee belemnite (VPDB) standard, that is \( \delta = R_s/R_{VPDB} - 1 \), where \( \delta \) represents either \( \delta_o \) or \( \delta_e \), and \( R_s \) and \( R_{VPDB} \) represent the carbon isotope ratio of the sample and VPDB standard, respectively. We determined \( \delta^{13}C_{\text{resp}} \) following Barbour et al. (2007a):

\[
\delta^{13}C_{\text{resp}} = \frac{\delta_o - \delta_e(1-p)}{p} \tag{2}
\]

where \( p \) equals \((c_o-c_e)/c_o\). We estimated the \( \delta^{13}C \) of assimilated sugars \( \delta^{13}C_s \) based on Farquhar et al. (1989) where \( \delta^{13}C_s = (\delta_e - \Delta_{\text{obs}})/(\Delta_{\text{obs}} + 1) \). All other reported gas
exchange values are calculated by the LI-6400 software following methods of Farquhar, Caemmerer & Berry (1980), after correcting for leaf area. We determined projected leaf area using a calibrated leaf area meter (LI-3100, LI-COR Biosciences) and all gas exchange calculations are reported on a projected leaf area basis.

*Model parameterization*

We incorporated our data into the comprehensive model of leaf $\Delta$ (Farquhar *et al.* 1982; Farquhar & Richards 1984):

$$
\Delta_{\text{comp}} = a_b \frac{p_a - p_s}{p_a} + a \frac{p_s - p_c}{p_a} + (b_s + a_w) \frac{p_i - p_c}{p_a} + b \frac{p_c - \frac{e R_d}{k} + f \Gamma^*}{p_a}
$$

(3)

where $a_b$, $a$, $a_w$, $b_s$, and $b$ are the fractionation factors associated with CO$_2$ diffusion through the leaf boundary layer (2.9‰), stomata (4.4‰), water (0.7‰), fractionation attributed with CO$_2$ entering solution (1.1‰), and the net fractionation attributed to phosphoenolpyruvate carboxylase and ribulose-1,5-bisphosphate carboxylase/oxygenase activity (estimated at 29‰; Roeske & O’Leary 1984), respectively. The variables $p_a$, $p_s$, $p_i$, and $p_c$ represent the partial pressure (Pa) of CO$_2$ in the atmosphere surrounding the leaf, at the leaf surface, in the intercellular spaces, and at the sites of carboxylation, respectively. The variables $\Gamma^*$, $R_d$, $k$, $f$, and $e$ represent the CO$_2$ compensation point (Pa) in the absence of day respiration, day respiration rate ($\mu$mol m$^{-2}$ s$^{-1}$), carboxylation efficiency ($\mu$mol m$^{-2}$ s$^{-1}$ Pa$^{-1}$), and fractionations associated with photorespiration and day respiration (‰; see Table 1 for values), respectively. We calculated $p_a$, $p_s$, and $p_i$ by incorporating mole fraction measurements of [CO$_2$] with atmospheric pressure in Los Alamos (mean = 79 kPa), and estimated $p_c$ following Farquhar & Sharkey (1982):
\[ p_e = p_i - A / g_i \]  \hspace{1cm} (4)

where \( g_i \) is internal conductance to \( \text{CO}_2 \) (\( \mu \text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1} \)). We chose a moderate \( g_i \) of 1.5 \( \mu \text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1} \) based on the range of \( g_i \) values observed over the study period.

Prevailing theory suggests \( \Gamma^* \) is highly conserved among \( C_3 \) species and previous work has demonstrated strong temperature dependence of the \( \text{CO}_2 \) photo-compensation point (Jordan & Ogren 1984; Brooks & Farquhar 1985), on which we based our calculations of diurnal \( \Gamma^* \). Our \( \Gamma^* \) calculations accounted for the reduced atmospheric pressure in Los Alamos and we confirmed our estimates of \( \Gamma^* \) with those calculated using the Sharkey et al. (2007) \( A/p_i \) estimating utility (Table 1). Strictly \( k \), the carboxylation efficiency, is \( A/p_e \); we used the initial slope of \( A/p_i \) response curves (\( n = 10 \)) as a surrogate estimate and confirmed these slope-based results with calculations presented in Ku & Edwards (1977) and Wingate et al. (2007) (Table 1). Much work has demonstrated an inhibitory effect of light on respiration rate, even at irradiance as low as 12 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (Atkin et al. 2000; Tcherkez et al. 2005; Tcherkez et al. 2008). To facilitate estimation of \( R_d \) we measured nocturnal respiration rate (PPFD = 0) on all three days for approximately 120 minutes after cessation of daytime measurements (see Results) and used these data to calculate an estimated \( R_d \) value for each day where \( R_d = 0.5R \) (Tcherkez et al. 2005) and \( R \) equals steady-state respiration rate 30–120 minutes post-illumination (Table 1). We parameterized the decarboxylation component of \( \Delta \text{comp} \) using constant \( f(8\%) \) (Rooney 1988; Tcherkez 2006) and \( e \) (\( -6\% \)) (Ghashghaie et al. 2003) values. Parameterizing \( e \) based on \( \delta^{13}\text{C}_{\text{resp}} \) (typically estimated at \( -6\% \)) may be problematic due to shifts in respiratory biochemistry under illuminated conditions (Tcherkez et al. 2008). We assessed the magnitude of uncertainty introduced at high and low \( A \) when varying \( e \) by
comparing \((R_d/A)(p_c/p_a)\) multiplied by values of \(e = -6\%\text{ and } -1\%\text{ and calculating the resulting variation in the } \Delta_{ef} \text{ term (see Eq. 10 below).}

We also ran model simulations following the recent revisions to the comprehensive model (eq. 4) put forward by Wingate et al. (2007):

\[
\Delta_{\text{revised}} = a_i \frac{p_a - p_s}{p_a} + a \frac{p_s - p_i}{p_a} + (b_s + a_w) \frac{p_i - p_c}{p_a} + b \frac{p_c}{p_a} - \frac{(e + e^*) R_d}{k} + f \Gamma^* \tag{5}
\]

where \(e^*\) represents apparent fractionation for day respiration expressing the difference between the isotopic composition of the respiratory substrate and photosynthetic assimilates at a given time (Table 1). We calculated an \(e^*\) value for each three minute isotopic measurement using the following equation:

\[
e^* = \delta^{13} p_a - \Delta_{\text{simple}} - \delta^{13} \text{C}_{\text{mean}} \tag{6}
\]

where \(\delta^{13} p_a\) is the carbon isotope ratio of atmospheric air in the leaf chamber and \(\delta^{13} \text{C}_{\text{mean}}\) equals the mean calculated from the \(\delta^{13} \text{C}_{\text{resp}}\) measurements for each measurement date (see Results). In \(\Delta_{\text{revised}}\) we used a constant \(e, f, R_d, g_i,\) and \(k\) and a temperature dependent \(\Gamma^*\) (Table 1). We estimated \(g_i\) in \(\Delta_{\text{revised}}\) and \(\Delta_{\text{comp}}\) as 1.5 \(\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \cdot \text{Pa}^{-1}\) based on observed morning values. Lastly, we modeled \(\Delta\) for comparison to \(\Delta_{\text{obs}}\) using the most simplified form of the Farquhar et al. (1982) model \(\Delta_{\text{simple}}\), which eliminates boundary layer, \(g_i,\) and decarboxylation contributions to CO\(_2\) flux and their associated fractionation factors:

\[
\Delta_{\text{simple}} = a + (b - a) \cdot \frac{p_i}{p_a} \tag{7}
\]
where $b = 27\%$ (Gessler et al. 2008). All modeling was performed in Microsoft Excel XP Professional (Microsoft Corp., USA).

**Estimation of $g_i$ and $\Delta_{ef}$**

We estimated $g_i$ following the slope-based approach ($g_{is}$) in Evans et al. (1986):

$$g_{is} = \frac{(b - b_s - a_w)}{r_i}$$

(8)

where $r_i$ is the internal resistance to CO$_2$ transfer estimated as the slope of predicted $^{13}$C discrimination minus $\Delta_{obs}$ versus $A/p_a$. In this application predicted discrimination ($\Delta_i$) was determined using equation 3 calculated with infinite $g_is$, i.e. $p_i = p_c$. In this study variation in $A/p_a$ was the result of natural variation in the leaf environment. We calculated slopes for each time period where new leaf material was enclosed in the leaf chamber and tested each slope using simple linear regression. All negative slopes were rejected because negative slopes result in negative $g_{is}$ estimates. All regression analyses were performed using JMP 5.0.1 (SAS Institute Inc., Cary, NC). We used significant ($P \leq 0.10$) slope values to estimate $g_{is}$ for each foliage measurement, and determined the viability of each $g_{is}$ estimate by comparing them to $A$ across the entire measurement period. If the $g_{is}$ estimate was too low to facilitate observed $A$ during any portion of the measurement period we deemed that estimate to be erroneous. Finally, based on theory developed by Evans et al. (1986) and Caemmerer & Evans (1991) we used the $y$-intercept of significant $g_{is}$ plots to estimate $\Delta_{ef}$.

We also estimated $g_i$ using the point based method ($g_{ip}$; Evans et al. 1986):

$$g_{ip} = \frac{(b - b_s - a_w)A / p_a}{(\Delta_{pred} - \Delta_{obs}) - \Delta_{ef}}$$

(9)

where $\Delta_{pred}$ represents a simplified predictive model of leaf $\Delta$. 

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\[ \Delta_{\text{pred}} = a_p \frac{p_a - p_i}{p_a} + a_p \frac{p_s - p_i}{p_a} + b \frac{p_i}{p_a} \tag{10} \]

and \( \Delta_{ef} \) is calculated as:

\[ \Delta_{ef} = \frac{eR_d}{k} + f \Gamma^* \tag{11} \]

where all factors are the same as described in \( \Delta_{\text{comp}} \) (Eq. 3).

\( g_i \) sensitivity analysis

We assessed the sensitivity of \( \Delta_{\text{comp}} \) to changes in \( g_i \) by holding all parameters listed in Table 1 constant and varying the \( g_i \) value used to calculate \( p_c \) over each day. We used \( g_i \) values ranging from 0.5–2.5 \( \mu \text{mol m}^{-2} \text{s}^{-1} \text{ Pa}^{-1} \) and applied each value uniformly across each measurement day.

Statistical analysis

We estimated the error in \( \Delta_{\text{obs}} \) and \( \delta^{13} \text{C}_{\text{resp}} \) by implementing the parametric bootstrap (Davison & Hinkley 1997); we describe the procedure for \( \Delta_{\text{obs}} \) but \( \delta^{13} \text{C}_{\text{resp}} \) can be substituted in the description. For each measurement cycle we used the sample mean and standard errors (SE) of the concentrations of \( ^{12} \text{CO}_2 \) and \( ^{13} \text{CO}_2 \) for the high WS tank, low WS tank, reference gas, and sample gas to define eight normal distributions. We drew eight random deviates of \( [^{12} \text{CO}_2] \) and \( [^{13} \text{CO}_2] \) from these distributions, calculated a bootstrap replicate of \( \Delta_{\text{obs}} \), and repeated this 10,000 times to provide a bootstrap sampling distribution of \( \Delta_{\text{obs}} \). This insured the variance measured with each isotopologue was propagated into each calculation of \( c_e, c_o, \xi, \delta_e, \) and \( \delta_o \) and, therefore, into \( \Delta_{\text{obs}} \) and
δ^{13}C_{resp}. The SE of the bootstrap replicates provides an estimate of the SE of $\Delta_{obs}$. We observed that the bootstrap sampling distributions of $\Delta_{obs}$ were roughly normal, so the estimated SE characterizes the variation in $\Delta_{obs}$. All bootstrap analyses were performed in R (R Development Core Team 2008).

For both $g_{is}$ and $g_{ip}$ the $g_i$ estimate is a reciprocal transformation of a normally distributed random variable. While the standard errors describe the normal distributions well they are not easily interpretable for the skewed distributions associated with $g_{is}$ and $g_{ip}$. $g_{is}$ is the reciprocal of $r_i$, estimated using the normally distributed regression slope (Table 3). For the slope-based $g_{is}$, we calculated $r_i$ and $r_i \pm 1SE$ and transformed these three values to the $g_i$ scale (eq. 8) to generate $g_i$ and an estimate of its error. Similarly, for the point-based $g_{is}$, we calculated the roughly normally distributed bootstrap mean $\Delta_{obs} \pm 1SE$ and transformed these to the $g_i$ scale (eq. 9). For these data, one SE on the $r_i$ or $\Delta_{obs}$ scales is asymmetric on the $g_i$ scale with the upper SE being roughly twice the lower SE.

To assess model performance we first used least squares regression analysis of predicted and observed values but found the residual analysis of data in all months and models exhibited a non-random distribution. Additionally, both the slope and intercept terms were significantly different from one and zero, respectively, and substantially different from one another, making model comparisons difficult to evaluate. We then modified the computation of the residuals so that all models conformed to a slope of one and intercept of zero (i.e. residuals = model prediction − observed data) and calculated the standard deviation (SD) of the residuals. These SD values represented the square root of the sum of the variance and squared model bias, or root mean square error (RMSE),
for each month and model, and facilitated direct comparison of the predictive performance between models within each month.

Results

Diurnal $\Delta_{\text{obs}}$

Juniper $\Delta_{\text{obs}}$ averaged (mean ± SE) 16.3 ± 0.2‰ in June, 17.2 ± 0.2‰ in July, and 19.0 ± 0.5‰ in August ($P \leq 0.0002$ between each). Leaf $\Delta_{\text{obs}}$ tended to be highest in the early morning in all three months, followed by mid-morning variability and a decline through much of the afternoon (Figure 1). The seasonal $\Delta_{\text{obs}}$ trend tracked the transition from low (June) to high (August) soil, leaf, and atmospheric water content (Table 2, Fig. 2D-F). Similarly, the diurnal trend towards lower $\Delta_{\text{obs}}$ observed in the afternoon reflects the transition from relatively high morning leaf $\psi_w$ to lower mid-day $\psi_w$ (Table 2). On July and August measurement days the variation in leaf $\Delta_{\text{obs}}$ reflects the stability of the light environment, with relatively stable PPFD in July concurrent with stable $\Delta_{\text{obs}}$ and a heterogeneous light environment in August resulting in fluctuating $\Delta_{\text{obs}}$ (Fig. 2). On 14 August we lack reliable isotopic data after 13:00 due to low ambient light (PPFD < 100 µmol m$^{-2}$ s$^{-1}$) preventing $A$ rates high enough to sustain reliable isotopic measurements.

We found a weak but significant correlation between leaf vapor pressure deficit ($VPD$) and $\Delta_{\text{obs}}$ ($r^2 = 0.20$, $P < 0.0001$; $F = 110.22$; Fig. 3), PPFD and $\Delta_{\text{obs}}$ ($r^2 = 0.20$, $P < 0.0001$; $F = 114.11$), and $A$ and $\Delta_{\text{obs}}$ ($r^2 = 0.11$, $P < 0.0001$; $F = 54.97$; Fig. 3) using data pooled across all three days. Excluding the seven very high $\Delta_{\text{obs}}$ values in the early August morning, there was a significant relationship between stomatal conductance ($g_s$) and $\Delta_{\text{obs}}$ ($r^2 = 0.03$, $P < 0.0001$; $F = 16.60$; Fig. 3).
Nocturnal $\delta^{13}C_{\text{resp}}$

The isotopic composition of nocturnal respiration was similar in June (mean = $-22.6 \pm 0.2\%$) and July (mean = $-22.7 \pm 0.2\%$; $P = 0.70$) (Fig. 4) while respiration rates were dissimilar ($2.6 \pm 0.04$ and $4.8 \pm 0.1$ µmol m$^{-2}$ s$^{-1}$, respectively; $P < 0.0001$). In August mean $\delta^{13}C_{\text{resp}}$ was more depleted (mean = $-23.5 \pm 0.1\%$) than values measured in June ($P < 0.0001$) and July ($P < 0.0001$), while respiration rate (mean = $3.7 \pm 0.004$ µmol m$^{-2}$ s$^{-1}$) was higher than observed in June ($P < 0.0001$) and lower than observed in July ($P < 0.0001$). These $\delta^{13}C_{\text{resp}}$ values were enriched compared with estimates of the composition of recently assimilated sugars, which were $-24.66 \pm 0.20\%$ in June, $-25.19 \pm 0.17\%$ in July, and $-25.97 \pm 0.30\%$ in August. The step change in $\delta^{13}C_{\text{resp}}$ observed approximately 50 minutes post-illumination in June and July was due to cessation of measurement on one group of foliage and the movement to new foliage.

Temporal variation in $g_i$ and $A_{ef}$

We tested 32 slopes and found seventeen were significant across the three days. These produced fourteen viable $g_{is}$ and $A_{ef}$ estimates based on comparisons to $A$, including two in June, six in July, and six in August (Fig. 5; Table 3). We also found three slopes in the August morning which failed our criteria for having a significant slope ($P \leq 0.1$), but whose estimates of $g_{is}$ fit the observed trend and are included in Figure 5 (Table 3). Other $g_{is}$ estimates failed to support observed $A$ or displayed negative slope relationships between $\Delta_i - A_{obs}$ and $A/p_a$ and were excluded from the analysis. Estimates of $g_{ip}$ produced non-viable values when $A_{obs}$ was larger than $A_{pred}$ in bootstrap resamples,
resulting in negative $g_{ip}$ estimates. These 98 negative values, representing 22% of all $g_{ip}$ estimates, were excluded from the analysis.

