Uncertainty in measurements of the photorespiratory CO2 compensation point and its impact on models of leaf photosynthesis

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Abstract Rates of carbon dioxide assimilation through photosynthesis are readily modeled using the Farquhar, von Caemmerer, and Berry (FvCB) model based on the biochemistry of the initial Rubisco-catalyzed reaction of net C3 photosynthesis. As models of CO2 assimilation rate are used more broadly for simulating photosynthesis among species and across scales, it is increasingly important that their temperature dependencies are accurately parameterized. A vital component of the FvCB model, the photorespiratory CO2 compensation point ($\Gamma^*$), combines the biochemistry of Rubisco with the stoichiometry of photorespiratory release of CO2. This report details a comparison of the temperature response of $\Gamma^*$ measured using different techniques in three important model and crop species (Nicotiana tabacum, Triticum aestivum, and Glycine max). We determined that the different $\Gamma^*$ determination methods produce different temperature responses in the same species that are large enough to impact higher-scale leaf models of CO2 assimilation rate. These differences are largest in N. tabacum and could be the result of temperature-dependent increases in the amount of CO2 lost from photorespiration per Rubisco oxygenation reaction.

Keywords Rubisco · Photorespiration · Temperature response · Modeling photosynthesis

Introduction

Biochemical models of leaf photosynthesis are increasingly important as we develop more sophisticated simulations of plant carbon budgets and search for new strategies to improve crop productivity (Zhu et al. 2008; Dufresne et al. 2013; Long et al. 2015; Kromdijk and Long 2016). The widely adopted biochemical model of leaf photosynthesis of Farquhar, von Caemmerer, and Berry (FvCB) has proven invaluable since its development over 35 years ago and continues to be employed to represent photosynthesis from the cell to global scale (Farquhar et al. 1980; von Caemmerer and Farquhar 1981; von Caemmerer 2000). This model is characterized by its elegant combination of Rubisco kinetics with the physiology of photosynthesis and photorespiration to simulate net CO2 assimilation rate in response to CO2 partial pressures, making it useful both for predicting rates of carbon uptake as well as probing plant physiology and metabolism.

The photorespiratory CO2 compensation point ($\Gamma^*$) is a critical parameter of the FvCB model. $\Gamma^*$ integrates Rubisco specificity for reaction with CO2 relative to O2.
(\(S_{\text{CO}}\)) with the stoichiometry of CO\(_2\) release per Rubisco oxygenation (\(\alpha\)) to quantify photorespiratory CO\(_2\) loss to net CO\(_2\) assimilation rate. \(\Gamma_*\) is measured in three main ways, which can be understood in light of the following equation:

\[
\Gamma_* = \frac{aO}{S_{\text{CO}}} = \frac{aC_o}{v_c},
\]

where \(O\), \(C_o\), \(v_o\), and \(v_c\) represent the oxygen partial pressure, chloroplastic CO\(_2\) partial pressure, rate of Rubisco oxygenation, and the rate of Rubisco carboxylation, respectively (Ruuska et al. 2000; von Caemmerer 2000; Walker and Cousins 2013). \(\Gamma_*\) has been measured in vivo using the common intersection method by measuring CO\(_2\) exchange under various CO\(_2\) partial pressures and irradiances and requires no assumed \(\alpha\) value (Laish 1977; Brooks and Farquhar 1985). \(\Gamma_*\) can also be calculated from in vitro determinations of \(S_{\text{CO}}\) values as a function of O\(_2\) partial pressure, assuming that \(\alpha\) equals 0.5 as predicted from the commonly accepted biochemical photorespiration (von Caemmerer 2000; Hermida-Carrera et al. 2016). \(\Gamma_*\) can also be determined from net oxygen fluxes in and out of the leaf using online mass spectroscopy (Badger; Ruuska et al. 2000; Walker and Cousins 2013). The oxygen exchange method determined by CO\(_2\) exchange involves the additional calculation of converting the CO\(_2\) photocompensation point to chloroplastic partial pressures using values of \(g_m\) to chloroplastic partial pressures using values of day respiration (\(R_d\)) and mesophyll conductance (\(g_m\); see

