# Letters

## The rapid $A/C_i$ response: a guide to best practices

### A response to Taylor & Long (2019) 'Phenotyping photosynthesis on the limit – a critical examination of RACiR'

The Rapid $A/C_i$ Response (RACiR) is a dynamic method of leaf-level gas exchange that allows the calculation of fundamental parameters for photosynthetic capacity in much shorter times than standard steady-state methods. This is accomplished by using $CO_2$ ramps instead of discrete values under steady-state conditions. Here, we present data describing potential pitfalls and provide a list of best practices that are important to follow in order to ensure the data acquired are of high quality.

Stinziano et al. (2017) demonstrated that the RACiR generates robust estimates of $V_{cmax}$ (maximal carboxylation rate) and $J$ (potential rate of electron transport) similar to the standard $A/C_i$, which can be very useful in phenotyping applications. They also suggested that the RACiR technique may generate new biological insights that are unattainable from slower measurements (Stinziano et al., 2017). Here, we respond to the Letter by Taylor & Long (2019; in this issue of New Phytologist, pp. 621–624), reiterating the points from Stinziano et al. (2017) and emphasizing that applying RACiR methodology outside of the specific conditions and recommendations of Stinziano et al. (2017) requires further testing.

Fig. 1(a,b) of Taylor & Long (2019) shows data using a ramp of 200 µmol mol$^{-1}$ min$^{-1}$, which Stinziano et al. (2017) stated as being a rate that may compromise the estimate of $J$. Therefore, it is not surprising to see examples at that speed that generate larger deviations from a standard $A/C_i$ than was in our data, provided in both figures and supplementary files of Stinziano et al. (2017) as well as in Fig. 1(c) of Taylor & Long (2019). Here, we present results demonstrating that higher ramp rates generate larger differences in the apparent compensation point (Fig. 1). These results also demonstrate that $CO_2$ ramping rate can result in apparent assimilation offsets. Such offsets may be due to instrumentation artifacts such as an imperfect empty chamber correction or other effects that are not yet demonstrated. At sufficiently high ramp rates, differences between standard steady-state and dynamic RACiR gas exchange methods would be expected and could reflect real differences in the underlying biology. When $CO_2$ can be changed faster than enzyme activation states, stomatal conductance, mesophyll conductance, or faster than some biochemical pools can respond, then gas exchange data may need reinterpretation, and this new approach may offer opportunities to test model assumptions. However, we note that higher $CO_2$ ramp rates are

complicated and we recommend limiting ramp rates to 100 ppm min$^{-1}$, unless one is explicitly examining the mechanisms behind high ramp rate offsets.

Taylor & Long (2019) extended data analyses to parameters that were beyond the scope of Stinziano et al. (2017). Based on potential procedural or biological concerns mentioned earlier, at high ramp rates RACiR may not generate compensation points similar to a standard $A/C_i$. However, we also point out that standard $A/C_i$ methods used to generate estimates of other parameters presented in Taylor & Long (2019), including $g_m$ (mesophyll conductance), $R_d$ (dark respiration), and $\Gamma^*$ (photorespiratory compensation point) are potentially problematic (Pons et al., 2009; Walker & Cousins, 2013; Walker & Ort, 2015; Farquhar & Busch, 2017) and we would not recommend using them.

Taylor & Long (2019) also combine data from the 500 to 0 and 300 to 800 µmol mol$^{-1}$ RACiRs of Stinziano et al. (2017) when performing model fits. While compiling a larger data set for curve fitting is understandable, we do not think it is appropriate here. Notably, the RACiRs in Stinziano et al. (2017) were not conducted in a way to maximize alignment between ramp ranges due to the way the $CO_2$ ramping loops were set up. The order of measurements is known to affect the results as evidenced by the common practice, in a standard $A/C_i$, of carefully returning to a common mid-point value between low and high $CO_2$ ranges. This is required because of the biological effects occurring during slow measurements, such as enzyme deactivation, in each $CO_2$ range. Therefore, we recommend against combining RACiRs from multiple ranges.