Internal conductance calculated from slope-based measurements ranged from 0.04–2.14 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ (mean ± SE = 1.06 ± 0.17 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$) across the three days. The 14 August $g_{is}$ measurements were obtained from one leaf area across the morning and early afternoon and demonstrated an increase in $g_{is}$ from 0.04–2.14 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ (Figure 5C). We observed a lower range of variability in July $g_{is}$, with afternoon values ranging between 0.92 and 1.3 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$. We did not find a significant relationship between leaf temperature ($T_l$) and $g_{is}$ ($r^2 = 0.003$, $P = 0.87$; $F = 0.028$).

Estimates of $g_{ip}$ ranged between 0.05–8.53 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ (mean ± SE = 1.89 ± 0.07 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$) across the three measurement days (Figure 5). Sensitivity analysis demonstrated a significant increase ($P < 0.0001$) in $g_{ip}$ estimates when varying $e = -6\%$ and $f = 8\%$ (mean ± SE = 1.60 ± 0.04 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$) to $e = -1\%$ and $f = 11\%$ (3.31 ± 0.14 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$). There was a small but significant relationship between $g_{ip}$ and $T_l$ ($r^2 = 0.03$, $P = 0.0003$; $F = 13.168$).

$\Delta_{ef}$ also exhibited diurnal variation, ranging between −22.2 and +1.34%. In August we observed a low $\Delta_{ef}$ value of −21.3% in the early morning, later morning values that were not significantly different from zero ($P \leq 0.10$), and afternoon values near −2.5% (Table 3). The morning value in July was not significantly different from zero, whereas the afternoon $\Delta_{ef}$ values were between −4.9% and −3.5%. Our single significant $\Delta_{ef}$ value in June was −10.56 ± 5.3%. The non-zero values of $\Delta_{ef}$ occur at early morning, mid-day, or late afternoon, when fluxes are small and errors are likely to be greatest (Table 3).
$\Delta_{\text{obs}}$ and $p_i/p_a$

First order linear relationships between $\Delta_{\text{obs}}$ and $p_i/p_a$ were significant in June ($r^2 = 0.25$, $P < 0.0001$; $F = 58.31$; Figure 7A), July ($r^2 = 0.51$, $P < 0.0001$; $F = 182.61$) and August ($r^2 = 0.72$, $P < 0.0001$; $F = 248.99$); however, second order polynomials described the relationships with greater predictive power in July ($r^2 = 0.64$, $P < 0.0001$; $F = 151.90$) and August ($r^2 = 0.88$, $P < 0.0001$; $F = 334.27$; Fig. 6B,C). The curvilinear relationship between $\Delta_{\text{obs}}$ and $p_i/p_a$ was most pronounced in the $p_i/p_a$ range between 0.75 and 0.85.

$g_i$ sensitivity analysis

Incorporation of variable $g_i$ into $\Delta_{\text{comp}}$ over diurnal periods produced variation in predictions of $\Delta_{\text{comp}}$. Sensitivity analysis demonstrated using low $g_i$ (0.5 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$) in $\Delta_{\text{comp}}$ resulted in a mean 6.9‰ underestimate of $\Delta_{\text{obs}}$ while relatively high $g_i$ (2.5 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$) resulted in a 0.70‰ overestimate of $\Delta_{\text{obs}}$ (Table 4). Pairwise comparisons of the residuals ($\Delta_{\text{obs}}-\Delta_{\text{comp}}$) resulting from $\Delta_{\text{comp}}$ predictions incorporating a $g_i$ value of 0.5 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ were significantly different from residuals produced when using $g_i$ values of 1.0, 1.5, 2.0, and 2.5 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ in $\Delta_{\text{comp}}$ ($P \leq 0.05$; Tukey’s HSD) within and across all three days. Similarly, all other $g_i$ inputs into $\Delta_{\text{comp}}$ (1.0, 1.5, 2.0, and 2.5 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$) produced significantly different residuals from one another within each day and across all three days (Table 4). The RMSE, a measure of the variance and squared bias associated with the residuals, largely followed the trend observed in the pairwise residual comparisons and was lower when residual differences were smaller; this demonstrates the importance of an accurate estimate of $g_i$ for model fit. Internal
conductance values of 1.5 and 2.0 µmol m\(^{-2}\) s\(^{-1}\) Pa\(^{-1}\) produced the best predictions, as determined by lowest pairwise residual differences and RMSE, when applied uniformly across each measurement day (Table 4).

**Model predictions: \(\Delta_{\text{comp}}, \Delta_{\text{revised}}, \text{and } \Delta_{\text{simple}}\)**

Model performance varied across the three measurement days (Figure 7). Assessing the error between model predictions and \(\Delta_{\text{obs}}\) in each month showed \(\Delta_{\text{simple}}\) had the lowest RMSE, 2.11‰, in June, \(\Delta_{\text{comp}}\) had the lowest error in July (RMSE = 1.50‰), and \(\Delta_{\text{revised}}\) exhibited the lowest error in August (RMSE = 3.15‰; Table 5). Substituting \(b = 25\%\) into \(\Delta_{\text{simple}}\) reduced model prediction bias (mean = 0.31 ± 0.12‰) but resulted in higher RMSE (mean = 2.65‰ versus 2.42‰ for \(b = 27\%\)) on all three days compared to using \(b = 27\%\). The estimated model prediction bias between \(\Delta_{\text{comp}}, \Delta_{\text{revised}}, \text{and } \Delta_{\text{simple}}\) and observed discrimination across all three dates was (mean ± SE) \(-0.62 ± 0.18\‰, -0.28 ± 0.19\‰, and 1.63 ± 0.18\‰, respectively. However, error assessment revealed the apparent close simulations suggested by the small model prediction bias between modeled and observed values masked substantial variance in all models’ predictions of \(\Delta_{\text{obs}}\) (Table 5).

At high \(A\), defined here as > 4.0 µmol m\(^{-2}\) s\(^{-1}\), uncertainty introduced into \(\Delta_{ef}\) by utilizing \(e = -6\%\) versus \(-1\%\) was equal to 2.21 ± 0.01‰ while at low \(A\), defined here as < 2.0 µmol m\(^{-2}\) s\(^{-1}\), the same uncertainty increased to 9.40 ± 1.51‰ (Table 6).

**Discussion**

The objectives of this study were to 1) examine the temporal variation in \(\Delta, \delta^{13}C_{\text{resp}}, g_i, \text{and } \Delta_{ef}\) under ambient field conditions, 2) test the hypothesis that \(g_i\) varies across the day,
3) test the hypothesis that $\Delta$ varies linearly in response to shifts in $p_i/p_a$ under field conditions, 4) test the influence of $g_i$ in a comprehensive leaf model of $\Delta$, and 5) test the predictive capabilities of three models: the comprehensive Farquhar et al. (1982) model of $\Delta$ ($\Delta_{\text{comp}}$), a recently suggested amendment to $\Delta_{\text{comp}}$ ($\Delta_{\text{revised}}$; Wingate et al. 2007) and the simplified form of the comprehensive model ($\Delta_{\text{simple}}$). We observed a large range of variation in $\Delta$, $g_i$, and $\Delta_{ef}$ over diurnal time periods and across the season. Seasonally, $\delta^{13}C_{\text{resp}}$ decreased as water availability increased. We found that $g_i$ varied across the day in August and that $g_i$ exerted substantial influence on $\Delta$ predictions. We found $\Delta_{\text{obs}}$ varied in a linear fashion in response to $p_i/p_a$ in June, but second order expressions better described the relationship in July and August. Finally, we found all models reasonably predicted $\Delta_{\text{obs}}$, but $\Delta_{\text{simple}}$ best predicted $\Delta_{\text{obs}}$ in June, $\Delta_{\text{comp}}$ best predicted $\Delta_{\text{obs}}$ in July, and $\Delta_{\text{revised}}$ best predicted $\Delta_{\text{obs}}$ in August.

**Diurnal $\Delta_{\text{obs}}$ & nocturnal $\delta^{13}C_{\text{resp}}$**

Diurnal $\Delta_{\text{obs}}$ in our juniper woodland varied between $12.0\%o$ and $27.4\%o$, which was similar in trend and magnitude to $\Delta$ observed in a tropical forest (Harwood et al. 1998) and a mesic *Picea* stand (Wingate et al. 2007) (Fig. 1). Variation in $\Delta_{\text{obs}}$ was generally related to environmental drivers such as PPFD and VPD (Figs. 1,2,3). The diurnal trend towards decreasing $\Delta_{\text{obs}}$ observed in June and July correlates with increasing leaf-to-atmosphere VPD observed both days, though low leaf $\psi_w$ and high air temperature likely contributed to low discrimination in June compared to July and August. In August, VPD was relatively low and cloudy conditions caused large variation in $\Delta_{\text{obs}}$. Cumulatively, these sensitivities to VPD and PPFD were similar to those seen in modeled canopy $\Delta$. 
(Baldocchi & Bowling 2003; Chen & Chen 2007). We also observed several high, but transient, discrimination values in all three months including mid-day values of 31.4‰ in June and 36.9‰ in July, and observations ranging from 29.7–44.9‰ in the early morning in August. These $\Delta_{\text{obs}}$ values were associated with greater uncertainty, but were similar to values observed in *Piper* and *Picea* (Harwood *et al.* 1998; Wingate *et al.* 2007).

Nocturnal $\delta^{13}\text{C}_{\text{resp}}$ for the juniper trees in our study ranged from $\sim -24$ to $-22$‰ and was moderately enriched compared to most observations in the literature (Bowling *et al.* 2002; Hymus *et al.* 2005; Prater, Mortazavi & Chanton 2005). $\delta^{13}\text{C}_{\text{resp}}$ values were similar in June and July, and were more enriched in $^{13}\text{C}$ compared to August (Fig. 4). The consistent 2-3‰ enrichment of $\delta^{13}\text{C}_{\text{resp}}$ compared to estimates of recently assimilated carbohydrate is consistent with previous reports (Duranceau *et al.* 1999; Ghashghaie *et al.* 2001) and may reflect respiratory fractionation, possibly combined with diverse respiratory substrate utilization (Tcherkez *et al.* 2003). This $\delta^{13}\text{C}_{\text{resp}}$ pattern is consistent with the temporal transition period from drought in June through the onset of summer monsoon in July to the strong monsoon in August.

**Temporal variation in $g_i$ & $\Delta_{\text{ef}}$**

We observed a diurnal increase in $g_i$ occurring in one leaf area across the August morning and early afternoon, and a range of variation in $g_i$ across the three months (Fig. 5). The physiological drivers of this variation in $g_i$ are unknown, but likely involved changes in protein activity facilitating the transfer of CO$_2$ across cell or chloroplast membranes (Flexas *et al.* 2006; Hanba *et al.* 2006; Uehlein *et al.* 2008). Previous work has demonstrated variability in $g_i$ in response to environmental variables such as temperature
(Bernacchi et al. 2002; Warren & Dryer 2006; Yamori et al. 2006) and water availability (Warren et al. 2004; Grassi & Magnani 2005; Galmés et al. 2007; Diaz-Espejo et al. 2007), both of which fluctuate in a field setting. We did not find a significant correlation between \( T_l \) and \( g_{is} \) but did find a significant relationship between \( T_l \) and \( g_{ip} \). It is possible that variable irradiance over each measurement period may have confounded any temperature effect on \( g_{is} \), but the higher temporal frequency of \( g_{ip} \) was closer to the frequency \( T_l \) was changing diurnally. Juniper displays anisohydric leaf hydraulic behavior and concurrent \( \psi_w \) measurements (Table 2) demonstrated a seasonal increase and diurnal decrease in xylem \( \psi_w \). The seasonal \( \psi_w \) pattern paralleled our seasonal \( g_i \) measurements, suggesting a linkage between leaf water status and the \( g_i \) patterns we observed, but are confounded by the increase in both \( g_{is} \) and \( g_{ip} \) in the August morning when \( \psi_w \) was decreasing. Notably, there was a distinct decrease in \( g_{is} \) in the upward morning trend that coincides with extended cloud cover (mean PPFD = 266 ± 46 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)). We speculate the large and prolonged drop in incident light played a regulatory role in the lower \( g_i \), similar to observations of other environmental regulators of \( g_i \) in controlled studies (Delfine et al. 1999; Bernacchi et al. 2002; Flexas et al. 2007). The July data exhibit modest variation in diurnal \( g_i \), but may reflect natural variation among branches. Given that our measurements were collected under ambient environmental conditions an accurate assessment of the factors driving the variation in \( g_i \) we observed is not possible and should be addressed in controlled studies.

The variation in \( g_{is} \) is potentially problematic for the slope-based method because it assumes \( g_i \) is constant over the period the slope data are collected. While rapid variation in \( g_i \) has been demonstrated in response to [CO\(_2\)] (Flexas et al. 2007), the rate
and magnitude of diurnal shifts in $g_i$ under field conditions has not been previously reported. Our 30–45 min $g_{is}$ measurement periods may have spanned too long and allowed time for $g_i$ to change in response to the environment. However, aside from periods where $\Delta_{obs}$ was highly variable, such as the July mid-day period, $g_{ip}$ values were generally stable around each $g_{is}$ value and show variation was low enough to provide valid $g_{is}$ estimates. Slope-based estimates of $g_i$ tended to underestimate $g_{ip}$ in June and July, but both trended together in August (Figure 5). $g_{ip}$ is sensitive to the parameterization of $e$ and $f$, and errors in estimating these values may have resulted in over- or under-estimation of $g_{ip}$.

Most of our $g_i$ estimates agree with values reported in other woody species (Lloyd et al. 1992; De Lucia, Whitehead & Clearwater 2003; Warren et al. 2003; Ethier et al. 2006) but we also found low $g_{is}$ estimates in the early morning and relatively high $g_{ip}$ estimates when $\Delta_{obs}$ was highly variable. We found a low $g_{is}$ estimate (0.03 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$) in the August early morning transition period from respiration to net $A$, where net CO$_2$ drawdown was between 6-10 µmol mol$^{-1}$, uncertainty in $\Delta_{obs}$ was higher, and measurements may have been more strongly influenced by the isotopic signature of CO$_2$ evolved during concurrent day respiratory processes. Though low, model simulations demonstrated the 0.03 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ conductance estimate was high enough to allow observed $A$ across the measurement period. Estimates from $g_{ip}$ during this period show consistently negative estimates of $g_i$ (data not shown). High and variable $g_{ip}$ estimates ranged between 4–8 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ during the mid-day period in July, driven by higher uncertainty in $\Delta_{obs}$ over this period.
Our measurements of $\Delta_{ef}$ suggest that fractionations attributed to decarboxylation activity may not be negligible at dawn and in the afternoon when rates of either respiration or photorespiration may be high (Table 3). Our early morning August measurement occurred during a time of low $A/p_a$ and generated a very negative $\Delta_{ef}$ value. If respiration had not fully deactivated to its daytime rate, then it may have had an unusually large impact during that time period (Gillon & Griffiths 1997). By mid-morning in July and August $A$ and $g_s$ had reached a plateau and $\Delta_{ef}$ was not significantly different from zero. However, in the June and July afternoons high temperature and PPFD created conditions conducive to higher photorespiration rates that may have contributed to greater variation in afternoon $\Delta_{ef}$ values. Further, compared to other C$_3$ species juniper exhibits high $R$, from which we estimated $R_d$, and thus the respiratory component of $\Delta_{ef}$ would have a larger impact on net $\Delta$ than would be expected for other species. Carefully controlled studies partitioning different components of the net flux will be necessary to elucidate the contribution of each component.

$A_{obs}$ and $p_i/p_a$

We observed significant first order linear relationships between $\Delta$ and $p_i/p_a$ in all months, but found second order models better described the curvilinear relationship between $\Delta$ and $p_i/p_a$ in July and August (Fig. 6). We propose that the curvilinear relationship is related to the increasing dominance of respiration and associated isotopic signatures on leaf exchanged CO$_2$ at high $p_i/p_a$ values. Previous work and theory have demonstrated a linear relationship between $\Delta$ and $p_i/p_a$ in C$_3$ plants (Farquhar et al. 1982b; Evans et al. 1986; Brugnoli et al. 1988; Farquhar et al. 1989), but unlike our study these data were
collected in controlled settings under steady-state conditions. In both July and August the curvilinear trend between \( \Delta \) and \( p_i/p_a \) was driven by high \( \Delta \) values. These high \( \Delta \) values correspond with conditions conducive to high respiratory and photorespiratory flux, notably the early morning and mid-day periods, and may reflect the isotopic signature of a highly enriched substrate (Tcherkez et al. 2005).