Differences among methods could indicate errors of some underlying physiological assumptions of the techniques. For example, it has been shown that \(\Gamma_*\) determined by CO\(_2\) exchange increases more with temperature than \(\Gamma_*\) determined by O\(_2\) exchange in Arabidopsis thaliana (Walker and Cousins 2013). Similar differences were observed between \(\Gamma_*\) determined in more extensive temperature response measurements in Nicotiana tabacum (Bernacchi et al. 2001, 2002, Fig. 1). Walker and Cousins (2013) suggested that the increased temperature response of \(\Gamma_*\) determined by CO\(_2\) exchange could be the result of an increase in \(\alpha\) with temperature, but this hypotheses could not be confirmed due to other possible explanations inherent to determining O\(_2\) exchange using online mass spectroscopy (Walker and Cousins 2013). Like the O\(_2\) exchange method, \(\Gamma_*\) determined from in vitro \(S_{\text{CO}}\) is also sensitive to the assumptions of \(\alpha\), so the secondary goal of this report was to observe if there were differences between \(\Gamma_*\) determined by CO\(_2\) exchange and in vitro \(S_{\text{CO}}\) consistent with an increase in \(\alpha\). Measuring \(\Gamma_*\) from CO\(_2\) exchange involves the additional complication of converting the CO\(_2\) photocompensation point as measured from the intercellular CO\(_2\) partial pressure (\(C_{i*}\)) to chloroplastic partial pressures using values of day respiration (\(R_d\)) and mesophyll conductance (\(g_m\); see

![Fig 1](https://example.com/fig1.png)

**Fig. 1** Comparison of the temperature response of the photorespiratory CO\(_2\) compensation point (\(\Gamma_*\)) measured from CO\(_2\) gas exchange using the common intersection method (closed circles and Bernacchi et al. 2001) and from O\(_2\) gas exchange (open circles and Bernacchi et al. 2002).
“Materials and methods” section). This conversion relies on the assumed values of \( g_m \) and could also play a role in explaining the differences in \( \Gamma_* \) as determined using various techniques.

In this report, the temperature response of \( \Gamma_* \) was measured using CO₂ exchange and in vitro \( S_{CO} \) in a C₃ model species (\( N. tabacum \)) and two major C₃ crop species (\( Triticum aestivum \) and \( Glycine max \)) to understand how comparable these methods are for use in simulating carbon assimilation at the leaf and canopy scale. This report demonstrates that there are differences between \( \Gamma_* \) determined by CO₂ exchange and in vitro \( S_{CO} \) that increase with temperature. These differences are most evident in \( N. tabacum \) and clearly present to a lesser extent in \( T. aestivum \) and \( G. max \). The differences in the \( \Gamma_* \) temperature response, particularly for \( N. tabacum \), are large enough to impact the output of leaf and canopy models of carbon assimilation parameterized with field data. Furthermore, differences in the \( \Gamma_* \) temperature response determined using CO₂ exchange, in vitro \( S_{CO} \), and O₂ exchange in \( N. tabacum \) are consistent with an increase in \( \alpha \) with temperature. These findings have important implications to how the FvCB model is parameterized and raise questions concerning one of its underlying assumptions that the stoichiometry of CO₂ release per Rubisco oxygenation (\( \alpha \)) is always 0.5.

**Results**

The common intersection measurements used to derive the slope–intercept regression values of \( C_{is} \) and \( R_d \) produced consistent intersection points for a given temperature and species and were highly reproducible (Supplemental 1a–c). The different light intensities where the CO₂ response of assimilation (\( A–C_i \)) was measured produced an even distribution of slopes and intercepts for each temperature and species, with the exception of 15 and 20°C in \( G. Max \) (Supplemental 1b). Additionally, due to the low values of CO₂ partial pressures used during the measurement, the linear regressions of each \( A–C_i \) curve used \( A–C_i \) data taken exclusively from the most linear region of the \( A–C_i \) curve. The common intersection point of these linear regressions showed typical variations for each temperature and species, but there was no consistent trend in how well the lines intersected as a function of temperature. When the slopes and the y-intercepts of these individual lines were used to determine \( C_{is} \) using slope–intercept regression (Walker and Ort 2015; Walker et al. 2016a), there was no clear pattern in the residuals of the slope values between the linear regression and the measured values (Supplemental 2). This lack of pattern in the residual plots indicates that the slope–intercept regression was not measurably non-linear, indicating that a single \( g_m \) term is adequate to describe CO₂ transfer to and from the chloroplast (Tholen and Zhu 2011; Tholen et al. 2012; Walker and Ort 2015; Walker et al. 2016a).