When we performed a re-analysis of the Stinziano et al. (2017) data using the Taylor & Long (2019) script, but restricted the fit to the 300–800 µmol mol$^{-1}$ RACiRs, most of the significant differences presented in Taylor & Long (2019) were no longer significant. However, we do not present the table here because we believe a larger data set is needed for proper statistical analyses. Every parameter estimate has some sort of error associated with it. When relatively small numbers of parameter estimates are compared using a standard statistical test (such as a t-test) this error is essentially ignored, and so we urge caution in declaring true differences between parameter estimates based on relatively limited data. Essentially, a larger data set that includes analyses of all errors would be helpful in order to more fully investigate these issues.

### Additional curve fitting considerations

Taylor & Long (2019) raise an excellent point: curve fitting approach matters. This issue was thoroughly examined by Gu et al. (2010) and has been repeatedly addressed by multiple groups for well over a decade (e.g. Ethier & Livingston, 2004; Dubois et al., 2007; Sharkey et al., 2007). It is important to recognize that different methods for fitting $A/C_i$ responses can yield different
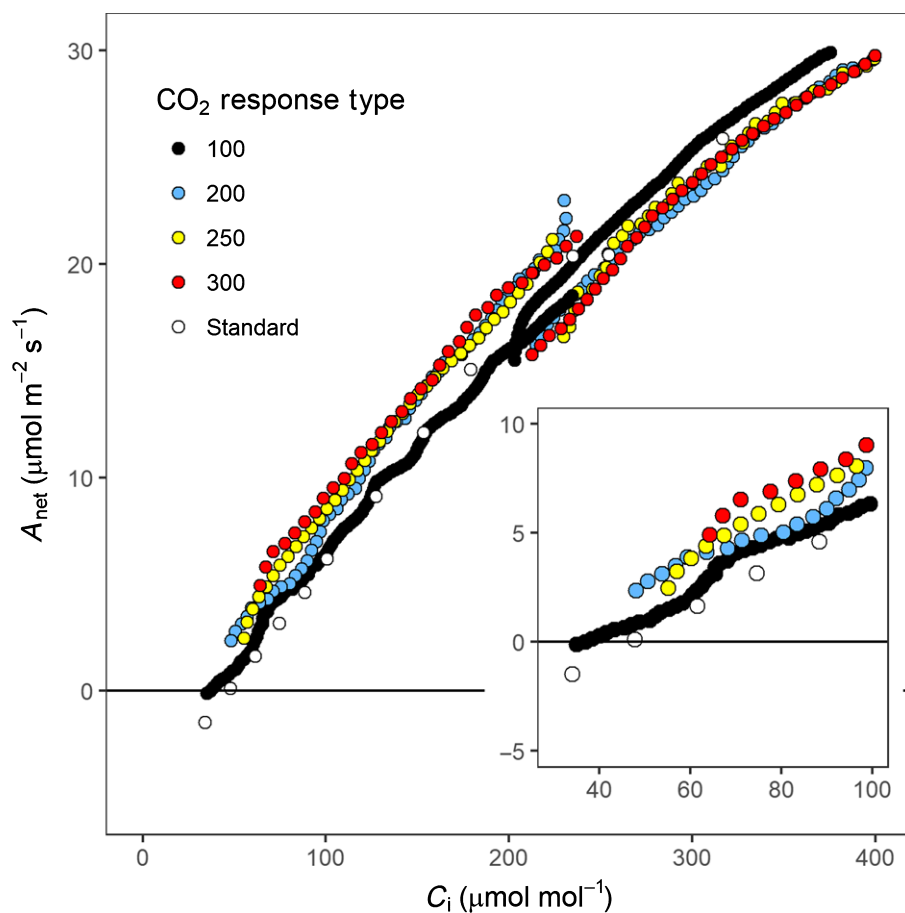
**Fig. 1** Effects of $CO_2$ ramp rates on data output from the Rapid $A/C_i$ Response (RACiR). Inset shows the region around the compensation point. RACiRs were run from 500 to 0 or 300 to 900 µmol $CO_2$ mol$^{-1}$ at 100, 200, 250, and 300 µmol $CO_2$ mol$^{-1}$ min$^{-1}$ on *Populus deltoides* leaves, paired with a standard $A/C_i$ response measured at 400, 300, 200, 150, 125, 100, 75, 50, 25, 400, 500, 600, 700, 800, 1000, 1200, 1500, 1800, and 2000 µmol $CO_2$ mol$^{-1}$. Cuvette settings were: leaf temperature of 25°C, overpressure of 0.2 kPa, flow rate of 600 µmol s$^{-1}$, fan speed of 10 000 rpm, reference [$H_2O$] of 19.5 mmol mol$^{-1}$, and saturating irradiance of 1000 µmol photons m$^{-2}$ s$^{-1}$. $A$, net $CO_2$ assimilation; $C_i$, intercellular $CO_2$ concentration.