\textit{g_i sensitivity analysis}

Incorporating variable internal CO\(_2\) conductance into \( \Delta_{\text{comp}} \) demonstrated \( g_i \) exerted substantial influence on predictions of diurnal discrimination. Average observed \( g_i \) was near 1.5 \( \mu \text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1} \) and our sensitivity analysis showed that relatively low (0.5 \( \mu \text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1} \)) and high (2.5 \( \mu \text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1} \)) values resulted in large deviations between model predictions and \( \Delta_{\text{obs}} \) (Table 4). However, we have shown that \( g_i \) can vary in a leaf over several hours and it is likely incorporating this diurnal variability into leaf and ecosystem models would improve discrimination predictions (McDowell et al. 2008a). Future studies should focus on assessing the diurnal variability in \( g_i \) independently and testing whether variable diurnal \( g_i \) significantly improves the accuracy and precision of predictions of \( \Delta \) in leaf models.

\textit{Model predictions:} \( \Delta_{\text{comp}}, \Delta_{\text{revised}}, \text{and} \ \Delta_{\text{simple}} \)

Our study supports the use of the more comprehensive models, \( \Delta_{\text{comp}} \) and \( \Delta_{\text{revised}} \), that incorporate fractionations associated with the diffusion pathway and decarboxylation activity, to describe leaf \( \Delta \) in our semi-arid system. The limitations of these models are that they require assumptions of the true value of fractionation during carboxylation and
decarboxylation, in addition to an accurate estimate of \( g_i \). Our sensitivity analysis showed that variation in \( e \) at low \( A \) resulted in \( \sim 9\% \) variation in \( \Delta_{ef} \), emphasizing the importance of \( e \) in plants like juniper that exhibit relatively high \( R \) compared to \( A \). Our estimate of \( e \) was based on the dark respiration fractionation, and we may have over- or under-estimated the true value of \( e \) or \( R_d \) and introduced model error. However, we have shown both models produced similar errors in their predictions of \( \Delta \).

The importance of decarboxylation activity in juniper \( \Delta \) is reflected both in the \( e^* \) values we calculated and the \( \Delta_{ef} \) estimates obtained from \( g_i \) plots. We calculated \( e^* \) values ranging from \(-12.5\%\) to \(+1.2\%\), values that suggest the isotopic disequilibria between recent photosynthate and the respiratory substrate being utilized was, at times, substantial. Further, our \( \Delta_{ef} \) estimates were mostly between \(-6.9\%\) and \(0\%\), whereas previous observations were close to \(0\%\) (Evans et al. 1986). It is also possible that other factors, such as stomatal patchiness, may not be fully captured in our estimates of \( p_i \), which could alter the \( p_i/p_a \) ratio important to all of the \( \Delta \) models (Farquhar 1989).

Despite lacking decarboxylation and \( g_i \) components \( \Delta_{simple} \) outperformed the more comprehensive models in June. Further, \( \Delta_{simple} \) exhibited modest error in predicting \( \Delta_{obs} \) compared to \( \Delta_{comp} \) and \( \Delta_{revised} \) in July and August but consistently overestimated \( \Delta_{obs} \), predicting \( \Delta \) values whose mean difference were \( > 1.0\% \) above \( \Delta_{obs} \) in all three months. This may represent a larger systematic bias than exists in the other models, though utilizing a lower \( b \) value reduced model bias while moderately increasing error. However, all of the models exhibited non-trivial RMSE, ranging from \(1.5–3.2\%\), suggesting that a significant amount of variability remains to be captured. Future field studies should aim to independently estimate the variability in diurnal \( \Delta_{ef} \) and \( g_i \) to ascertain their impacts on
diurnal leaf isotopic exchange. Similarly, future controlled studies should partition the net flux to assess \( g_i \) and \( \Delta_{ef} \), as well the regulatory influence of environmental variables such as temperature and PPFD on these components of carbon discrimination.

**Conclusions**

Our study demonstrates the diurnal variation in \( \Delta \) in our semi-arid conifer ecosystem was of similar trend and magnitude to that observed in ecosystems as diverse as tropical forest and mesic conifer forest. Additionally, we demonstrated that \( \Delta \) varies rapidly in response to shifts in environmental conditions and that the comprehensive Farquhar *et al.* (1982) model and its descendents are capable of capturing a wide range of diurnal variation in leaf \( \Delta \). Our observations are consistent with previous results showing low \( \Delta \) during conditions of low soil water availability and elevated \( VPD \) and PPFD, and higher \( \Delta \) when soil water was more abundant, PPFD was variable, and \( VPD \) was low. We observed a linear relationship between \( \Delta \) and \( p_i/p_a \) in June, but found a strong curvilinear relationship in July and August. Future studies might be strengthened by testing this relationship in other species over a wide range of \( p_i/p_a \) and environmental conditions. Our findings support the inclusion of \( g_i \) and decarboxylation activity to attain the most accurate and precise predictions of \( \Delta \) from leaf models, and evolving technologies such as TDL make these improvements more easily achievable. Lastly, the magnitude of diurnal variation in \( g_i \) of other C3 species needs to be quantified, as do the environmental and physiological drivers of this variation, so that \( g_i \) can be more accurately parameterized in future ecosystem process models.
Acknowledgements

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References


Tables

Table 1. Parameters used in model simulations of observed discrimination using the comprehensive model ($\Delta_{\text{comp}}$) and the revised model ($\Delta_{\text{revised}}$). The fractionation factors associated with day respiration, $e$, and photorespiration, $f$, were assumed based on literature values while all the other terms are derived from our data.

<table>
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<th>Day</th>
<th>$k$</th>
<th>$R_d$</th>
<th>$\Gamma^*$</th>
<th>$e$</th>
<th>$f$</th>
<th>$g_i$</th>
<th>$e^*$</th>
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<td>2.86 - 5.23</td>
<td>-6</td>
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<td>3.17 - 5.17</td>
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<td>-12.5 to -0.9</td>
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<td>2.43 - 4.29</td>
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<td>8</td>
<td>1.5</td>
<td>-10.5 to 1.2</td>
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Table 2. Mean xylem water potential with standard error (SE) on all three measurement days. Mid-day values from McDowell et al. (2008b).

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<tr>
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Table 3. Slope and intercept statistics from linear regressions used to calculate $g_{is}$ and estimate $\Delta_{ef}$. Cut-off values for the test of slope significance within each regression was $P \leq 0.10$, but three marginal slopes are also represented (*). Most intercepts were not significantly different from zero, but significant intercepts ($P \leq 0.10$) deviated substantially from zero.

<table>
<thead>
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<th>campaign</th>
<th>Time</th>
<th>slope</th>
<th>SE</th>
<th>P</th>
<th>$\Delta_{ef}$</th>
<th>SE</th>
<th>P</th>
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Table 4. Results from a sensitivity analysis utilizing variable $g_i$ values within $\Delta_{\text{comp}}$ and applied across each measurement day. $\Delta_{\text{obs}} - \Delta_{\text{comp}}$ represents the pairwise residual difference (‰) between observed discrimination ($\Delta_{\text{obs}}$) and model predictions ($\Delta_{\text{comp}}$). $\Delta_{\text{comp}}$ predictions using each of the $g_i$ values produced residuals significantly different from one another within each day and across days. As determined by lowest root mean square error (RMSE; ‰) and pairwise residual difference, $g_i$ of 1.5 and 2.0 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ performed best in predicting $\Delta_{\text{obs}}$.

<table>
<thead>
<tr>
<th>$g_i$</th>
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<th>July $n = 176$</th>
<th>August $n = 97$</th>
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</tbody>
</table>
Table 5. Comparison of model performance in predicting $\Delta_{\text{obs}}$. Means represent the difference between model predictions and $\Delta_{\text{obs}}$ and RMSE, the root mean square error. $\Delta_{\text{simple}}$ consistently overestimated $\Delta_{\text{obs}}$ but showed lower error in predicting $\Delta_{\text{obs}}$ in June compared to $\Delta_{\text{comp}}$ and $\Delta_{\text{revised}}$. $\Delta_{\text{comp}}$ exhibited the lowest error in July, while $\Delta_{\text{revised}}$ exhibited lower error and mean difference between predicted and observed values in August compared to $\Delta_{\text{simple}}$ and $\Delta_{\text{comp}}$.

<table>
<thead>
<tr>
<th></th>
<th>June $n = 177$</th>
<th>July $n = 176$</th>
<th>August $n = 97$</th>
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</thead>
<tbody>
<tr>
<td>$\Delta_{\text{simple}}$</td>
<td>2.23</td>
<td>1.32</td>
<td>1.2</td>
</tr>
<tr>
<td>$\Delta_{\text{comp}}$</td>
<td>0.28</td>
<td>-1.58</td>
<td>1.50</td>
</tr>
<tr>
<td>$\Delta_{\text{revised}}$</td>
<td>0.79</td>
<td>-0.68</td>
<td>1.61</td>
</tr>
</tbody>
</table>
Table 6. Results from a sensitivity analysis assessing the variation in $\Delta_{ef}$, the decarboxylation term in $\Delta_{\text{comp}}$, when parameterized with $e = -6\%$ and $e = -1\%$. The uncertainty introduced into the decarboxylation term at low to high net photosynthetic rate ($A$) when varying $e$ from $-6\%$ to $-1\%$ is represented in $\Delta_{ef}$ ($\%$). This demonstrates $\Delta_{ef}$ is very sensitive to variation in $e$ at low $A$; in this study < 4% of all measurements were at $A < 2.0$ µmol m$^{-2}$ s$^{-1}$.

<table>
<thead>
<tr>
<th>$A$ (µmol m$^{-2}$ s$^{-1}$)</th>
<th>$\Delta_{ef}$ (%)</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 2.00</td>
<td>9.40</td>
<td>1.51</td>
</tr>
<tr>
<td>2.00-3.99</td>
<td>2.64</td>
<td>0.04</td>
</tr>
<tr>
<td>4.00-9.15</td>
<td>2.21</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Figure Legends

**Figure 1.** Diurnal variation in carbon isotope discrimination (●; $\Delta_{\text{obs}}$) on 12 June, 11 July, and 14 August. Error bars represent one standard error. Note change of y-axis scaling in panels.

**Figure 2.** Environmental parameters on each measurement day. Panels A-C depict incident photosynthetic photon flux density (PPFD) trends across each measurement day. Panels D-F show leaf temperature, as measured by energy balance (□) and vapor pressure deficit ($VPD$; ▼) across each measurement day.

**Figure 3.** The relationship between observed discrimination ($\Delta_{\text{obs}}$) and net photosynthetic rate ($A$; A), leaf-to-atmosphere vapor pressure deficit ($VPD$; B), and stomatal conductance ($g_s$; C). $\Delta_{\text{obs}}$ exhibited a significant correlation with pooled leaf $A$ ($r^2 = 0.11$, $P < 0.0001$) and $VPD$ ($r^2 = 0.20$, $P < 0.0001$). Excluding seven high August morning values, $\Delta_{\text{obs}}$ exhibited a significant relationship with $g_s$ ($r^2 = 0.03$, $P < 0.0001$).

**Figure 4.** The ratio of $^{13}$CO$_2$ to $^{12}$CO$_2$ in post-illumination nocturnal respiration (●; $\delta^{13}C_{\text{resp}}$) on the evening of 12 June (A), 11 July (B) and 14 August (C). $\delta^{13}C_{\text{resp}}$ was similar in June and July ($P = 0.70$) but August was more significantly more $^{13}$C depleted than in June ($P < 0.0001$) and July ($P < 0.0001$). Error bars represent one standard error.

**Figure 5.** Diurnal variation in internal conductance of CO$_2$ estimated using sloped-based methods (■; $g_{is}$) and point-based methods (○; $g_{ip}$) on 12 June (A), 11 July (B), and 14
August (C). Internal conductance values derived from non-significant slopes (P ≥ 0.10) on 14 August are also represented (■); all \( g_i \) estimates from 14 August were measured on one leaf area. Error bars represent one SE and are presented with grey (\( g_{ip} \)) and black (\( g_{is} \)) lines.

**Figure 6.** The relationship between observed discrimination (\( \Delta_{\text{obs}} \)) and \( p_i/p_a \). First order linear relationships were observed in June (A; \( r^2 = 0.25, P < 0.0001 \)), July (B; \( r^2 = 0.51, P < 0.0001 \)), and August (C; \( r^2 = 0.72, P < 0.0001 \)) though 2\(^{nd} \) order polynomial relationships better described the data in July (\( r^2 = 0.64, P < 0.0001 \)) and August (\( r^2 = 0.88, P < 0.0001 \)).

**Figure 7.** The relationship between observed discrimination (\( \Delta_{\text{obs}} \)) and discrimination values predicted using \( \Delta_{\text{revised}} \) (▲), \( \Delta_{\text{comp}} \) (○), and \( \Delta_{\text{simple}} \) (■) relative to the 1:1 \( \Delta_{\text{obs}} \) line (solid line). Note: axes are unequal among panels to enhance resolution. \( \Delta_{\text{revised}} \) and \( \Delta_{\text{comp}} \) utilized a \( b = 29\% \), while \( \Delta_{\text{simple}} \) was fit with a \( b = 27\% \); other parameters are listed in Table 1. \( \Delta_{\text{simple}} \) exhibited the lowest overall error in predicting \( \Delta_{\text{obs}} \) in June, \( \Delta_{\text{comp}} \) exhibited the lowest error in July and \( \Delta_{\text{revised}} \) exhibited the lowest error in August.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Chapter 3

Influence of diurnal variation in internal conductance on modeled $^{13}$C discrimination: results from a field study

CHRISTOPHER P. BICKFORD$^1$

$^1$University of New Mexico, Department of Biology, MSC03-2020, Albuquerque, NM

87131
Abstract

Internal CO\textsubscript{2} conductance ($g_i$) can limit carbon assimilation and influence carbon isotope discrimination ($\Delta$) under some environmental conditions but environmental regulation of $g_i$ is not well understood. We used high frequency field measurements to test the importance of $g_i$ in predicting $\Delta$ using the comprehensive Farquhar, O’Leary & Berry (1982) model of $\Delta$ ($\Delta_{\text{comp}}$) when $g_i$ was parameterized using three different methods based on: mean $g_i$, the relationship between stomatal conductance ($g_s$) and $g_i$, and the relationship between time of day (TOD) and $g_i$. Incorporating mean $g_i$ and TOD-based $g_i$ improved $\Delta_{\text{comp}}$ predictions compared to the simple model of $\Delta$ ($\Delta_{\text{simple}}$) that omits fractionation factors associated with $g_i$ and decarboxylation, but predictions using $g_s$-based $g_i$ did not outperform $\Delta_{\text{simple}}$. Sensitivity tests suggest $b$, the fractionation due to carboxylation, was lower (24‰) than the value commonly used in $\Delta_{\text{comp}}$ (29‰). These results demonstrate the limits of $\Delta_{\text{simple}}$ while reinforcing the need for improved parameterization of $\Delta_{\text{comp}}$ by showing both $g_i$ and $b$ impact $\Delta$ and that variability in both terms should be accounted for to better predict $\Delta$.

Keywords: carbon isotopes, mesophyll conductance, Farquhar model

Introduction

Low internal CO\textsubscript{2} conductance from substomatal cavities to sites of carboxylation ($g_i$) can reduce the partial pressure of CO\textsubscript{2} (pCO\textsubscript{2}) at the site of carboxylation, limit photosynthesis ($A$), and affect carbon isotope discrimination ($\Delta$). $g_i$ varies on numerous time-scales in response to environmental drivers, from rapid variation in response to
changes in intercellular [CO₂] (Flexas et al. 2007) to shifts in response to temperature (Bernacchi et al. 2002), water stress (Ethier & Livingston 2004), light gradients (Piel et al. 2002; Flexas et al. 2007a), and others (see Flexas et al. 2008 for a review). Scaling relationships between $g_i$ and photosynthetic capacity have been shown (Evans & Caemmerer 1996; Le Roux et al. 2001; Ethier et al. 2006) and challenged (Warren & Adams 2006). Similarly, a linkage between $g_i$ and $g_s$ has been demonstrated (Loreto et al. 1992; Lauteri et al. 1997; Hanba et al. 2003; Flexas et al. 2002; Ethier et al. 2006) and is intriguing because of the potential for high frequency modeling of $g_s$ and subsequent estimates of $g_i$. Internal 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, Kogami & Terashima 2003; Warren & 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. $\Delta$ is a strong regulator of $\delta^{13}$C$_L$ and $\delta^{13}$C$_{resp}$ (Bowling, Pataki & Randerson 2008), and therefore a better understanding of $g_i$ in leaf-level predictions of discrimination may improve interpretation of $\delta^{13}$C signals from multiple sources. Studies testing the role of $g_i$ in $\Delta$ predictions are limited, but differ by showing the influence of $g_i$ was either negligible (Wingate et al. 2007) or important (Le Roux et al. 2001; Bickford et al. 2009).