The temperature response of \( \Gamma_* \) was steeper when measured using CO₂ exchange as compared to that calculated using Rubisco specificity in \( N. tabacum, T. aestivum, \) and \( G. max \) (Fig. 2). The differences were most pronounced in \( N. tabacum \) as compared to \( T. aestivum \)

![Fig. 2 Temperature response of the photorespiratory CO₂ compensation point (\( \Gamma_* \)) measured from CO₂ gas exchange using the common intersection method (\( \text{solid triangle} \)), calculated from Rubisco specificity values measured using the O₂ oxygen electrode method (\( \text{solid circles} \)) and from O₂ exchange (\( \text{open circles} \)) assuming CO₂ release per oxygenation =0.5. Shown are the results from \( N. tabacum \) (a), \( T. aestivum \) (b), and \( G. max \) (c). Bars represent the means of \( n=5–7 \) for the CO₂ gas exchange data and \( n=5–16 \) for the in vitro assays ± SE.](image)
and *G. max* with the greatest differences being observed at 35 °C, the highest temperature measured. There was a close agreement between the temperature response of $\Gamma_*$ calculated from Rubisco specificity and measurements from O$_2$ exchange in *N. tabacum* (Bernacchi et al. 2002, Fig. 2a).

A sensitivity analysis was performed to determine if the differences in $\Gamma_*$ measured using CO$_2$ exchange as compared to $\Gamma_*$ measured from Rubisco specificity could be explained by errors in the values of $R_d$ and $g_m$ used to convert $C_{i,i}$ to $\Gamma_*$ (see “Materials and methods” section). This sensitivity analysis revealed that in *N. tabacum*, *G. max*, and *T. aestivum*, the values of $g_m$ or $R_d$ would have to be negative to explain the differences between $\Gamma_*$ measured using CO$_2$ exchange and $\Gamma_*$ measured from Rubisco specificity at all temperatures, i.e., 25°C and above (Table 2). Furthermore, $g_m$ or $R_d$ would have to be negative or reduced by an order of magnitude to explain the differences in $\Gamma_*$ measured using the two techniques at temperatures below 25°C. Since the negative values of $g_m$ and $R_d$ are not possible, it follows that the temperature-dependent differences in $\Gamma_*$ measured using the two techniques cannot be explained by incorrect assumptions of $g_m$ or measurements of $R_d$. Thus, the differences in the values of $\Gamma_*$ measured from CO$_2$ exchange vs. in vitro Rubisco specificity are both much too large and in the wrong direction to be explained by errors in $g_m$ or $R_d$.

Alternatively, increases in $\alpha$ with increasing temperature could explain the differences between $\Gamma_*$ observed when measured using CO$_2$ exchange, Rubisco specificity, or O$_2$ exchange (Fig. 3). The required increase in $\alpha$ necessary to explain the difference was largest in *N. tabacum* and consistent when calculated using the values from Rubisco specificity or O$_2$ exchange. An increase in $\alpha$ of 54% between 15 and 35°C would be required to explain the difference in $\Gamma_*$ derived by the different determination techniques. Putative increases in $\alpha$ were less pronounced, but still large, in *T. aestivum* using the values from Rubisco specificity amounting to a 30% increase in $\alpha$ between 15 and 35°C; however, some of that increase was observed only at 35°C. When the 35°C value was removed, the differences in *T. aestivum* and *G. max* were explained by a 22 and 30% increase between 15 and 30°C, respectively. We next explored how these different $\Gamma_*$ values from the different determination techniques impact higher-scale models of leaf photosynthesis using the values from *N. tabacum*, since these *N. tabacum* parameters are most commonly used to parameterize the FvCB model.

Differences in modeled CO$_2$ response curves at the leaf level reflected the difference in $\Gamma_*$ values (Fig. 4). The modeled gas exchange using $\Gamma_*$ values measured from CO$_2$ exchange were lower than those measured from Rubisco specificity or using O$_2$ exchange at 25 and 35°C by 5 to >40%. The difference increased substantially at 35 °C. The modeled differences were largest at lower CO$_2$ partial pressures, where Rubisco kinetics most limit photosynthesis and the model is most sensitive to differences in Rubisco kinetics. The rapid increase in the percent differences at 25 and 35°C occur during the transition between Rubisco and RuBP regeneration-limited photosynthesis.