parameter estimates. This makes assigning 'truth' more difficult since the method used can affect the outcome. This is especially true for values like $g_m$, $R_d$, and $\Gamma^*$ such that it is not widely accepted that these are optimally derived from a single standard $A/C_i$ (see methods Pons *et al.*, 2009; Walker & Cousins, 2013; Walker & Ort, 2015; Farquhar & Busch, 2017). The most widely accepted use for the $A/C_i$ is to estimate $V_{cmax}$ and $J$ (with much less certainty about $J$) and those values are in 'reasonably close agreement' between the standard and RACiR approaches in Taylor & Long (2019) and in Stinziano *et al.* (2017). While it is logical and valuable to compare standard $A/C_i$ and RACiR approaches for fitting other parameters as Taylor & Long (2019) have done, assigning truth becomes more difficult given the uncertainty of model fitting as well as the methodological points we have raised earlier. Given the importance of careful consideration of ramping conditions and preliminary testing with a species of interest, we are providing a more detailed and explicit procedural guide for new users.

## Best practices procedural guide

RACiR requires characterization of the species in question to determine the best way to setup the $CO_2$ ramps, *especially* the ramping rate. To date, a ramping rate of 100 µmol mol$^{-1}$ min$^{-1}$ has provided the best comparisons with steady-state $V_{cmax}$ and $J$. RACiR empty-chamber calibration curves are specific to flow rate, temperature, and ramp rate, and may be sensitive to large

changes in water mole fraction during the ramp. As such, we are clarifying key recommendations whenever RACiR is used:

• First, determine if RACiR is suitable for your research question. For example, are you assessing variation in $V_{cmax}$ and $J$, or other parameters? To date, Stinziano *et al.* (2017) have provided evidence that $V_{cmax}$ and $J$ are substantially similar between RACiR and standard $A/C_i$ curves. We reiterate our caution that applying RACiR approaches to other parameters requires further investigation.

• On each measurement day, and for each instrument, ensure that empty-chamber RACiR calibration curves are run for every flow rate, temperature, ramp range, direction, and ramp rate that is to be used. Only high chamber mixing speeds should be used.

• The same match should be used for the empty chamber and leaf curves. In other words, the exact same IRGA match – in both concentration and time – should be used for the empty chamber curve(s) and the leaf curves they are correcting. When a new IRGA match is needed due to IRGA drift, the passage of time, or changes in environmental conditions, a new empty-chamber correction must be run as well. Between individual RACiR curves, we recommend *only checking* the match at the original conditions used for matching. At the original conditions, an empty chamber should have $A_{net}$ values close to zero (within a few tenths) and sample and reference $CO_2/H_2O$ mole fractions should be nearly identical.