$\Delta$ is influenced by numerous environmental and physiological regulators and well correlated with key physiological indicators. The ratio of intercellular to ambient pCO₂ ($p_i/p_a$) is a physiological parameter that succinctly describes the variability in the pCO₂ 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. 1982; Farquhar,
Ehleringer & Hubick 1989; Brugnoli & Farquhar 2000). \( \frac{p_i}{p_a} \) 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 diffusion 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 \( g_i \) 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 \( \frac{p_i}{p_a} \) interacting with key model parameters (Farquhar et al. 1982b). In addition to \( \frac{p_i}{p_a} \), the key drivers of \( \Delta_{\text{simple}} \) include 1) the carboxylation term, \( b \), that represents net fractionation associated with phosphoenolpyruvate (PEP) carboxylase and Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and 2) the fractionation associated with diffusion in air and through stomata (\( a; 4.4\% \)) (Farquhar et al. 1989). \( b \) is typically estimated at \(~27\%\) in \( \Delta_{\text{simple}} \), or \(~2\%\) lower than early measurements of the full Rubisco fractionation (\(~29\%\); Roeske & O’Leary 1984), to account for omitted fractionation factors (Farquhar & Richards 1984). Recent work suggests net Rubisco fractionation may be between 25-30\% (Tcherkez & Farquhar 2005) and gross Rubisco fractionation may be as low as 27.4\% in tobacco (\( \text{Nicotiana tabacum} \); McNevin et al. 2007) while \( b \) is estimated to be 26\% in \( \text{Senecio} \) (Lanigan et al. 2008).

The comprehensive mechanistic \( \Delta \) model incorporates the factors discussed above plus fractionation associated with \( \text{CO}_2 \) diffusion, including \( g_is \) and decarboxylation
activity. As previously discussed, $g_i$ is dynamic and may influence $\Delta$ by reducing the diffusion rate from stomatal 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 $g_i$ and $\Delta$ (Evans et al. 1986, Caemmerer & Evans 1991) but recent evidence suggests these may be non-negligible variables (Ghashghaie et al. 2003), with $f$ values ranging from $\sim +7$–$13\%$ (Tcherkez 2006, Lanigan et al. 2008). $R_d$ is difficult to measure and not well understood, but existing studies demonstrate inhibition of respiration rate under illuminated conditions (Tcherkez et al. 2005) and biochemical differences between $R_d$ and dark respiration ($R$; Tcherkez et al. 2008). Similarly, $e$ is very difficult to estimate and no direct 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 we used tunable diode laser spectroscopy ($TDL$) coupled to infra-red gas analyzers ($IRGA$) to measure $g_i$ and $\Delta$ of Juniperus monosperma 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 1) measure the diurnal variation of $g_i$, 2) quantify the relationship between diurnal $g_i$ and i) $g_s$ and ii) time of day ($TOD$), 3) assess model sensitivity to variation in $eR_d$ and $b$, 4) measure the diurnal variation in $\Delta$ and examine the relationship between $\Delta$ and environmental and physiological drivers and 5) assess the performance of $\Delta_{\text{comp}}$, when fitted with diurnally variable $g_i$, compared to predictions from $\Delta_{\text{simple}}$. 
Methods

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

Leaf gas exchange measurements

We conducted two simultaneous measurements of leaf gas exchange: 1) on the crowns of three mature juniper trees (j_ambient) which we rotated through from ~0600–1800 on each day with measurements conducted maintaining the chamber environment similar to ambient conditions and, 2) on an adjacent mature juniper tree (j_manipulate) measured continuously throughout each day but subject 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 we measured leaf gas exchange and \(^{13}\)C discrimination in response to ambient conditions. For both j_ambient and j_manipulate we engaged temperature regulation in the chamber when leaf temperature (\(T_L\)), measured by energy balance, \(\geq 35^\circ\)C. We manipulated incoming irradiance in j_manipulate by using a plastic shade to reduce incident light by \(~50\%\) one or two times per hour to regulate net photosynthetic rate (\(A; \mu\text{mol m}^{-2} \text{s}^{-1}\)). Shading was maintained for 15–25 minute intervals to induce sufficient variation in \(A\) within each hour across the diurnal measurement period. Natural variation in irradiance occurred during both shaded and un-shaded periods and contributed to a wide range of \(A\). While all light manipulations were
performed on one tree ($j_{\text{manipulate}}$), we did measure different leaves over the course of each day and across the season including two on 1 June, three on 20 June, two on 19 July, and three on 23 August.

We measured leaf gas exchange by providing buffered air, via two 50L buffer volumes, to two LICOR 6400 portable photosynthesis systems (IRGA; 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) for measurement of the $^{12}\text{C}\text{O}^{16}\text{O}$ and $^{13}\text{CO}_2$ within each gas stream. Lines connecting each IRGA and the TDL were different lengths, resulting in different lag times, and we accounted for the 33 s and 50 s lag between the two IRGA’s and the TDL when summarizing data between the instruments. We used three minute TDL measurement cycles 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 $[\text{CO}_2]$ measurements used to calibrate each measurement cycle were (mean ± standard error (SE)) 548.648 ± 0.04 µmol/mol ($^{12}\text{C}\text{O}^{16}\text{O}$): 5.920 ± 0.0005 µmol/mol ($^{13}\text{C}\text{O}^{16}\text{O}$): 2.212 ± 0.0001 µmol/mol ($^{12}\text{C}^{18}\text{O}^{16}\text{O}$) for the high WS tank and 347.248 ± 0.25 µmol/mol ($^{12}\text{C}\text{O}^{16}\text{O}$): 3.747 ± 0.003 µmol/mol ($^{12}\text{C}^{16}\text{O}^{16}\text{O}$): 1.399 ± 0.001 µmol/mol ($^{12}\text{C}^{18}\text{O}^{16}\text{O}$) 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 535.972 ± 0.32 µmol/mol (¹²C¹⁶O¹⁶O): 5.785 ± 0.003 µmol/mol (¹³C¹⁶O¹⁶O): 2.161 ± 0.001 µmol/mol (¹²C¹⁸O¹⁶O) while the low WS tank was the same as described above. All WS calibration tanks were calibrated for four hours monthly 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, 539.568 µmol/mol (¹²C¹⁶O¹⁶O): 5.933 µmol/mol (¹³C¹⁶O¹⁶O): 2.208 µmol/mol (¹²C¹⁸O¹⁶O) and for the low tank, 339.433 µmol/mol (¹²C¹⁶O¹⁶O): 3.764 µmol/mol (¹³C¹⁶O¹⁶O): 1.401 µmol/mol (¹²C¹⁸O¹⁶O). Measurements of [CO₂] concentration occasionally exceeded the lower span of the WS calibration tanks (maximum deviation: 42.6 µmol/mol), but post-hoc tests of the TDL demonstrated a linear measurement response beyond lowest the range of CO₂ values observed in this study (Bickford et al. 2009).

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

Model parameterization
We tested whether variable $g_i$ improved model predictions of $\Delta_{\text{obs}}$ in $j_{\text{ambient}}$ using a comprehensive model of $\Delta$ ($\Delta_{\text{comp}}$; Farquhar et al. 1982b),

$$\Delta_{\text{comp}} = a_b \frac{P_a - P_s}{p_a} + a \frac{P_s - P_i}{p_a} + (b_s + a_w) \frac{P_i - P_c}{p_a} + b \frac{P_c}{p_a} - \frac{e R_d}{k} + \Gamma^*$$

(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 with 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. $\Gamma^*$, $R_d$, $k$, $f$, and $e$ represent the CO$_2$ compensation point in the absence of day respiration (Pa), day respiration rate ($\mu$mol m$^{-2}$ s$^{-1}$), carboxylation efficiency ($\mu$mol 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$_2$]. We estimated $R_d$ at 1.5 $\mu$mol m$^{-2}$ s$^{-1}$ based on reported measurements of dark respiration in juniper (Bickford et al. 2009), calculated $k$ as $A/p_c$ for each three minute cycle, and calculated $\Gamma^*$ based on $T_L$ (Brooks & Farquhar 1985). The photorespiratory, $f$, and day respiratory fractionation, $e$, 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. Because uncertainty in \( e, R_d, \) and \( b \) could contribute to model uncertainty we tested the sensitivity of \( \Delta_{comp} \) to variation in each and compared model predictions to \( \Delta_{obs} \). In these sensitivity tests \( \Delta_{comp} \) was fitted with a \( g_i = 0.71 \mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1} \) and both \( \Delta_{comp} \) and \( \Delta_{simple} \) were tested against all \( \Delta_{obs} \) values (\( n = 705 \)), where \( \Delta_{simple} \) is:

\[
\Delta_{simple} = a + (b - a) \cdot \frac{p_i}{p_a}
\]

(2)

and \( b \) is equal to 27\% to account for omitted fractionation factors (Farquhar & Richards 1984).

We parameterized \( g_i \) in \( \Delta_{comp} \) in three ways for inter-model testing, calculating \( \Delta_{comp} \) using \( g_{i1} \) (\( \Delta_{comp1} \)), \( g_{i2} \) (\( \Delta_{comp2} \)), and \( g_{i3} \) (\( \Delta_{comp3} \)). All three variations of \( \Delta_{comp} \) and \( \Delta_{simple} \) were tested against \( \Delta_{obs} \), but \( \Delta_{obs} \) values occurring outside the range of conditions of regression parameters associated with \( g_{i2} \) and \( g_{i3} \) were excluded from all inter-model comparisons (see Results). 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 one and intercept of zero (i.e. residuals = model prediction − \( \Delta_{obs} \)). The mean of these residuals represents model bias, while the standard deviation of the residuals represents the RMSE (Bickford et al. 2009).

\( \Delta \) and Diurnal \( g_i \)

We calculated leaf carbon isotope discrimination (\( \Delta_{obs} \)) from TDL generated data:

\[
\Delta_{obs} = \frac{\xi (\delta_o - \delta_e)}{1 + \delta_o - \xi (\delta_o - \delta_e)}
\]

(3)
where $\delta_e$ and $\delta_o$ equal the $\delta^{13}$C of the entering and outgoing chamber gas streams, respectively, and $\xi$ equals $c_e/(c_e-c_o)$ and $c_e$ and $c_o$ are the $[CO_2]$ of the gas entering and exiting the leaf chamber, respectively. Measurement error in $\Delta_{obs}$ was estimated following Bickford et al. (2009). We estimated $g_i$ in $j_{manipulate}$ from 40–80 minute periods of leaf gas exchange and isotopic data using slope-based methods (Evans et al. 1986),

$$g_i = \frac{(b-b_s-a_w)}{r_i}$$

(4)

where $r_i$ is the internal resistance to CO$_2$ diffusion and is proportional to the slope of the linear regression between $A/p_a$ and predicted discrimination ($\Delta_i$) minus $\Delta_{obs}$ (Figure 1); $\Delta_i$ is $\Delta_{simple}$ with $b = 29\%$. We determined the significance of each slope from zero ($P \leq 0.05$) using simple linear regression (SLR), and used these $g_{is}$ estimates to quantify $g_i$ three ways for model testing. First we calculated a mean $g_i$ from all $g_{is}$ estimates ($g_{i1}$). Second, we fit a SLR between time of day (TOD) and $g_i$ measured within each day. On days when a significant relationship was found between TOD and $g_i$ the data were pooled across dates, analyzed using SLR, and the resulting expression was used to estimate $g_i$ ($g_{i2}$). Thirdly, we transformed each $g_i$ estimate expressed in partial pressure ($\mu$mol m$^{-2}$ s$^{-1}$ Pa$^{-1}$) to a flux density (mol CO$_2$ m$^{-2}$ s$^{-1}$). Calculations showed incorporating partial pressure resulted in 21.1% higher $g_{is}$ estimates at 79 kPa so we added 21.1% to each flux density estimate of $g_i$ to account for underestimation due to these pressure considerations. These transformed $g_i$ were then compared to stomatal conductance of CO$_2$ ($g_{sCO2}$; mol CO$_2$ m$^{-2}$ s$^{-1}$) data using SLR. $g_{sCO2}$ was calculated as stomatal conductance of H$_2$O ($g_{sH2O}$) divided by 1.6 to account for differences in diffusivity between water vapor and CO$_2$ (Farquhar & Sharkey 1982). This relationship was tested to determine if slopes were significantly different from zero ($P \leq 0.05$; SLR) on each measurement date. The
expression resulting from all dates where there was a significant \( g_{\text{CO2}} - g_i \) relationship was used to estimate \( g_i \) \((g_{i3})\). All statistical tests were performed in JMP 5.0.1 (SAS Institute Inc., Cary, NC).

**Results**

*Diurnal* \( g_i \)

\( g_i \) ranged between 0.11–1.97 \( \mu \text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1} \) in \( f_{\text{manipulate}} \) across the four measurement days (Figure 2). Mean \( g_i \) was different between 1 June (mean \( \pm \) SE = 1.12 \( \pm \) 0.65 \( \mu \text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1} \)) and 20 June (0.60 \( \pm \) 0.33 \( \mu \text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1} \); \( P = 0.04 \)), but not between other dates \((P > 0.05)\). There was a significant relationship between \( g_{\text{CO2}} \) and \( g_i \) \((P \leq 0.03; \text{Figure } 3)\) and \( \text{TOD} \) and \( g_i \) \((P \leq 0.01)\) on 20 June and 19 July, but not on other dates. The linear expression \( g_i = -0.043 + 2.455g_{\text{CO2}} \) described the \( g_{\text{CO2}} - g_i \) relationship \((P = 0.0002, R^2 = 0.58, F = 22.22)\) between \( g_{\text{CO2}} \) values of 0.02–0.06 \( \mu \text{mol CO}_2 \text{m}^{-2} \text{s}^{-1} \), thus excluding periods when \( g_{\text{CO2}} \) fell below 0.02 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) from model testing (see *Model performance* below). The linear expression \( g_i = 1.623 - 2.138\text{TOD} \) described the \( \text{TOD} - g_i \) relationship \((P \leq 0.0001, R^2 = 0.74, F = 45.02)\) across the day between 06:00–17:00, excluding time periods beyond 17:00 on 1 June from model testing (see *Model performance* below). Linear slopes used to estimate \( g_i \) showed strong relationships between \( \Delta_r - \Delta_{\text{obs}} \) and \( A/p_a \) (Table 1, Figure 1).

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

Mean \( \Delta_{\text{obs}} \) in \( f_{\text{ambient}} \) was 14.3 \( \pm \) 0.2\% on 1 June, 16.3 \( \pm \) 0.2\% on 20 June, 17.6 \( \pm \) 0.4\% on 19 July, and 15.4 \( \pm \) 0.3\% on 23 August. \( \Delta_{\text{obs}} \) was similar on the 20 June and 23 August
measurement dates (Tukey’s Honestly Significant Differences (HSD), P > 0.05) but was significantly different on all other dates (P < 0.0001; Figure 4). When pooled across months physiological parameters exhibited significant but weak linear relationships with ∆_{obs} including A (P < 0.0001, R^2 = 0.22, F = 194.81), g_s (P < 0.0001, R^2 = 0.03, F = 20.30), and p_i/p_a (P < 0.0001, R^2 = 0.26, F = 247.46) (Figure 5). One measurement date, 19 July, showed a curvilinear trend between ∆_{obs} and p_i/p_a that was better described by a second order polynomial (P < 0.0001, R^2 = 0.84, F = 380.18) compared to a linear regression (P < 0.0001, R^2 = 0.71, F = 338.97) (data not shown). We attribute the diffuse pattern seen at higher p_i/p_a (> 0.7) to variation among measured trees (data not shown). A was not significantly different between dates (Tukey’s HSD, P > 0.05; Table 2); g_s was similar on 20 June and 19 July, but was different on all other days (P ≤ 0.05; Table 2).

There were weak but significant relationships between ∆_{obs} and T_L on 1 June (P = 0.02, R^2 = 0.04, F = 8.92) and 19 July (P = 0.01, R^2 = 0.05, F = 6.81) but not other dates (P ≥ 0.05). Similarly, there were weak but significant relationships between ∆_{obs} and VPD on each day (P ≤ 0.04), but not when VPD data were pooled across months (P = 0.06, R^2 = 0.005). VPD was significantly higher on 1 June and lower on 23 August compared to other days (Tukey’s HSD, P ≤ 0.05), but was similar on remaining days (P > 0.05; Table 2). Finally, there was a weak but significant linear relationship between ∆_{obs} and PPFD across all dates (P < 0.0001, R^2 = 0.09), but a second order polynomial better described the relationship (P < 0.0001, R^2 = 0.25). Soil water content at 200mm 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) ranged between −0.62 ± 0.06 (23 May) and −3.4 ± 0.33 MPa (25 July), before increasing to
−2.75 ± 0.34 MPa (23 August). The relationship between Ψ<sub>w</sub> and Δ<sub>obs</sub> was not significant (P = 0.15, R² = 0.75).