To understand how using these different temperature responses of $\Gamma_*$ would impact the larger-scale models
parameterized with field conditions, we next incorporated each method’s temperature response into a well-validated multilayer canopy model of soybean (MLCan, Drewry et al. 2010a, b, Fig. 5). Since the impact of different $\Gamma_*$ functions are influenced by temperature and CO$_2$ concentrations, we ran the model using field data modified according to the current and future climate predictions from the IPCC (Table 1) to produce a realistic range of the present and future conditions. Consistent with the CO$_2$ response curve modeling, simulations using $\Gamma_*$ from Rubisco specificity and O$_2$ exchange simulate higher net assimilation rates under all conditions. Under the current and RCP 2.6 conditions, simulations using $\Gamma_*$ from Rubisco specificity and O$_2$ exchange were 9% greater. Under RCP 8.5 the differences were 7% greater.

Discussion

The differences among $\Gamma_*$ values measured using the different methods revealed an apparent inconsistent temperature response in a critical parameter of photosynthesis that impacts leaf- and canopy-scale simulations of carbon assimilation. Measurements of $\Gamma_*$ derived from CO$_2$ gas exchange were the most sensitive to physiological temperature ranges (Fig. 2), and these differences were large enough to result in lower simulated photosynthetic rates as compared to the $\Gamma_*$ values determined from Rubisco specificity or O$_2$ exchange. Simulated photosynthesis was especially lower in leaf-level simulations using CO$_2$ exchange-based $\Gamma_*$ values under decreased CO$_2$ partial pressures at 35 °C (Fig. 4). The differences among methods resulted in more modest differences in photosynthesis when simulated

![Fig. 4 Simulated impact of different assumptions of the photorespiratory CO$_2$ compensation point ($\Gamma_*$) on the net CO$_2$ assimilation rate at 25 °C (a, c) and 35 °C (b, d). Lines were modeled using the standard biochemical FvCB model of leaf photosynthesis, the temperature response of Rubisco kinetics, the maximum rate of electron transport determined in Bernacchi et al. (2001, 2002), and $\Gamma_*$ assuming the temperature response measured in this study from CO$_2$ exchange using the common intersection method (solid lines) and from in vitro Rubisco specificity measured using the O$_2$ electrode method (dashed lines). Shown are the percent differences between net CO$_2$ assimilation rate simulated using $\Gamma_*$ measured from CO$_2$ exchange and in vitro Rubisco specificity measured using the O$_2$ electrode method (dashed lines, c, d).](https://example.com/fig4.jpg)
under field conditions at the current and future predictions of climate (Fig. 5). Since intercellular CO₂ partial pressure is reduced following stomatal closure, the differences in simulated photosynthesis would be greater under stress conditions including drought (Farquhar and Sharkey 1982). These simulations illustrate the sensitivity of the model to parameters including drought (Farquhar and Sharkey 1982). An increase in α could arise through non-enzymatic decarboxylation reactions in the peroxisome of photorespiratory intermediates such as glyoxylate and/or hydroxypyruvate previously suggested to explain all of the photorespiratory CO₂ loss (Zelitch 1972; Halliwell and Butt 1974; Grodzinski 1978, 1979). This theory was later discounted by numerous lines of genetic and physiological evidence, but only at optimal temperatures (Ogren 1984). Alternatively, excess CO₂ could be released enzymatically, for example during the generation of carbon skeletons through starch degradation in a proposed glucose 6-phosphate shunt around the Calvin–Benson cycle or an as yet undescribed reaction(s) (Sharkey and Weise 2016).

A recent series of isotopic labeling and fluxomic experiments on detached leaves support a stoichiometry of 0.5 in Helianthus annuus L. at 21 °C (Abadie et al. 2016), but this value has not yet been confirmed under elevated temperatures or in additional species. Interestingly, the trend in the calculated increases in α were not as pronounced in T. aestivum or G. max (Fig. 3b, c), indicating a potential improvement in photorespiratory efficiency with temperatures above 35 °C (Bernacchi et al. 2002; Evans et al. 1992; Tazoe et al. 2011). While many of these methods have been used to measure the temperature response of N. tabacum with similar results, they do show variation, especially at temperatures above 35 °C (Bernacchi et al. 2002; Evans and von Caemmerer 2012; Walker et al. 2013). Given this uncertainty, is it possible that the differences among $\Gamma_*$, measuring techniques result from erroneous assumptions of $g_m$?