Model performance

The performance of Δ<sub>comp</sub> and its comparison to Δ<sub>simple</sub> varied depending on how g<sub>i</sub> was parameterized. To facilitate model comparison all periods of gs<sub>CO2</sub> or TOD outside the range of parameterization for g<sub>i2</sub> and g<sub>i3</sub> were excluded from all four models during testing (n = 137 Δ<sub>comp</sub> values removed, n = 568 used in each Δ<sub>comp</sub> and Δ<sub>simple</sub> analysis; see Diurnal g<sub>i</sub> above). As determined by lowest RMSE, Δ<sub>comp1</sub> and Δ<sub>comp2</sub> performed better than Δ<sub>comp3</sub> and Δ<sub>simple</sub> throughout the study. Δ<sub>comp1</sub> performed best on 20 June and 19 July and Δ<sub>comp2</sub> performed best on 1 June and 23 August (Table 3). Δ<sub>comp3</sub> showed lower error than Δ<sub>simple</sub> on all days after 1 June (Table 3; Figure 7). Model predictions were also pooled across the whole study and compared to pooled Δ<sub>obs</sub> data. Among pooled data Δ<sub>comp1</sub> and Δ<sub>comp2</sub> still exhibited relatively lower error than Δ<sub>comp3</sub> and Δ<sub>simple</sub> (Table 3), though model bias was higher in Δ<sub>comp1</sub> (bias = 3.45‰ vs. 3.27‰ for Δ<sub>comp2</sub>; Table 3). A primary conclusion from Table (3) is that all models consistently over-predicted Δ by at least 1‰.

Sensitivity tests showed reduced model bias and RMSE when eR<sub>d</sub> and b were set to low values (compare Tables 3 and 4). Model bias increased 60% and error decreased 7.4% as eR<sub>d</sub> shifted from more positive (−1‰) to more negative (−9‰) values when b was 29‰. Across tested eR<sub>d</sub> values the use of lower b values in Δ<sub>comp</sub> consistently reduced model bias and error. Δ<sub>simple</sub> showed a 94% reduction in model bias and 1.6% reduction in error when fit with b = 22‰ instead of b = 27‰ (Table 4). Excluding 19
July, all variations of $\Delta_{\text{comp}}$ and $\Delta_{\text{simple}}$ overestimated $\Delta_{\text{obs}}$ by 2–6‰, as determined by model bias, though accounting for the variance, as in the RMSE term, reduced total error to between 1.0–2.4‰ on individual days. The pooled data were skewed by the high bias and error in the 23 August data but reveal better performance by $\Delta_{\text{simple}}$ than seen on individual dates, including modest improvement in model error compared to $\Delta_{\text{comp}}$
. Using RMSE as the metric, the best fit to $\Delta_{\text{obs}}$ was found using $\Delta_{\text{comp}}$ with $eR_d = -9$‰ and $b = 24$‰.

**Discussion**

**Diurnal $g_i$**

Two diurnal $g_i$ trends were evident across the study. On 1 June $g_i$ increased through most of the morning period to relatively high values ($\sim 2$ µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$) and then decreased in the afternoon period. This trend of low to high $g_i$ over the early morning to midday period resembles previous observation of diurnal $g_i$ in juniper (Bickford *et al.* 2009). Predawn $\Psi_w$ was relatively high during both periods, and higher leaf water status may have contributed to the morning increase. In the other three days, however, a different pattern was observed: the highest $g_i$ ($\sim 1$ µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$) was observed in the early morning, with a linear decline across two of the three days (Fig. 2). On 23 August the decline occurs in the morning, with $g_i$ stabilizing around $\sim 0.5$ µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ for the remainder of the day. The diurnal decline in $g_i$ is consistent with previous work showing reduced $g_i$ under water stressed conditions (Warren, Livingston & Turpin 2004; Flexas *et al.* 2004), however, the range of $\Psi_w$ seen during this period of the study would be characterized as moderate water stress in juniper (Linton, Sperry & Williams 1998;
McDowell et al. (2008). \( g_i \) was significantly related to \( g_s \) and \( TOD \) on two of four days. The predictive power of the \( g_i-TOD \) could likely be improved by accounting for variation in the early evening time period. Below \( g_s \) of \(-0.035 \text{ mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \), \( g_i \) was limiting CO\(_2\) transfer to the sites of carboxylation; most \( g_s \) measurements were above 0.035 mol CO\(_2\) m\(^{-2}\) s\(^{-1}\) and thus stomatal limitations often provided the greatest diffusion resistance. Our findings agree with the strong \( g_s-g_i \) relationship among 15 species shown by Loreto et al. (1992), where \( g_i \) was 1.4\( g_s \), and in \textit{Nicotaina} (Galmes et al. 2006), but differ from data in other species showing generally lower \( g_i \) compared to \( g_s \) (Hanba et al. 2003). Our \( g_s-g_i \) data deviate from the 1:1, likely due to different regulatory processes between stomatal and internal conductance to CO\(_2\), but others have observed nearly 1:1 \( g_s-g_i \) relationships (Lauteri et al. 1997).

\( \Delta \), \textit{environmental & physiological parameters}

Diurnal patterns across the study were consistent with previous studies showing environmental regulation of \( \Delta_{\text{obs}} \). As previously observed in model and empirical studies, \( VPD \) and PPFD acted as environmental drivers of \( \Delta \) (Baldocchi & Bowling 2003; Chen & Chen 2007; McDowell et al. 2008b; Bickford et al. 2009), likely through their strong influence on \( A \) and \( g_s \). \( \Psi_w \) co-varied with \( \Delta \), decreasing when \( \Delta \) was increasing from 1 June to 19 July, and \( \Delta \) decreased when \( \Psi_w \) again increased in August. \( \Delta \) was comparable to previous observations in juniper during the same months in 2006, but was lower on 23 August (Bickford et al. 2009). Predawn \( \Psi_w \) was substantially more negative in August 2007 (−2.75 MPa) compared to August 2006 (−0.58 MPa; McDowell et al. 2008b) and
may have contributed to the seasonal $\Delta$ pattern. The non-significant relationship between $\Psi_w$ and mean $\Delta_{\text{obs}}$ was likely due to low sample size (n=4).

The variation in the physiological parameters $A$, $g_s$, and $p_i/p_a$ was correlated with $\Delta_{\text{obs}}$. $\Delta_{\text{obs}}$ was generally higher when $A$ was low and $g_s$ and $p_i/p_a$ were high (Figure 5). Conversely, $\Delta_{\text{obs}}$ tended to be lower when $A$ was high and $p_i/p_a$ was low. A large range of $\Delta_{\text{obs}}$ was seen at low $g_s$, consistent with previous work showing relatively high $\Delta$ when $g_s$ and $A$ are low (Bickford et al. 2009). It is likely that isotopic measurements indicating moderate to high $\Delta_{\text{obs}}$ (~25–35‰) occurring at low $g_s$ (< 0.05 mol m$^{-2}$ s$^{-1}$) are being more strongly influenced by respiratory and/or photorespiratory activity (Bickford et al. 2009).

**Model performance**

Overall $\Delta_{\text{comp}}$ performed best in predicting $\Delta_{\text{obs}}$ when fitted with $g_{i1}$ and $g_{i2}$, while $\Delta_{\text{comp3}}$ and $\Delta_{\text{simple}}$ produced poorer predictions of $\Delta_{\text{obs}}$ (Table 3), and supports recent work showing improved model fit when including $g_i$ in model predictions versus using simpler models (Cai et al. 2008; Bickford et al. 2009). Our results demonstrate no substantial improvement when using $\Delta_{\text{comp2}}$ compared to $\Delta_{\text{comp1}}$, indicating the validity of using a mean $g_i$ value to predict juniper $\Delta$ over the diurnal periods and across the seasonal gradient in this study. This finding supports previous work showing improved model fit when utilizing a mean $g_i$ in $\Delta_{\text{comp}}$ across diurnal and seasonal timescales (Bickford et al. 2009), but contrasts with recent evidence showing improved model predictions of respired $\delta^{13}$C values when $g_i$ was linked to variation in $g_s$ compared to using a static $g_i$ in model predictions (Cai et al. 2008). The discrepancy between our observation of diurnal shifts in $g_i$ and the null effect of incorporating this variability into model predictions may
be due to the use of a mean \( g_i \) that was high enough so that resistance in the diffusion pathway was minimized to an extent that did not substantially effect model predictions. Alternatively, our model assessment method may have lacked sufficient sensitivity to discern improvements brought about by using \( \Delta_{\text{comp2}} \). The predictive performance of \( \Delta_{\text{simple}} \) and \( \Delta_{\text{comp3}} \) was similar enough that their performance ranking varied depending on the temporal scale of the analysis, with \( \Delta_{\text{comp3}} \) showing lower error on most days but \( \Delta_{\text{simple}} \) outperforming when data were pooled across the whole study. This shows that improper parameterization can override the expected predictive advantage of \( \Delta_{\text{comp}} \) and produce inferior results compared to a more parsimonious model. Model bias was relatively high on most days (Figure 6), particularly 23 August, and in the pooled data (Table 3), showing all models consistently overestimated \( \Delta_{\text{obs}} \). The most likely reason for this is model parameterization error (discussed below in our sensitivity analysis). Viewed from the whole study perspective there was lower model bias and error in \( \Delta_{\text{comp1}} \) and \( \Delta_{\text{comp2}} \) compared to \( \Delta_{\text{simple}} \), supporting the use of a carefully parameterized \( \Delta_{\text{comp}} \) for leaf-level predictions of \( \Delta \).

Sensitivity tests showed that, in addition to \( g_i \), variation in \( eR_d \) and \( b \) improved model performance. Lowering \( eR_d \) resulted in reduced error for a given \( b \) value, but consistently increased model bias. Step-change reductions in \( b \) from the value we used (29‰), however, resulted in consistently lower model bias and error. Two factors could explain these findings, namely that the fractionation associated with \( b \) is lower than has been reported until recently or that \( R_d \) was higher and \( e \) more negative than we estimated. The simultaneous reduction in model bias and error we observed when reduced \( b \) values were implemented suggests \( b \) is the stronger regulator of model performance, but without
assays of PEP and Rubisco activity only limited conclusions can be made. A lower \( b \)
could be explained by relatively high PEP carboxylation activity proportional to Rubisco
activity (Farquhar & Richards 1984; Lanigan et al. 2008) or a lower intrinsic isotope
effect of the carboxylases comprising \( b \) (Raven & Farquhar 1990; Brugnoli & Farquhar
2000). PEP carboxylation is typically associated with C4 photosynthesis and results in
low discrimination against \(^{13}\)C (~ −5.7‰; Farquhar et al. 1989), but the extent of PEP
carboxylase activity in C3 photosynthesis is not well understood. Alternatively, the
influence of respiratory activity may have been higher than we estimated in this study.
We based our estimates on previous work showing high dark respiration rate, which we
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 we did not account 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 is not yet understood.

\( \Delta_{\text{simple}} \) was less sensitive to variation in \( b \) compared to \( \Delta_{\text{comp}} \), but sensitivity tests
demonstrated variability in \( b \) may be greater than currently assumed. Previous studies
using \( \Delta_{\text{simple}} \) have shown \( b \) values < 27‰ resulting in the best fit of observed \( \Delta \) (Brugnoli
& Farquhar 2000), and this is usually attributed to the reduced \( b \) value accounting for
omitted fractionation factors. We tested \( \Delta_{\text{comp}} \) and \( \Delta_{\text{simple}} \) with the same \( \Delta_{\text{obs}} \) dataset,
however, and found improvement in both models when lower \( b \) values were used,
supporting the use of species specific \( b \) values in \( \Delta_{\text{comp}} \) to improve model predictions.
Further studies of the net carboxylation fractionation in other groups of higher plants (i.e.
conifers and deciduous woody species) are needed to better understand variation in $b$.

Overall, the results of our model tests and sensitivity analysis show non-negligible model error in predicting leaf $\Delta$, but suggest better understanding and incorporation of the variability in key parameters such as $g_i$, $b$, $e$, and $R_d$ may aid in more accurate and precise model fits. In the interim, modelers interested in predicting diurnal $\Delta$ across seasonal and annual time scales and at larger organizational scales should consider the relative sensitivity of $\Delta_{\text{comp}}$ to proper parameterization versus results from the parsimonious $\Delta_{\text{simple}}$.

Acknowledgements

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Tcherkez Guillaume. 2006. How large is the carbon isotope fractionation of the


Tables

Table 1. Correlation coefficients and $P$ values from the linear regressions used to calculate all slopes for estimation of internal CO$_2$ conductance across each measurement day.

<table>
<thead>
<tr>
<th>Time</th>
<th>$R^2$</th>
<th>$P$</th>
<th>Time</th>
<th>$R^2$</th>
<th>$P$</th>
<th>Time</th>
<th>$R^2$</th>
<th>$P$</th>
<th>Time</th>
<th>$R^2$</th>
<th>$P$</th>
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<td>19 July</td>
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<td>23 August</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7:00</td>
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<td>&lt;0.0001</td>
<td>7:00</td>
<td>0.32</td>
<td>0.005</td>
<td>6:30</td>
<td>0.68</td>
<td>&lt;0.0001</td>
<td>6:30</td>
<td>0.63</td>
<td>&lt;0.0001</td>
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<td>0.36</td>
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<td>9:00</td>
<td>0.71</td>
<td>&lt;0.0001</td>
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Table 2. Mean diurnal net photosynthetic rate ($A$; µmol m$^{-2}$ s$^{-1}$), stomatal conductance to H$_2$O ($g_s$; mol m$^{-2}$ s$^{-1}$), and vapor pressure deficit ($VPD$; kPa), each reported with one standard error (SE) and sample size ($n$). $A$ was not different across dates ($P > 0.05$); $g_s$ and $VPD$ were both different on 1 June and 23 August ($P < 0.05$) from all other days, but 20 June and 19 July were not different from one another ($P > 0.05$).

<table>
<thead>
<tr>
<th>Date</th>
<th>$A$</th>
<th>SE</th>
<th>$g_s$</th>
<th>SE</th>
<th>$VPD$</th>
<th>SE</th>
<th>$n$</th>
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<td>0.06</td>
<td>0.002</td>
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<td>0.05</td>
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<tr>
<td>23-August</td>
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<td>0.11</td>
<td>0.002</td>
<td>1.34</td>
<td>0.05</td>
<td>158</td>
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</table>
Table 3. Results from model prediction tests of observed discrimination ($\Delta_{\text{obs}}$). $\Delta_{\text{simple}}$ represents the simplified model of discrimination and $\Delta_{\text{comp}}$ represents the comprehensive model of discrimination, with different forms of $\Delta_{\text{comp}}$ indicating parameterization with different internal conductance ($g_i$) values. Here $\Delta_{\text{comp}1}$ uses a seasonal mean $g_i$ value of 0.71 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$, $\Delta_{\text{comp}2}$ uses $g_i$ derived from a regression describing the relationship between $g_i$ and time of day, and $\Delta_{\text{comp}3}$ uses $g_i$ calculated based on the regression between $g_i$ and stomatal conductance of CO$_2$. Model bias (‰) ranged between 1.0–6.75‰ and error (RMSE; ‰) ranged from 1.0–2.4‰ across individual measurement dates, but showed reduced variation in the whole study assessment. Assessed monthly and across the whole study $\Delta_{\text{comp}1}$ and $\Delta_{\text{comp}2}$ best predicted $\Delta_{\text{obs}}$. $\Delta_{\text{comp}3}$ outperformed $\Delta_{\text{simple}}$ on individual days, but $\Delta_{\text{simple}}$ outperformed $\Delta_{\text{comp}3}$ across the whole study. Bolded values highlight the best performing model in each month and across the study.

<table>
<thead>
<tr>
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<th>1 June bias</th>
<th>1 June RMSE</th>
<th>20 June bias</th>
<th>20 June RMSE</th>
<th>19 July bias</th>
<th>19 July RMSE</th>
<th>23 August bias</th>
<th>23 August RMSE</th>
<th>Whole study bias</th>
<th>Whole study RMSE</th>
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<td>$\Delta_{\text{comp}1}$</td>
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<td>1.09</td>
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<td>3.45</td>
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<td>2.35</td>
<td>3.88</td>
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Table 4. Results from sensitivity tests where the parameters representing the day respiration fractionation ($e$; ‰), day respiration rate ($R_d$; µmol m$^{-2}$ s$^{-1}$), and fractionation during carboxylation ($b$) were adjusted in the comprehensive model of carbon discrimination ($\Delta_{\text{comp}}$; eq. 1), and $b$ was adjusted in the simplified version of carbon discrimination ($\Delta_{\text{simple}}$; eq. 2). $g_i$ was held constant at 0.71 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$, all other variables are as described in Model parameterization. More negative $eR_d$ and/or lower $b$ values reduced $\Delta_{\text{comp}}$ model bias (‰) and root mean squared error (RMSE; ‰) when compared to observed discrimination ($\Delta_{\text{obs}}$). Similarly, lower $b$ values reduced $\Delta_{\text{simple}}$ model bias and RMSE when compared to $\Delta_{\text{obs}}$.

<table>
<thead>
<tr>
<th>$eR_d$</th>
<th>$b$ (%)</th>
<th>$\Delta_{\text{comp}}$ bias</th>
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<th>$b$ (%)</th>
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Figure 1. Regression slopes of the relationship of predicted discrimination ($\Delta_i$) minus observed discrimination ($\Delta_{obs}$) in relationship to the ratio of net photosynthetic rate ($A$) to the partial pressure of CO$_2$ in the atmosphere surrounding the leaf ($p_a$) used to estimate the internal CO$_2$ conductance. See Table 1 for $P$ values and correlation coefficients associated with each slope.

Figure 2. Diurnal variation in internal CO$_2$ conductance ($g_i$) across the four measurement dates. $g_i$ was significantly different between 1 June and 20 June ($P < 0.05$), but not between other dates.

Figure 3. The relationship between stomatal conductance to CO$_2$ ($g_{sCO2}$) and internal CO$_2$ conductance ($g_i$) on 20 June and 19 July. $g_{sCO2}$ and $g_i$ data on each date were tested for significance ($P \leq 0.05$, simple linear regression); significant relationships were pooled and the regression used to estimate $g_i$ based on $g_{sCO2}$ when $g_{sCO2}$ was $> 0.02$ mol m$^{-2}$ s$^{-1}$.

Figure 4. Diurnal variation in carbon isotope discrimination ($\Delta$;●) and photosynthetic photon flux density (PPFD;○) on the four measurement dates. Error bars represent one SE. The abrupt shifts in $\Delta$ mid-day on 1 June can be attributed to variation among trees, but variation seen on other dates results from plant environmental response. There was a significant relationship between PPFD and $\Delta$ best described by a second order polynomial ($P < 0.0001$, $R^2 = 0.25$).
Figure 5. The relationship between observed discrimination ($\Delta_{obs}$) and net photosynthetic rate ($A$), stomatal conductance to H$_2$O ($g_s$), and the ratio of partial pressure of CO$_2$ in intercellular spaces and the environment around the leaf ($p_i/p_a$). When pooled across months these parameters exhibited significant linear relationships with $\Delta_{obs}$ including $A$ ($P < 0.0001$, $R^2 = 0.22$), $g_s$ ($P < 0.0001$, $R^2 = 0.03$), and $p_i/p_a$ ($P < 0.0001$, $R^2 = 0.26$).

Figure 6. Model tests of observed discrimination ($\Delta_{obs}$). Four models were tested against $\Delta_{obs}$ including the simple model of discrimination ($\Delta_{simple}$; ●), the comprehensive model of discrimination using a mean internal CO$_2$ conductance ($g_i$) of 0.71 µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$ ($\Delta_{comp1}$; ●), the comprehensive model of discrimination using a $g_i$ estimated from the regression between diurnal $g_i$ and time of day (TOD) ($\Delta_{comp2}$; □), and the comprehensive model of discrimination using a $g_i$ estimated from the regression describing the relationship between stomatal conductance of CO$_2$ and $g_i$ ($\Delta_{comp3}$; ▲). $\Delta_{predicted}$ represents discrimination predictions of any of the four models. In individual months and across the whole study $\Delta_{comp1}$ and $\Delta_{comp2}$ performed best, exhibiting lower model bias and error than either $\Delta_{comp3}$ or $\Delta_{simple}$. These results support the use of a mean $g_i$ value or $g_i$ based on TOD in $\Delta_{comp}$ to predict diurnal carbon discrimination.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Chapter 4

Linkages between leaf water potential and internal conductance during drought in two isohydric species

CHRISTOPHER P. BICKFORD

1University of New Mexico, Department of Biology, MSC03-2020, Albuquerque, NM 87131

Running title: $g_i$ in isohydric species
Abstract

Water deficit is known to reduce many leaf gas exchange characteristics, including the internal conductance of CO$_2$ from substomatal cavities to sites of carboxylation ($g_i$). In this study we imposed soil water deficit (SWD) in two isohydric species, *Populus fremontii* (poplar) and *Quercus gambelii* (oak), to investigate whether static leaf water potential during SWD would influence $g_i$ activity. Using tunable diode laser spectroscopy we measured instantaneous carbon isotope discrimination ($\Delta$) and estimated $g_i$ from gas exchange data. Results show no statistically significant reduction in leaf water potential ($\Psi_w$) or $g_i$ among droughted poplar and oak individuals in response to SWD. These non-significant differences in poplar $g_i$, however, may have generated significant changes in the relationship between $\Delta$ and the CO$_2$ partial pressure in intercellular airspaces and at the site of carboxylation relative to CO$_2$ in the ambient atmosphere, providing some evidence for an effect of SWD on CO$_2$ diffusion in leaves. Based on these data, it appears that maintenance of a constant $\Psi_w$ diminishes the response of $g_i$ to SWD and thus $\Psi_w$ may have a regulatory role in $g_i$.

Keywords: mesophyll conductance, $p_c/p_a$, water stress, carbon isotope discrimination, decarboxylation, isohydry

Introduction

Drought has a detrimental effect on plant productivity globally. Many plant responses to soil water deficit (SWD) are well understood, including reduced stomatal conductance ($g_s$) that limits H$_2$O loss and carbon uptake (Lawlor & Cornic 2002, Flexas *et al.*, 2004)
and biochemical impairment under severe water stress that reduces photosynthetic rate (Tezara et al., 1999, Flexas et al., 2006). Leaf water potential ($\Psi_w$) is a widely used indicator of plant water stress (Jones 2007), but plants exhibit different strategies for regulating $\Psi_w$ in response to soil drought. Isohydric plants tightly regulate $g_s$ to maintain a mid-day $\Psi_w$ ‘set point’ that is largely invariant in response to moderate to severe SWD, whereas anisohydric plants exhibit less stringent regulation of $g_s$ and vary $\Psi_w$ as water availability and/or vapor pressure deficit ($VPD$) changes (Tardieu and Simmoneau 1998). Mechanisms underlying this regulatory framework are still poorly understood, though membrane aquaporin regulation may be important (Sade et al., 2009). Functionally, isohydric and anisohydric behavior may play a substantial role in drought survival by driving different gas exchange patterns during drought and drought recovery (McDowell et al., 2008; West et al., 2008).

In addition to stomatal control of carbon assimilation, several studies have shown that water deficit reduces the internal conductance of CO$_2$ from substomatal cavities to sites of carboxylation ($g_i$; Ridolfi and Dreyer 1997, Scartazza et al., 1998; Flexas et al., 2002; Warren, Livingston & Turpin 2004, Grassi and Magnani 2005; Galmes et al., 2007). Most found that $g_i$ was reduced when water stress occurred over time periods ranging from minutes to days or weeks (Warren et al., 2004, Grassi and Magnani 2005) though reports exist showing no significant reductions in $g_i$ under transient water stress (Monti et al., 2006) or even longer term SWD (Delfine et al., 2001). The proportion of reported species exhibiting decreased $g_i$ during drought that are isohydric is unclear, though some reports suggest a correlation between variable $g_i$ and anisohydric behavior (Warren et al., 2004) and others suggest little change in $g_i$ in some isohydric plants.
during SWD (Galmes et al., 2007). These reductions in $g_i$ impact photosynthetic rate ($A$; Flexas et al., 2002, 2006) and measurement (Scartazza et al., 1998) and modeling of carbon isotope discrimination ($\Delta$) by regulating the CO$_2$ diffusion pathway (Le Roux et al., 2001; Bickford et al., 2009). Recently, evidence has been put forward demonstrating that aquaporin proteins are facilitating the movement of CO$_2$ across cell membranes (Flexas et al., 2006b, Uehlein et al., 2008) and play a regulatory role under drought conditions (Miyazawa et al., 2008), however, environmental regulation of aquaporin activity is not well understood (Kaldenhoff et al., 2008).

Carbon isotope discrimination is the primary regulator of the $^{13}$CO$_2$/^{12}$CO$_2$ ($\delta^{13}$C) signature fixed into sugars and other plant C products (Farquhar, Ehleringer & Hubick 1989), though post-photosynthetic fractionations can induce variation among different organs and substrate pools (Bowling et al., 2008; Gessler et al., 2008). $\Delta$ is known to respond to environmental change as it is linearly related to the ratio of CO$_2$ partial pressure ($p$CO$_2$) in intercellular air spaces and the atmosphere ($p_i/p_a$) (Farquhar et al., 1989, Brugnoli and Farquhar 2000). The strong influence of drought and/or high VPD conditions on $g_s$ rapidly affects $\Delta$ by restricting CO$_2$ diffusion from the atmosphere to intercellular air spaces (Farquhar et al., 1989). Many studies have used the linear relationship between $\Delta$ and $p_i/p_a$ to make inferences about stomatal conductance or photosynthetic rate ($A$), nevertheless, $\Delta$ occurs at the carboxylase and thus the ratio of $p$CO$_2$ at the site of carboxylation and in the atmosphere ($p_c/p_a$) is the more accurate parameter for correlation (Seibt et al., 2008).

The net flux and apparent isotopic fractionation associated with respiratory and photorespiratory activity ($\Delta_{ef}$) during light reactions may also be important for
interpreting $\Delta$. Recent work suggests the isotopic fractionation associated with photorespiration ($f$) is between 10–12‰ (Tcherkez 2006, Lanigan et al., 2008). Dark respiration ($R$) is inhibited in the light (Atkin et al., 2000, Tcherkez et al., 2005), and biochemically distinct from day respiration ($R_d$) processes as only portions of the dark respiration pathway are fully active in the light (Tcherkez et al., 2008). Currently, the isotopic fractionation associated with $R_d$ ($e$) is not well understood and, consequently, measurements of the fractionation occurring during $R$ are often used as a surrogate estimator. Studies have demonstrated isotopic enrichment occurring during $R$ in response to drought (Duranceau et al., 1999), temperature (Tcherkez et al., 2003), and light exposure (Barbour et al., 2007). The cumulative effect of $\Delta_{ef}$ can be estimated from isotopic gas exchange data (Evans et al., 1986) and recent evidence suggests it may be important for predicting leaf $\Delta$ in some species (Wingate et al., 2007; Bickford et al., 2009), though the effects of drought on the interaction of $R_d$ and photorespiratory processes are not well understood.

In this study we coupled a portable photosynthesis system to a tunable diode laser to obtain high frequency measurements of the concentration and composition of leaf gas exchange occurring in two isohydric species: *Populus fremontii* S. Watson (poplar) and *Quercus gambelii* Nuttall (oak). The aims of this study were to 1) test the hypothesis that $\Psi_w$ has a regulatory role in $g_i$ among isohydric plants during SWD, 2) test whether drought had a significant effect on $\Delta_{ef}$, and 3) examine the relationship between observed carbon isotope discrimination ($\Delta_{obs}$) and $VPD$, $p_i/p_a$ and $p_c/p_a$. 

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Methods

The study was conducted in two experiments, hereafter referred to as the poplar and oak experiments. In both experiments we measured the concentration and isotopic composition of leaf gas exchange to assess variation in $g$, in response to SWD and $\Psi_w$. We coupled a portable photosynthesis system ($IRGA$; LICOR 6400, LICOR Biosciences Inc., Lincoln, NE, USA) fitted with a custom leaf chamber to a tunable diode laser ($TDL$; TGA100A, Campbell Scientific Inc., Logan, UT, USA) as described in Bickford et al., (2009). The custom leaf chamber has a glass top and is capable of illuminating up to 75 cm$^2$ of leaf area when used with the external white LED light source (Photon Systems Instruments SL3500-W-D, Brno, Czech Republic). Boundary layer conductance in the chamber is $\geq 1.8$ mol m$^{-2}$ s$^{-1}$. For the poplar experiment, the working standard (WS) calibration tanks spanning the range of expected sample $\text{[CO}_2\text{]}$ used to calibrate each 3 minute measurement cycle were (mean $\pm$ standard error (SE)) 535.972 $\pm$ 0.32 µmol/mol ($^{12}\text{C}^{16}\text{O}^{16}\text{O}$): 5.785 $\pm$ 0.003 µmol/mol ($^{13}\text{C}^{16}\text{O}^{16}\text{O}$): 2.161 $\pm$ 0.001 µmol/mol ($^{12}\text{C}^{18}\text{O}^{16}\text{O}$) for the high WS tank and 347.248 $\pm$ 0.25 µmol/mol ($^{12}\text{C}^{16}\text{O}^{16}\text{O}$): 3.747 $\pm$ 0.003 µmol/mol ($^{12}\text{C}^{16}\text{O}^{16}\text{O}$): 1.399 $\pm$ 0.001 µmol/mol ($^{12}\text{C}^{18}\text{O}^{16}\text{O}$) for the low WS tank. The WS calibration tanks were calibrated for four hours monthly against WMO-certified tanks that were filled and $\delta^{13}\text{C}$ 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 $\text{[CO}_2\text{]}$ of the WMO traceable tanks used in this study were, for the high tank, 539.568 µmol/mol ($^{12}\text{C}^{16}\text{O}^{16}\text{O}$): 5.933 µmol/mol ($^{13}\text{C}^{16}\text{O}^{16}\text{O}$): 2.208 µmol/mol ($^{12}\text{C}^{18}\text{O}^{16}\text{O}$) and for the low tank, 339.433 µmol/mol ($^{12}\text{C}^{16}\text{O}^{16}\text{O}$): 3.764 µmol/mol ($^{13}\text{C}^{16}\text{O}^{16}\text{O}$): 1.401
µmol/mol (\(^{12}\text{C}^{18}\text{O}^{16}\text{O}\)). Measurements of \([\text{CO}_2]\) concentration occasionally exceeded the lower span of the WS calibration tanks in the poplar experiment (maximum deviation: 78.0 µmol/mol), but post-hoc tests of the TDL (Bickford et al. 2009) demonstrated a linear measurement response to 247.43 µmol/mol, a \([\text{CO}_2]\) lower than observed in this study. Ambient air was provided to the IRGA via a 50L buffer volume. For the oak experiment working standard (WS) calibration tanks spanning the range of expected \([\text{CO}_2]\) measurements used to calibrate each 2 minute measurement cycle were (mean ± standard error (SE)) 473.336 ± 0.25 µmol/mol (\(^{12}\text{C}^{16}\text{O}^{16}\text{O}\)): 5.18321 ± 0.003 µmol/mol (\(^{13}\text{C}^{16}\text{O}^{16}\text{O}\)): 1.938 ± 0.001 µmol/mol (\(^{12}\text{C}^{18}\text{O}^{16}\text{O}\)) for the high WS tank and 243.47378 ± 0.10 µmol/mol (\(^{12}\text{C}^{16}\text{O}^{16}\text{O}\)): 2.66630 ± 0.001 µmol/mol (\(^{13}\text{C}^{16}\text{O}^{16}\text{O}\)): 0.996 ± 0.001 µmol/mol (\(^{12}\text{C}^{18}\text{O}^{16}\text{O}\)) for the low WS tank. These WS tanks were calibrated for three hours with the previously described WMO-traceable standard tanks. During the oak experiment air provided to the IRGA came from disposable \(\text{CO}_2\) gas cylinders filled from a natural well (\(\delta^{13}\text{C} = -4\%_o\); Liss America, Macedon, New York, USA).

We performed the poplar experiment in a greenhouse located at Los Alamos National Laboratory in Los Alamos, NM, USA (elev. 2140m; atmospheric pressure = ~79 kPa). Daytime temperature across the growth period ranged between 21.5 and 33.4° C, and a shade cloth covering the greenhouse reduced the maximum photosynthetic photon flux (PPF) to ~1050 µmol photons m\(^{-2}\) s\(^{-1}\). Plants were started from cuttings and transferred to 7L pots, where they grew between May and August 2007, when measurements commenced. Pots were filled with Metro-Mix 300 growing medium (Sun-Gro Horticulture Distribution Inc., Bellevue, WA, USA) and fertilized 3 times weekly with 20-20-20 solution (Fertilome, Voluntary Purchasing Group). We withheld water
from seven trees for 2 d prior to gas exchange measurements to induce soil water deficit. 500mL H₂O was added to all droughted plant pots whose soil water content (SWC) fell below 15% at the end of day 1 measurements to bring SWC up to ~25%. During the two measurement days we measured volumetric SWC hourly on all plants using a soil water content measurement system (Hydrosense, Campbell Scientific Inc., Logan, UT, USA). During gas exchange measurements leaf temperature (Tₗ) was regulated between 26-31°C. Tₗ was measured using a thermocouple temperature sensor (Type E, Omega Engineering Inc., Stamford, CT, USA) in contact with lower side of the leaf. PPF was varied in step-change reductions from ~1550 to 200 µmol m⁻² s⁻¹. Immediately following gas exchange measurements we collected a leaf punch from the portion of the lamina measured and placed the punch in a calibrated leaf psychrometer (C-52 sample chamber, Wescor Environmental Products Division, Logan, UT, USA) coupled to a CR-7 datalogger (Campbell Scientific Inc., Logan, UT, USA) for measurement of Ψᵩ. We determined leaf area using a leaf area meter (LI-3100; LICOR Biosciences Inc., Lincoln, NE, USA), and present leaf area corrected gas exchange data.