It does not seem probable that errors in the assumptions of $g_m$, or $R_d$ for that matter, can explain the differences in $\Gamma_*$ for several reasons. First, $\Gamma_*$ values from CO₂ exchange were higher than those calculated from Rubisco specificity as the temperature increased (Fig. 2), even though $\Gamma_*$ decreases with the inclusion of $g_m$ in the calculation (Eq. 2). This means that $g_m$ or $R_d$ would need to decrease with temperature to explain the direction of the differences among $\Gamma_*$ measurements, which has not been observed in

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**Table 1** Current and future representative concentration pathways (RCP) of mean global CO₂ and temperatures according to the 2014 IPCC report

| Scenario       | Ambient CO₂ (ppm) | Temp. increase (°C) |
|----------------|-------------------|---------------------|
| Current        | 400               | 0.0                 |
| 100 years RCP 2.6 | 450       | 1.0                 |
| 100 years RCP 8.5 | 1000    | 3.7                 |

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**Fig. 5** Simulated impact of using different photorespiratory CO₂ compensation point ($\Gamma_*$) temperature response functions on canopy-level photosynthesis. A multilayer root–canopy model was parameterized with field data from 2002–2005 Bondville, Illinois AmeriFlux eddy covariance experiment assuming the current atmospheric CO₂ and temperature (400 PPM, no change to air temperature as measured in Bondville), IPCC scenario RCP 2.6 (450 PPM, +1 °C), and IPCC scenario RCP 8.5 (1000 PPM, +3.7 °C). Shown are the total simulated net moles of CO₂ fixed during the three modeled growing seasons.
any reported temperature responses (Bernacchi et al. 2002; Warren and Dreyer 2006; Walker et al. 2013; von Caemmerer and Evans 2014). Furthermore, even if \( g_m \) were assumed to be a negligible value, \( \Gamma^* \) in \( N. \) tabacum at 35 °C would only be reduced from 7.8 to 7.6 Pa, which is insufficient to approach the value of 5.2 Pa determined from Rubisco specificity. This point is further illustrated in the sensitivity analysis of \( \Gamma^* \) calculations, where the impossibility of negative values of \( g_m \) or \( R_d \) are required to explain the differences between \( \Gamma^* \) measured from \( CO_2 \) exchange and in vitro Rubisco specificity (Table 2). Together, these observations and calculations indicate that the differences between methods of measuring \( \Gamma^* \) are not the result of incorrect assumptions or measurements of \( R_d \) and/or \( g_m \).

Recent work concerning the validity of assumptions necessary for \( \Gamma^* \) measurements using the common intersection approach raise important considerations to ensure accurate determinations of \( \Gamma^* \). One such concern is the appropriateness of using linear fits to determine the intersection point of non-linear \( A-C_1 \) curves, which, according to simulations, results in underestimates of \( \Gamma^* \) and, by extension, \( \Gamma^* \) (Gu and Sun 2014). To prevent assumptions of linearity from biasing our common intercept determinations, we used measurement \( CO_2 \) partial pressures below 10 Pa \( CO_2 \) that further simulations demonstrate result in <1% underestimation of \( C_{i*} \) (Walker and Ort 2015). Additionally, the common intersection measurements did not show the “staggered” interceptions expected if their determination was biased by improper assumptions of linearity (Supplemental 1a–c). Finally, even if improper assumptions of linearity resulted in underestimates of \( C_{i*} \), measured in the temperature response of \( N. \) tabacum, \( G. \) max, and \( T. \) aestivum, this would only serve to increase the differences between the \( \Gamma^* \) values measured using the common intersection method and those from in vitro Rubisco specificity.

It has also been suggested that the current understanding of \( g_m \) needs to be revised, since it is commonly assumed that all \( CO_2 \) released from the mitochondria passes through the chloroplast, and multiple conductances of \( CO_2 \) between organelles and cytosol need to be considered (Tholen et al. 2012). A new method of interpreting \( \Gamma^* \) measurements from \( CO_2 \) exchange indicates that the relationship between the slope and intercepts of a common intersection measurement would be non-linear in the presence of multiple inter-organellar fluxes from photorespired \( CO_2 \) (Walker and Ort 2015; Walker et al. 2016a). Non-linearity in the slope and intercept relationships was not observed under our growth and measurement conditions, suggesting that an assumption of a simple linear \( g_m \) was justified in this case (Table 2).

### Table 2 Intercellular \( CO_2 \) partial pressure of the common intersection measurements (*\( C_{i*} \) \( ; \) \( Pa \) \( CO_2 \)), the corresponding rates of day respiration \( (R_d; \mu mol \ CO_2 \ m^{-2} s^{-1}) \), the assumed mesophyll conductance \( (g_m; \ mu m \ m^{-2} s^{-1} \ MPa^{-1}) \), and the final \( CO_2 \) photorespiration compensation point \( (\Gamma^*; \ Pa \ CO_2) \) calculated from \( C_{i*} \), \( R_d \), and \( g_m \)

| \( T \) | \( C_{i*} \) | \( R_d \) | \( R_d/S_C/O \) | \( g_m \) | \( g_m/S_C/O \) | \( \Gamma^* \) | \( \Gamma^*_{S_C/O} \) |
|---|---|---|---|---|---|---|---|
| **N. tabacum** | | | | | | | |
| 15 | 2.58 ± 0.14 | 0.55 ± 0.14 | 0.09 | 3.32 | 0.54 | 2.75 ± 0.32 | 2.87 ± 0.02 |
| 25 | 4.27 ± 0.25 | 1.34 ± 0.10 | −0.10 | 5.69 | −0.42 | 4.51 ± 0.57 | 3.70 ± 0.06 |
| 35 | 7.59 ± 0.45 | 2.32 ± 0.24 | −0.27 | 9.01 | −1.04 | 7.85 ± 1.01 | 5.18 ± 0.03 |
| **G. max** | | | | | | | |
| 15 | 2.92 ± 0.27 | 0.05 ± 0.07 | −0.10 | 2.63 | −5.17 | 2.94 ± 0.50 | 2.66 ± 0.02 |
| 25 | 4.27 ± 0.14 | 0.89 ± 0.15 | −0.14 | 4.85 | −0.77 | 4.45 ± 0.31 | 3.58 ± 0.02 |
| 35 | 5.04 ± 0.17 | 1.17 ± 0.24 | −0.17 | 5.40 | −0.78 | 5.26 ± 0.39 | 4.13 ± 0.04 |
| **T. aestivum** | | | | | | | |
| 15 | 2.59 ± 0.12 | 0.63 ± 0.16 | 0.02 | 3.21 | 0.09 | 2.79 ± 0.27 | 2.65 ± 0.02 |
| 25 | 3.95 ± 0.18 | 0.88 ± 0.20 | −0.10 | 3.94 | −0.43 | 4.17 ± 0.40 | 3.57 ± 0.04 |
| 35 | 4.67 ± 0.20 | 1.17 ± 0.17 | −0.11 | 4.01 | −0.37 | 4.96 ± 0.46 | 4.23 ± 0.11 |
| 45 | 6.08 ± 0.14 | 1.88 ± 0.15 | −0.38 | 3.76 | −0.77 | 6.58 ± 0.32 | 4.64 ± 0.01 |

Also shown are the \( \Gamma^* \) value calculated from in vitro Rubisco specificity \( (\Gamma^*_{S_C/O}; \ Pa \ CO_2) \), the \( R_d \) value necessary to explain the differences between \( C_{i*} \) and \( \Gamma^*_{S_C/O} \) \( (R_d/S_C/O) \), and the \( g_m \) value necessary to explain the differences between \( C_{i*} \) and \( \Gamma^*_{S_C/O} \) \( (g_m/S_C/O) \) all according to Eqs. 1 and 2. All data are shown for leaf temperatures \( (T; \) °C) between 15 and 35 °C. The \( g_m \) values were determined according to the temperature responses measured previously for these species (von Caemmerer and Evans 2014). Shown are the means of \( n=5–7 \) for the \( CO_2 \) gas exchange data and \( n=5–16 \) for the in vitro assays ± \( SE \).
An alternative intriguing possibility is that the assumptions used to derive $C_i$ are not always appropriate and result in a systematic error in the estimation of $\Gamma_s$ in common intersection measurements. This argument rests on the assumption of the FvCB model that the vast majority of water loss occurs through the stomata and through the same path as CO$_2$ diffusion (Moss and Rawlins 1963). This assumption has recently been challenged after an analysis of its impact on gas exchange measurements, especially the ones sensitive to small fluxes as in $\Gamma_s$ determination (Hanson et al. 2016). This re-evaluation of a common assumption is supported by the work demonstrating that water diffuses 20–40 times faster across the cuticle than CO$_2$ (Boyer 2015a). The impact of cuticular water loss on $\Gamma_s$ determination (Han-son et al. 2016). This re-evaluation of a common assumption is supported by the work demonstrating that water diffuses 20–40 times faster across the cuticle than CO$_2$ (Boyer 2015a) and that many leaves transmit significant amounts of water through the cuticle, resulting in an over-estimation of stomatal conductance and consequently $C_i$, especially at lower rates of leaf water loss (Boyer 2015a). The impact of cuticular water loss on $C_i$ estimation would be complex and require additional specialized measurements to determine if these effects could explain the differences observed using CO$_2$ gas exchange to measure $\Gamma_s$. Despite the added complexity, the possibility remains that cuticular water loss could explain the differences observed between the $\Gamma_s$ values determined using CO$_2$ exchange and those determined based on in vitro Rubisco specificity.