The oak study was conducted at the University of New Mexico in Albuquerque, NM, USA (elev. 1524m; ~ atmospheric pressure = 84.8 kPa) on September 17 and 19, 2008. We grew oak plants from seed in 2.5L pots in a greenhouse between October 2007 and September 2008, when we conducted the study. Pots were filled with Metro-Mix 360 growing medium (Sun-Gro Horticulture Distribution Inc., Bellevue, WA, USA) and fertilized weekly with 20-20-20 N-P-K solution (Jack’s 20-20-20; J.R. Peters, Inc., Allentown, PA, USA). Greenhouse temperature ranged between 18 and 27°C and maximum daytime irradiance was ~1100 µmol photons m⁻² s⁻¹. We withheld water from
eight plants for 10 d prior to measurements on 17 September, except for providing ~100mL to droughted plants three days prior to 17 September measurements. Similarly, we administered ~200mL to remaining droughted plant pots at the end of 17 September measurements to maintain SWC at ~25% for the 19 September measurements. We determined SWC gravimetrically by measuring pot, soil, and plant mass at the time of gas exchange measurements \( W_m \), then bringing them to field capacity and measuring mass again \( W_{fc} \), and finally measuring the dry mass \( W_d \). To quantify SWC we used the equation \( \text{SWC} = \frac{W_m - W_d}{W_{fc} - W_d} \).

\( T_L \) was measured and maintained as in the poplar experiment. PPF was varied in step-change reductions from ~1300 to 200 µmol m\(^{-2}\) s\(^{-1}\), and following light measurements a dark cloth was placed over the chamber to facilitate measurement of the dark respiration rate and \( \delta^{13}\text{C} \) of dark respired CO\(_2\) \( (\delta^{13}\text{C}_{\text{resp}}) \).

Immediately following gas exchange measurements the measured leaf and petiole were excised from the stem for measurement of xylem \( \Psi_w \) using a Scholander-type pressure bomb (PMS Instruments Inc., Corvallis, OR, USA). We determined leaf area using by scanning measured leaves and calculating leaf using Scion Image for Windows (Scion Corporation, Frederick, MD, USA).

We calculated five parameters from our leaf isotopic gas exchange data: \( \Delta_{\text{obs}} \), \( \delta^{13}\text{C} \), \( g_{is}, g_{ip} \) and \( \Delta_{ef} \). We determined \( \Delta_{\text{obs}} \) following Evans et al. (1986),

\[
\Delta_{\text{obs}} = \frac{\xi (\delta_o - \delta_{oe})}{1 + \xi (\delta_o - \delta_{oe})} 
\]

(1)

where \( \xi = c_o/(c_e - c_o) \) is the ratio of the reference CO\(_2\) concentration entering the chamber \( (c_e) \) relative to the sample CO\(_2\) concentration exiting the chamber \( (c_o) \), and \( \delta_e \) and \( \delta_o \) are the \( \delta^{13}\text{C} \) of the reference and sample gas, respectively. All variables incorporated in \( \Delta_{\text{obs}} \) and \( \delta^{13}\text{C}_{\text{resp}} \) (below) are derived from TDL measurements of \(^{12}\text{CO}_2\) and \(^{13}\text{CO}_2\). We
calculated $\delta_o$ and $\delta_e$ from the molar abundance of each isotopologue and present them in ratio to the Vienna Pee Dee belemnite (VPDB) standard, that is $\delta = R_s/R_{VPDB} - 1$, where $\delta$ represents either $\delta_o$ or $\delta_e$, and $R_s$ and $R_{VPDB}$ represent the carbon isotope ratio of the sample and VPDB standard, respectively. We calculated mixing ratios of total $[\text{CO}_2]$ and $\delta^{13}\text{C}_{\text{resp}}$ following Barbour et al. (2007),

$$\delta^{13}\text{C}_{\text{resp}} = \frac{\delta_o - \delta_e (1-p)}{p}$$  \hspace{1cm} (2)

where $p$ equals $(c_o - c_e)/c_o$. We calculated $g_i$ using slope-based methods in Evans et al. (1986),

$$g_{is} = \frac{(b - b_s - a_o)}{r_i}$$  \hspace{1cm} (3)

where $b$, $b_s$, and $a_o$ are the isotopic fractionation factors associated with carboxylation (29‰), CO$_2$ entering solution (1.1‰), and diffusion in the aqueous phase (0.7‰), respectively, and $r_i$ is the internal resistance to CO$_2$ diffusion from substomatal cavities to sites of carboxylation. $r_i$ is proportional to the slope of the relationship between $A/p_a$ and $\Delta_i - \Delta_{obs}$ (Evans et al., 1986), where $A$ is photosynthetic rate, $p_a$ is the $p$CO$_2$ in the leaf chamber, and $\Delta_i$ is the predicted discrimination,

$$\Delta_i = a_o \frac{P_a - P_s}{P_a} + a_s \frac{P_s - P_l}{P_a} + b \frac{P_l}{P_a} \hspace{1cm} (4).$$

Variables $a_o$, $p_s$ and $p_l$ represent fractionation associated with diffusion through air (2.9‰), $p$CO$_2$ at the leaf surface, and $p$CO$_2$ in intercellular spaces, respectively. We used positive $r_i$ slopes that were significantly different from zero ($P \leq 0.05$) to calculate $g_i$, excluding any slope that displayed a negative relationship between $A/p_a$ and $\Delta_i - \Delta_{obs}$ because negative slopes produce negative $g_i$ estimates. We estimated $\Delta_{ef}$ from significant ($P \leq 0.05$) $y$-intercepts of the regressions used to calculate $r_i$, following theory developed
in Evans et al. (1986). We used point-based methods of calculating $g_i$ ($g_{ip}$) to determine if $g_i$ varied with $A/p_a$ across range of values we used in $g_{is}$ where $g_{ip}$ is estimated following Evans et al. (1986),

$$g_{ip} = \frac{(b - b_e - a_e)A/p_a}{\Delta - \Delta_{obs} - \Delta_{ef}}$$

and $\Delta_{ef}$ is calculated as:

$$\Delta_{ef} = \frac{e R_d + f \Gamma^*}{k P_a}$$

Variables $e$ and $f$ represent fractionation associated with day respiration (estimated at $-3\%$) and photospiration ($11.6\%$; Lanigan et al., 2008), respectively, and $R_d$, $k$, and $\Gamma^*$ represent day respiration ($\mu$mol m$^{-2}$ s$^{-1}$), carboxylation efficiency ($\mu$mol m$^{-2}$ s$^{-1}$ Pa$^{-1}$), and the photo-compensation point in the absence of day respiration (Pa), respectively.

Variability in $R_d$ is not well understood among species or in response to stressors but has previously been shown to be approximately $0.5R$ (Tcherkez et al., 2005) where $R$ is dark respiration rate; here we estimate $R_d = 0.5 \mu$mol m$^{-2}$ s$^{-1}$ based on oak $R$. We estimated $k$ by calculating a mean $A/p_c$ value from oak ($0.71 \mu$mol m$^{-2}$ s$^{-1}$ Pa$^{-1}$) and poplar ($2.3 \mu$mol m$^{-2}$ s$^{-1}$ Pa$^{-1}$) gas exchange measurements. We calculated $\Gamma^*$ based on $T_L$ (Brooks and Farquhar 1985); $\Gamma^*$ ranged between 3.57 and 4.80 Pa in poplar and between 4.01 and 4.99 Pa in oak leaves.

**Statistical analysis**

We assessed potential error in our calculations of $\Delta_{obs}$ and $\delta^{13}C_{resp}$ using bootstrap methods and in $g_i$ and $\Delta_{ef}$ using regression statistics. We used the standard deviation (SD) of the $[^{12}\text{CO}_2]$ and $[^{13}\text{CO}_2]$ measurements to generate 10000 bootstrap resamples of each
Δ_{obs} and δ^{13}C_{resp} value following methods in Bickford et al. (2009) and used the standard error (SE) of the variation in bootstrap resamples as an estimate of the SE in Δ_{obs} or δ^{13}C. We estimated the uncertainty in g_{is} by transforming the SE associated with r_i to the g_{is} scale (Eqn. 3; Bickford et al., 2009) and the uncertainty in Δ_{ef} using the SE associated with the y-intercept of the regression. Uncertainty in g_{ip} was determined by incorporating Δ_{obs} ± SE for each point and transforming these to the g_{ip} scale (eq. 5). All error propagation was performed in R (R Core Development Team 2008); all other statistical tests were performed in JMP 5.0.1 (SAS Institute Inc., Cary, NC, USA).

Results

Soil water deficit did not significantly reduce g_{is} in droughted poplar (drought g_{is} mean ± SE = 6.62 ± 1.03 µmol m^{-2} s^{-1} Pa^{-1} versus control g_{is} = 7.55 ± 0.84 µmol m^{-2} s^{-1} Pa^{-1}; P = 0.5, t = 0.702) or droughted oak (1.56 ± 0.35 µmol m^{-2} s^{-1} Pa^{-1} versus control = 1.96 ± 0.20 µmol m^{-2} s^{-1} Pa^{-1}; P = 0.35, t = 1.07), nor did it significantly reduce Ψ_w in droughted poplar (drought mean ± SE = −1.35 ± 0.06 MPa versus control = −1.24 ± 0.05 MPa; P = 0.2, t = 1.308) or droughted oak (mean drought Ψ_w = −1.85 ± 0.18 MPa versus control Ψ_w = −1.96 ± 0.16 MPa; P = 0.65, t = −0.47) (Figure 1, Table 1). Slopes used to calculate poplar and oak g_{is} were generally strong (mean R^2 = 0.74; Table 2). SWC was significantly higher in control poplar (48.8 ± 3.0%) compared with droughted poplar (23.7 ± 1.6%; P = 0.0002, t = 6.29, n = 10) and in control oak (75.7 ± 0.86%) compared with droughted oak (22.9 ± 2.32%; P < 0.0001, t = 24.12, n = 12). 33% of droughted poplars experienced shoot dieback as a result of the drought treatment. Leaf temperature was significantly higher in droughted poplar plants compared to control poplar plants
(drought $T_L = 29.01 \pm 0.19^\circ C$ vs. control $T_L = 27.62 \pm 0.13^\circ C$; $P < 0.0001$, $t = -6.11$) but not in droughted oak ($T_L = 29.19 \pm 0.16^\circ C$) compared to control oak plants ($29.18 \pm 0.10^\circ C$; $P = 0.97$).

There were significant negative relationships between $g_{ip}$ and $A/p_a$ in control ($P < 0.0001$, $F = 68.59$, $n = 99$, slope $= -4.23$) and droughted poplar ($P = 0.04$, $F = 4.28$, $n = 77$, slope $= -1.74$) but no significant relationship between $g_{ip}$ and $A/p_a$ in control ($P = 0.11$, $F = 2.63$, $n = 99$) or droughted oak plants ($P = 0.46$, $F = 0.56$, $n = 86$). Consequently, we also present estimates of poplar $g_{ip}$ calculated under saturating PPF conditions ($> 1000 \mu mol \text{ m}^{-2} \text{s}^{-1}$). $g_{ip}$ estimates were higher than $g_{is}$ estimates ($P = 0.001$, paired t-test, $n = 10$) with mean $g_{ip}$ equal to $11.33 \pm 1.2 \mu mol \text{ m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ compared with a mean $g_{is}$ of $7.17 \pm 0.63 \mu mol \text{ m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ (Table 3). In contrast to tests between $g_{is}$ and SWC, there was a significant decrease in $g_{ip}$ among droughted poplar plants (mean $\pm$ SE $= 7.87 \pm 0.97 \mu mol \text{ m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$) compared to controls ($13.44 \pm 1.1 \mu mol \text{ m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$; $P = 0.004$, $t = 3.79$, $n = 12$).

Water deficit reduced $\Delta_{obs}$, $A$ and $g_s$ in droughted poplar ($P < 0.0001$ for all) and droughted oak ($P < 0.05$ for all; Table 1) compared to controls. The relationship between $\Delta_{obs}$ and $VPD$ was negative and linear in control ($P < 0.0001$, $F = 110.37$, $R^2 = 0.50$) and droughted poplar plants ($P < 0.0001$, $F = 92.21$, $R^2 = 0.50$) but was better described by a log transformed second order polynomial when control and drought data were pooled ($P < 0.0001$, $F = 257.97$, $R^2 = 0.74$; Figure 2a). The relationship between $g_s$ and $VPD$ was also negative and linear in control ($P < 0.0001$, $F = 41.77$, $R^2 = 0.28$) and droughted poplar plants ($P < 0.0001$, $F = 66.14$, $R^2 = 0.41$) but when control and drought data were pooled the relationship was better described by a log transformed second order
polynomial ($P < 0.0001$, $F = 292.88$, $R^2 = 0.76$; Figure 2b). A negative linear relationship existed between $\Delta_{\text{obs}}$ and $VPD$ in droughted oak plants ($P = 0.002$, $F = 10.97$, $R^2 = 0.17$), but control oaks exhibited a positive linear relationship between $\Delta_{\text{obs}}$ and $VPD$ ($P < 0.0001$, $F = 19.05$, $R^2 = 0.19$; Figure 2c). Significant negative relationships existed between $g_s$ and $VPD$ in both control ($P = 0.0007$, $F = 12.54$, $R^2 = 0.14$) and droughted oak ($P < 0.0001$, $F = 246.76$, $R^2 = 0.82$; Figure 2d). Mean $p_i/p_a$ was higher in control poplar plants (0.81 ± 0.01) compared with droughted poplar plants (0.61 ± 0.01; $P < 0.0001$, $t = 11.69$; Figure 3a); mean $p_c/p_a$ was also higher in control poplar plants (0.61 ± 0.01) compared with drought plants (0.53 ± 0.02; $P < 0.0001$, $t = 3.98$; Figure 3b). Similarly, mean $p_i/p_a$ was higher in control oak (0.70 ± 0.01) versus droughted oak plants (0.64 ± 0.02; $P = 0.002$, $t = 3.17$; Figure 3c) and $p_c/p_a$ was higher in control (0.52 ± 0.02) versus droughted plants (0.46 ± 0.02; $P = 0.03$, $t = 2.14$; Figure 3d). There were significant linear relationships between $\Delta_{\text{obs}}$ and $p_i/p_a$ in both control and droughted poplar ($P < 0.0001$) and oak ($P < 0.0001$) as well as significant relationships between $\Delta_{\text{obs}}$ and $p_c/p_a$ among all poplar ($P < 0.0001$) and oak plants ($P < 0.0001$; Figure 3). As determined by overlapping 95% confidence intervals, the slopes representing the relationship between $\Delta_{\text{obs}}$ and $p_i/p_a$ and $\Delta_{\text{obs}}$ and $p_c/p_a$ did not differ between control and droughted poplar plants or control and treatment oak plants.

Patterns in $\Delta_{ef}$ differed between poplar and oak. Half of the poplar $\Delta_{ef}$ values were not significantly different from zero ($P > 0.05$, Table 1), but droughted poplar exhibited significantly higher $\Delta_{ef}$ (mean ± SE = 2.85 ± 0.84‰) compared to control poplar plants ($-0.34 ± 0.84‰$; $P = 0.04$, $t = -2.69$, $n = 12$; Table 4). All oak $\Delta_{ef}$ values were significantly less than zero ($P \leq 0.03$; Table 2), but were not significantly different
between control (−4.88 ± 0.75‰) and droughted oak plants (−5.07 ± 1.12‰, P = 0.88, t = 0.167, Table 4). Pooled by species, poplar showed more positive Δ_{ef} (1.26 ± 0.74‰) than oak (−4.96 ± 0.55‰; P < 0.0001, t = −6.72, n = 24).

\[ R \text{ in oak two minutes post-illumination (} R_{2\text{min}} \text{) was not different between control (mean ± SE = 0.43 ± 0.08 µmol m}^{-2}\text{s}^{-1} \text{) and droughted oak (0.37 ± 0.08 µmol m}^{-2}\text{s}^{-1}; P = 0.62, t = 0.51, n = 14), but was higher four minutes post-illumination (} R_{4\text{min}} \text{) in control (1.26 ± 0.07 µmol m}^{-2}\text{s}^{-1} \text{) versus droughted oak (0.97 ± 0.11 µmol m}^{-2}\text{s}^{-1}; P = 0.05, t = 2.21, n = 14; Table 4). \]

This near 3-fold increase in \( R \) between \( R_{2\text{min}} \) and \( R_{4\text{min}} \) was significant (\( P = 0.006, t = −3.21, n = 9 \)). Due to low CO\(_2\) flux during the transition from net \( A \) to stable \( R \) most \( \delta^{13}\text{C}_{\text{resp}} \) measurements collected two minutes post-illumination were associated with high uncertainty (mean = 13.3‰) and are not shown. \( \delta^{13}\text{C}_{\text{resp}} \) measurements collected 4–6 minutes post-illumination showed no significant difference between control (−27.92 ± 2.26‰) and droughted oak plants (−31.61 ± 1.03‰; P = 0.25, t = 1.26, n = 10; Table 4).