There are two primary methods used to determine the in vitro Rubisco specificity. These alternatively monitor O$_2$ consumption via oxygenation of RuBP in an O$_2$ electrode system (Parry et al. 1989), or determine the ratio of $^3$H-glyceraldehyde/$^3$H-glycolate produced from the consumption of $^3$H-RuBP (Kane et al. 1994). While the absolute values produced do differ, there is consistency across methods as to the comparisons across species (e.g., both methods maintain that wheat Rubisco has a higher specificity than N. tabacum at 25°C). Both methods have been employed in model species and a number of crop species under standard temperatures, and datasets incorporating temperature response are available for both methods (e.g., Galmés et al. 2005; Perdomo et al. 2015; Hermida-Carrera et al. 2016; Orr et al. 2016; Prins et al. 2016; Sharwood et al. 2016). However, the difference between methods has not been directly compared with temperature responses, due to a slight overlap of species with temperature response data using both methods. Recent efforts to compile and normalize in vitro Rubisco catalysis data (including $S_{CO2}$) from the available literature suggest that the methods available largely agree on the extent of temperature response once in vitro data were calculated accounting for the variation in equilibrium CO$_2$ concentration and the ionic strength of buffers (Galmés et al. 2016). This observation suggests that our findings should be relatively consistent with those from the other in vitro methods. The close agreement between $\Gamma_s$ values determined from O$_2$ exchange and using Rubisco specificity determined using the O$_2$ electrode is remarkable. Clearly, if in vitro specificities are to be used in the modeling efforts of CO$_2$ exchange, the method used to collect them should be reported and carefully considered.

In this report, we demonstrate that there are significant differences in the temperature response of $\Gamma_s$ dependent on the measurement method used and that these differences are large enough to impact leaf and canopy models of photosynthesis. While we have limited our discussion to the impact of these different $\Gamma_s$ values to net CO$_2$ uptake, similar analysis could be performed to determine the impact to the measurements of $g_m$ or carbon isotope exchange (Far-quhar et al. 1989; Harley et al. 1992; Tholen et al. 2012; Gu and Sun 2014). Given the growing use of biochemical models of leaf photosynthesis to calculate carbon balance and productivity at all scales, it is critical to next reveal the mechanism for these differences in order to determine which methods should be used to accurately parameterize future work or explore novel physiology. The intent of this work is thus not to invalidate the measurements of $\Gamma_s$ using the common intersection method, but rather to determine if more complete physiology can be learned by carefully comparing the $\Gamma_s$ values measured using different techniques. Additionally, the source of these differences could provide insight into the efficiency of photorespiration in response to temperature or the biochemistry of Rubisco.

**Materials and methods**

**Plant growth conditions**

Plant material used for in vitro measurements was grown in a glasshouse at Rothamsted Research with a 16/8 h day/night cycle and accompanying diurnal temperatures of 26/19°C. Plants were kept well watered. Young healthy leaves were collected, snap frozen immediately in liquid nitrogen, and then stored at −80°C until analysis. For CO$_2$ gas exchange determination of $\Gamma_s$, at the University of Illinois, N. tabacum, T. aestivum, and G. max seeds were grown in 2-L pots for 3–5 weeks until large enough for gas exchange. Plants were grown in a climate-controlled cabinet (Conviron, Winnipeg, Manitoba, Canada) set to mimic conditions in the Rothamsted glasshouse with day/night cycles of 16/8 h at 26/19°C under an irradiance of 800 µmol m$^{-2}$ s$^{-1}$.

**In vitro Rubisco specificity measurements**

Rubisco was purified from each species using the material grown in glasshouse conditions at Rothamsted Research, using the method described by Prins et al. (2016), and with
alterations as in Orr et al. (2016). The oxygen electrode method of Parry et al. (1989) was used to make a minimum of 12 replicate measurements of \( S_{\text{CO}_2} \) for each species, at 15 and 35°C, and normalized to a known value for \( T. \text{aestivum} \) at each temperature, as described previously (Parry et al. 1989). For 20, 25, and 30°C, the values from Orr et al. (2016) were used.

\( \Gamma_* \) and \( R_d \) measurements using the common intersection method

The youngest fully expanded leaves of 3- to 5-week-old plants were used for gas exchange. Gas exchange was performed using a LI-COR 6400 XT modified to reach low \( \text{CO}_2 \) partial pressures (LI-COR Biosciences 2010) using a 6 cm² chamber with a red/blue light source (LI-COR Biosciences, Lincoln, NE, USA). Assimilation measurements were corrected for \( \text{CO}_2 \) leakage according to the manufacturer’s instruction. \( \Gamma_* \) was measured using the common intersection method by measuring the \( \text{CO}_2 \) response of photosynthesis under various sub-saturating irradiances (Laisk 1977; Brooks and Farquhar 1985). The common intersection was determined using slope–intercept regression to produce more accurate and consistent values of \( C_{\text{is}} \) and \( R_d \) (Walker and Ort 2015; Walker et al. 2016a).

To determine irradiances that would result in an even distribution of photosynthetic rates for \( \Gamma_* \) determinations, the photosynthetic light response of each species was first measured at 20 Pa \( \text{CO}_2 \). Prior to \( \Gamma_* \) determinations using the common intersection method, plants were acclimated under 250 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) at 39 \( \text{Pa} \) \( \text{CO}_2 \) until photosynthesis reached steady state to activate Rubisco. Following initial acclimation, plants were measured at 15, 12, 9, 7, 5, and 3 Pa \( \text{CO}_2 \) under irradiances of 250, 165, 120, 80, and 50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) for \( N. \text{tabacum} \), 250, 160, 100, 60, and 30 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) for \( T. \text{aestivum} \), and 250, 165, 120, 80, and 50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) for \( G. \text{max} \). The \( x \)-intersection point represents \( C_{\text{is}} \) which can be converted to \( \Gamma_* \) according to

\[
\Gamma_* = C_{\text{is}} + R_d / g_m, \tag{2}
\]

where \( R_d \) is the \( y \)-intersection point (von Caemmerer 2000; Furbank et al. 2009). Species-specific temperature responses were used at each temperature for \( g_m \) (von Caemmerer and Evans 2014).

Leaf- and canopy-scale modeling of photosynthesis

Leaf-level modeling of the \( \text{CO}_2 \) response of net photosynthesis was modeled at 25 and 35°C using the standard FvCB model of leaf photosynthesis. For 25°C, the model was parameterized with \( V_{\text{max}} = 80 \mu \text{mol m}^{-2} \text{s}^{-1} \), \( K_c = 26.7 \text{ Pa} \), \( K_o = 16.3 \text{ kPa} \), \( R_d = 1 \mu \text{mol m}^{-2} \text{s}^{-1} \), and \( J_{\text{max}} = 120 \mu \text{mol m}^{-2} \text{s}^{-1} \). \( \Gamma_* \) was assumed to be 4.74, 3.78, and 3.7 Pa for the common intersection method, \( \text{O}_2 \) exchange, and in vitro determinations, respectively. For 35°C, the model was parameterized with \( V_{\text{max}} = 187 \mu \text{mol m}^{-2} \text{s}^{-1} \), \( K_c = 77.1 \text{ Pa} \), \( K_o = 22.2 \text{ kPa} \), \( R_d = 2 \mu \text{mol m}^{-2} \text{s}^{-1} \), and \( J_{\text{max}} = 211 \mu \text{mol m}^{-2} \text{s}^{-1} \), respectively. \( \Gamma_* \) was assumed to be 7.88, 5.15, and 5.2 Pa for the common intersection method, \( \text{O}_2 \) exchange, and in vitro determinations, respectively.

For canopy-level implementation, we used a well-validated multilayer canopy–root–soil model (MLCan, Drewry et al. 2010a, b) with minor additions to include \( g_m \) (Walker et al. 2016b). The model was parameterized with field data from the Bondville, Illinois, AmeriFlux eddy covariance site measured during the 2002, 2004, and 2006 growing seasons (available from the AmeriFlux Database; http://ameriflux.lbl.gov/data/download-data). Full-field data can also be obtained from B. J. W. upon request.

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