**Discussion**

These findings show that soil water deficit does not necessarily reduce \( g_i \) if leaves exhibit isohydric leaf behavior. Both poplar and oak exhibited isohydric regulation and did not show significant differences in \( \Psi_w \) or \( g_i \), based on \( g_{is} \) estimates, between droughted and control plants even though the drought was severe enough to cause large declines in \( A \). This provides initial support for the hypothesis that \( \Psi_w \) has a regulatory role in \( g_i \) that contrasts with most reports showing no linkage between \( \Psi_w \) and \( g_i \). Previous studies providing data on both \( \Psi_w \) and \( g_i \) show reduced \( g_i \) corresponding with reductions in \( \Psi_w \) in
Pseudotsuga (Warren et al., 2004), Beta vulgaris during persistent drought (Monti et al., 2006) and a variety of Mediterranean plants (Galmes, Medrano & Flexas 2007). Among three isohydric species examined in Galmes et al. (2007) one (Diplotaxis ibicensis) showed substantial declines in $g_i$ while two Limonium species exhibited modest decreases in $g_i$ in response to moderate-to-severe water stress. In Beta vulgaris, however, transient drought did not reduce $\Psi_w$, suggesting isohydric tendencies, and no significant reductions in $g_i$ were observed compared to controls (Monti et al., 2006). The variation in B. vulgaris responses to drought duration and the discrepancies between our results and those observed in D. ibicensis demonstrate a need for further investigation of the $g_i$ response to drought among other isohydric species.

The current consensus posits aquaporin activity as the primary regulator of $g_i$ by facilitating CO$_2$ transport across cell membranes, as shown in Nicotiana tabacum (Flexas et al., 2006b, Uehlein et al., 2008), but the relationship between aquaporin activity and $\Psi_w$ remains poorly understood. One recent study found linkages between PIP2 plasma membrane (PM) aquaporin gating patterns and leaf water status that directly affected $g_i$ in Nicotiana by reducing CO$_2$ diffusion during drought (Miyazawa et al., 2008). Existing studies suggesting some linkage between $\Psi_w$ and $g_i$ include a report of PIP2 PM protein phosphorylation being partially dependent on apoplastic water potential (Johannson et al., 1996) and recent work proposing a role for the tonoplast aquaporin SITIP2;2 in regulating isohydric and anisohydric behavior (Sade et al., 2009). Further study of the interaction between aquaporin activity and $\Psi_w$, specifically for those proteins shown to facilitate CO$_2$ transfer, are needed.
Drought did significantly reduce other leaf gas exchange characteristics, confirming previous studies. As expected, SWD reduced Δ$_{\text{obs}}$, $g_s$ and $A$ in both poplar and oak (Lawlor and Cornic 2002, Flexas et al., 2006, Monti et al., 2006). We also examined the relationship between Δ$_{\text{obs}}$ and atmospheric water deficits, or VPD, and found that both drought and control poplar, as well as droughted oak, exhibited the expected negative relationship between these two parameters but that control oak plants showed a weakly positive relationship between Δ$_{\text{obs}}$ and VPD (Figure 2c). VPD during oak control measurements was generally < 2 kPa, low enough to facilitate moderate $g_s$ in this semiarid adapted species, and thus not a large constraint on $A$ and Δ$_{\text{obs}}$ across the VPD range we observed. The curvilinear relationships between VPD and both Δ$_{\text{obs}}$ and $g_s$ in drought and control plants demonstrates the strong regulatory importance of $g_s$ on poplar Δ$_{\text{obs}}$ at higher VPD (> 1.0 kPa; Figure 2). In contrast, oak plants exhibited relatively weak relationships between VPD and Δ$_{\text{obs}}$ and $g_s$, possibly due to the small range of $g_s$ we observed in both control and droughted plants. Both $p_i/p_a$ and $p_c/p_a$ were higher among control poplar and oak compared to drought plants, but the slopes describing their relationships with Δ$_{\text{obs}}$ were similar across species. Our $p_i/p_a$ and $p_c/p_a$ estimates were mostly higher, but still comparable, to those observed in other Quercus and Populus species (Roupsard et al., 1996). Among droughted plants most lower $p_i/p_a$ and $p_c/p_a$ values could be attributed to lower $g_s$, and not necessarily lower $g_i$. The relationship between Δ$_{\text{obs}}$ and $p_c/p_a$ among control and droughted poplar plants, however, extends across a similar $p_c/p_a$ range and show lower Δ$_{\text{obs}}$ in droughted plants when $p_c/p_a < 0.65$. This could be due to positive Δ$_{ef}$ influencing discrimination or it could be a biologically
significant reduction in $g_i$ among droughted poplar plants that was not captured in our statistical tests, a finding which agrees with our poplar $g_{ip}$ estimates.

It is possible that our $g_i$ estimates do not accurately reflect the internal conductance of CO$_2$ in poplar leaves. Our $g_{is}$ estimates depended on variation in $A$, which we manipulated using variable PPF. Recent evidence suggests $g_i$ can vary rapidly in response to changes in PPF and other environmental variables (Flexas et al., 2007) and this may have confounded our $g_{is}$ results. The significant variation in poplar $g_{ip}$ that occurred over the range of $A/p_a$ we used in this study lends support to this conclusion. The estimates we calculated using $g_{ip}$, however, were much higher than $g_i$ values reported in the literature for other woody deciduous angiosperms (Flexas et al., 2008) and should be interpreted conservatively. Alternatively, the $g_{ip}$ estimates may have accurately reflected differences in poplar $g_i$ between droughted and control plants but overestimated the actual internal conductance of CO$_2$.

Decarboxylation activity differed between poplar and oak, and among oak treatments. Overall $\Delta_{ef}$ was lower in oak compared with poplar, for reasons that were not made clear by our data. $\Delta_{ef}$ was similar among oak plants, being $\sim -5\%$ in both droughted and control plants, but was different between droughted and control poplar. Among droughted poplar most $\Delta_{ef}$ values were positive, and may have had a negative forcing effect that resulted in a lower net $\Delta_{obs}$ compared with control plants. Using mechanistic models as a framework for conceptualizing the interactions occurring during diffusion, carboxylation, and decarboxylation that influence fractionation, $\Delta_{ef}$ (eq. 6) is subtracted from the sum of fractionations due to diffusion and carboxylation processes (Farquhar et al., 1989), and thus positive $\Delta_{ef}$ could result in more negative $\Delta_{obs}$. Two high
Δef values stood out (Table 2), and made differences between poplar treatments significant. These high measurements were collected from severely drought stressed plants, and this stress level may have resulted in stomatal patchiness that could have adversely affected our p1 estimates (Farquhar 1989), and thus impacted Δi calculations used to estimate Δef. The low Δef among the oak plants highlights two points. Quantitatively, these low values show that Δi, the simplified predictive model of discrimination, largely under-predicted Δobs. Functionally, this suggests accounting for Rd and photorespiration, as well as their associated fractionation factors, may be important in oak to fully describe leaf isotopic exchange, as observed in juniper (Bickford et al., 2009). In oak, Rd showed evidence of up-regulation of dark respiration activity by exhibiting a 3-fold increase in Rd in the minutes following illumination. There was lower Rd and, unexpectedly, lower δ13Cresp among droughted oak. Lower Rd during short-term water deficit has been observed previously (Atkin et al., 2005), but lower δ13Cresp is typically associated with well-watered conditions (McDowell et al., 2004). It is possible that supplemental watering prior to day 2 oak measurements briefly increased gas exchange activity, resulting in assimilate being formed that was isotopically similar to control plants and that was subsequently decarboxylated during measurements.

Conclusions
This study provides a new view on the correlation between leaf water relations and gi, and supports the existence of a linkage between Ψw and CO2 conductance to sites of carboxylation. Because they display static Ψw in response to soil water deficit, isohydric plants provide a unique platform to separate the effects of SWD and leaf water potential.
In contrast to numerous studies showing reduced \( g_i \) in response to drought, our study found, based on slope-based estimates, no significant reduction in \( g_i \) during SWD in these isohydric species. Functionally, however, either the \( g_i \) we observed in droughted poplar did affect \( p_c/p_a \) differently than control plants, or \( \Delta_{ef} \) exerted stronger influence on \( \Delta_{obs} \) at lower \( p_c/p_a \). Given the minor discrepancies between our data and the few existing data sets exploring \( g_i \) in other isohydric species it is important to document \( g_i \) in other plants with similar leaf hydraulic behavior to see whether this is a widespread phenomenon. The recent work linking \( g_i \) and aquaporin activity seems a promising avenue to further investigate linkages with leaf water potential, and such study should aid our understanding of \( g_i \) in both isohydric and anisohydric plants.

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References


**Flexas J, Ribas-Carbo M, Hanson DT, Bota J, Otto B, Cifre J, McDowell N, Medrano H, Kaldenhoff R. 2006.** Tobacco aquaporin NtAQP1 is involved in mesophyll


Tables

Table 1. Summary of results for internal conductance of CO₂ ($g_i$), leaf water potential ($\Psi_w$), soil water content (SWC), observed carbon discrimination ($\Delta_{obs}$), net assimilation rate ($A$) and stomatal conductance to H₂O ($g_s$) in poplar and oak plants. Values are represented as means ± one SE.

<table>
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<th>$g_i$ (µmol m⁻² s⁻¹ Pa⁻¹)</th>
<th>$\Psi_w$ (MPa)</th>
<th>SWC (%)</th>
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$P < 0.0001$
Table 2. Summary of slope and intercept statistics from slope-based estimates ($g_{is}$) of internal conductance to CO$_2$ where T and C represent droughted and control poplar plants, respectively, and D and W represent droughted and control oak plants, respectively. $\Delta_{ef}$ represents the estimate of the total fractionation attributed to both respiratory and photorespiratory activity (‰). SE represents one standard error.

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<td>0.68</td>
<td>&lt; 0.0001</td>
<td>0.85</td>
</tr>
<tr>
<td>W7</td>
<td>10.97</td>
<td>2.52</td>
<td>0.003</td>
<td>-3.32</td>
<td>1.24</td>
<td>0.03</td>
<td>0.70</td>
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</table>
Table 3. Estimates of internal conductance of CO$_2$ in poplar calculated using a slope-based method ($g_{is}$) and a point-based method ($g_{ip}$) where C and T represent control and droughted poplar plants, respectively. $g_{ip}$ estimates were significantly higher than $g_{is}$ estimates ($P = 0.001$). There was a significant difference between droughted and control poplar plants using $g_{ip}$ ($P = 0.004$, $n = 12$) but not when using $g_{is}$ ($P = 0.50$).

<table>
<thead>
<tr>
<th></th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>$g_{is}$ (µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$)</td>
<td>7.13 ± 0.85</td>
<td>7.19 ± 0.38</td>
<td>6.19 ± 0.39</td>
<td>6.30 ± 0.55</td>
<td>11.63 ± 1.49</td>
</tr>
<tr>
<td></td>
<td>$g_{ip}$ (µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$)</td>
<td>16.25 ± 1.72</td>
<td>12.67 ± 0.38</td>
<td>11.53 ± 0.39</td>
<td>10.72 ± 2.03</td>
<td>17.34 ± 0.40</td>
</tr>
<tr>
<td>T</td>
<td>$g_{is}$ (µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$)</td>
<td>9.64 ± 1.37</td>
<td>5.37 ± 1.67</td>
<td>5.19 ± 1.27</td>
<td>6.27 ± 1.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$g_{ip}$ (µmol m$^{-2}$ s$^{-1}$ Pa$^{-1}$)</td>
<td>12.11 ± 0.79</td>
<td>8.85 ± 1.54</td>
<td>6.36 ± 0.57</td>
<td>5.34 ± 0.23</td>
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</table>
Table 4. Summary of the mean fractionation attributed to all decarboxylation activity ($\Delta_{ef}$), dark respiration rate two minutes ($R_{2\text{min}}$) and four minutes post-illumination ($R_{4\text{min}}$), and the $^{13}\text{C}/^{12}\text{C}$ ratio of dark-respired CO$_2$ ($\delta^{13}\text{C}_{\text{resp}}$) four minutes post-illumination. Values are represented as means ± one SE.

<table>
<thead>
<tr>
<th>Oak</th>
<th>$\Delta_{ef}$ (%)</th>
<th>$P$</th>
<th>$R_{2\text{min}}$</th>
<th>$P$</th>
<th>$R_{4\text{min}}$</th>
<th>$P$</th>
<th>$\delta^{13}\text{C}_{\text{resp}}$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>-4.88 ± 0.75</td>
<td>0.88</td>
<td>0.43 ± 0.08</td>
<td>0.62</td>
<td>1.26 ± 0.07</td>
<td>0.05</td>
<td>-27.92 ± 2.26</td>
<td>0.25</td>
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<tr>
<td>drought</td>
<td>-5.08 ± 0.89</td>
<td>0.37</td>
<td>0.37 ± 0.08</td>
<td>0.97</td>
<td>0.97 ± 0.11</td>
<td>0.05</td>
<td>-31.61 ± 1.03</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Figure captions

**Figure 1.** The relationship between internal conductance of CO$_2$ ($g_i$) and leaf water potential ($\Psi_w$; Panel a) and soil water content (SWC; Panel b) in poplar and oak plants. $g_i$ was not different between droughted and control poplar ($P = 0.5$) or oak plants ($P = 0.35$), nor was $\Psi_w$ different between droughted and control poplar ($P = 0.2$) or oak plants ($P = 0.65$). This demonstrates that soil water deficit does not necessarily reduce $g_i$ in isohydric plants like poplar and oak.

**Figure 2.** The relationship between vapor pressure deficit ($VPD$) and observed carbon discrimination ($\Delta_{obs}$) and stomatal conductance to H$_2$O ($g_s$). Panels a and b represent droughted (□) and control (■) poplar plants; panels c and d represent droughted (▲) and control (▲) oak plants. Error bars in all panels represent one SE. Linear and negative relationships between $VPD$ and both $\Delta_{obs}$ and $g_s$ were significant among both droughted and control poplar plants ($P < 0.0001$). In oak, however, linear negative relationships existed between $VPD$ and $g_s$ among treatments ($P \leq 0.0007$) and between $VPD$ and $\Delta_{obs}$ and droughted oak ($P = 0.002$), but a positive linear was found among control oak plants ($P < 0.0001$).

**Figure 3.** The relationship between observed carbon discrimination ($\Delta_{obs}$) and the ratio of intercellular to atmospheric CO$_2$ partial pressure ($p_i/p_a$), and the relationship between $\Delta_{obs}$ and the ratio of CO$_2$ at the site of carboxylation to atmospheric CO$_2$ partial pressure ($p_c/p_a$). Panels a and b represent droughted (○) and control (●) poplar plants; panels c and d represent droughted (■) and control (■) oak plants. Error bars in all panels represent one SE. Significant linear relationships existed between $\Delta_{obs}$ and both $p_i/p_a$ and $p_c/p_a$ ($P < 0.0001$).
but slopes representing these relationships in drought and control treatments were not different (P > 0.05) in either poplar or oak plants.
Figure 1.
Figure 2.
Figure 3.
Chapter 5

Conclusions

The aims of this study were to utilize a novel gas exchange system to explore leaf isotopic exchange at high frequency under field conditions, assess the importance internal CO$_2$ conductance ($g_i$) in predicting carbon isotope discrimination ($\Delta$), and explore linkages between leaf water potential and $g_i$. Previous methods for measuring instantaneous $\Delta$ in the field were cumbersome and expensive, involving flask collection of gases and complex distillation processes followed by expensive analysis using mass spectrometry. These burdens limited our understanding of the dynamic nature of $\Delta$ in response to diurnal environmental shifts. In chapter two I detailed the first steps towards measuring $\Delta$ in the field at high frequency, allowing us to better understand the relationship between environmental drivers such as light, vapor pressure, and water availability impact $\Delta$ process in a natural setting. Beyond these observations, however, in chapters three and four I put forward an assessment of the importance of $g_i$ in predicting $\Delta$ using existing models. I found that, in juniper, accounting for $g_i$ was important to improve our predictions of $\Delta$ compared with simpler models that omit $g_i$ and other variables. This needs to be assessed in other species, and if verified as an important component then further steps should be taken to account for $g_i$ in large scale models of $\Delta$, possibly by utilizing mean $g_i$ based on vegetation type. The current understanding of environmental regulation of $g_i$ is limited by a lacking mechanistic understanding of the aquaporin regulation underlying the passage of CO$_2$ across cell walls. Linkages between $\Psi_w$ and $g_i$ discussed in chapter 4 may provide insight into another aspect of coordinated
regulation of water and carbon relations and hopefully will provoke further study on the topic.

Field observations of $\Delta$ provided an opportunity to validate the most widely used models of leaf discrimination under both steady state and non-steady state conditions. As discussed in chapters 2 and 3, the widely used simple model of $\Delta$ performs well given its few components, however, with the increased use of isotopes to enhance understanding of ecosystem processes the discrepancies between measured and modeled $\Delta$ have substantial implications for prediction and interpretation and need to be recognized and improved upon. The variability in several of the components of the comprehensive model of $\Delta$ allow for just such improvements as we gain better understanding of large drivers like $g_i$, $b$, and decarboxylation components.

This research presented in chapter 4 provides yet another linkage in the intricate balance between carbon and water relations in leaves. We now have evidence that leaf water potential may be related to $g_i$, though the underlying mechanism is poorly understood. This phenomena needs to be explored in other isohydric species to determine if this is restricted to a limited group of plants, and if this contributes to the variability in $g_i$ among anisohydric plants. In particular, does the water potential in cell walls or other cell subunits have regulatory influence over aquaporin activity? Future studies will be necessary to address this question.