• Determine which polynomial best fits the empty-chamber calibration curve, ranging from first to fifth order. IRGA factory

calibrations use up to fifth order polynomials, which is why we have selected this range. We have found that third and fourth order polynomials most commonly provide the best fits. We have recently made an R script freely available to automate this process (Stinziano, 2018).

• Determine what range of $[CO_2]$ is useable. These are the data that are fit well and are consistent between empty chamber tests. This often requires discarding the first and last 30 s of data, where the rates of change in $[CO_2]$ are not constant.

• Minimize differentials in water mole fraction between the calibration and measurement runs. This involves preliminary testing with each species under each condition, and may require control of reference or sample $[H_2O]$ rather than leaf vapor pressure deficit when running RACiR or the introduction of humidity into the empty chamber.

• Run preliminary tests on each species and each environmental condition of interest to determine an appropriate range in $[CO_2]$ and $CO_2$ ramp rate for parameters of interest. The importance of this step cannot be over-stated. This is similar to running preliminary tests of the light response, which needs to be done before any $A/C_i$ to determine saturation intensity.

Taken together, we think the main conclusions of Stinziano *et al.* (2017) still hold in that RACiR can generate estimates of $V_{cmax}$ and $J$ that are substantially similar to estimates derived from the standard approach, and that RACiR is a useful screening tool for rapid phenotyping. The work of Taylor & Long (2019) is helpful in determining where differences in parameter estimates between the two methods may exist, and points to the need for additional research as RACiR is further developed.

## ORCID

David T. Hanson (ID) http://orcid.org/0000-0003-0964-9335
Dayle K. McDermitt (ID) http://orcid.org/0000-0001-9823-6852
Joseph R. Stinziano (ID) http://orcid.org/0000-0002-7628-4201

Joseph R. Stinziano[1] (ID) , Dayle K. McDermitt[2] (ID) ,
Douglas J. Lynch[3], Aaron J. Saathoff[3,4], Patrick B. Morgan[4,5]
and David T. Hanson[1]* (ID)

[1]Department of Biology, The University of New Mexico,
Albuquerque, NM 87104, USA;
[2]Department of Agronomy and Horticulture, University of
Nebraska-Lincoln, Lincoln, NE 68583, USA;
[3]Li-Cor Inc., Lincoln, NE 68504, USA;
[4]School of Natural Resources, University of Nebraska-Lincoln,
Lincoln, NE 68583, USA;
[5]Monsanto, Chesterfield, MO 63017, USA
(*Author for correspondence: tel +1 505 277 6681;
email dthanson@unm.edu)

## References

**Dubois J-JB, Fiscus EL, Booker FL, Flowers MD, Reid CD. 2007.** Optimizing the statistical estimation of the parameters of the Farquhar–von Caemmerer–Berry model of photosynthesis. *New Phytologist* **176**: 402–414.

**Ethier GJ, Livingston NJ. 2004.** On the need to incorporate sensitivity to $CO_2$ transfer conductance into the Farquhar–von Caemmerer–Berry leaf photosynthesis model. *Plant, Cell & Environment* **27**: 137–153.

**Farquhar GD, Busch FA. 2017.** Changes in the chloroplastic $CO_2$ concentration explain much of the observed Kok effect: a model. *New Phytologist* **214**: 570–584.

**Gu L, Pallardy SG, Tu K, Law BE, Wullschleger SD. 2010.** Reliable estimation of biochemical parameters from $C_3$ leaf photosynthesis-intercellular carbon dioxide response curves. *Plant, Cell & Environment* **33**: 1852–1874.

**Pons TL, Flexas J, von Caemmerer S, Evans JR, Genty B, Ribas-Carbo M, Brugnoli E. 2009.** Estimating mesophyll conductance to $CO_2$: methodology, potential errors, and recommendations. *Journal of Experimental Botany* **60**: 2217–2234.

**Sharkey TD, Bernacchi CJ, Farquhar GD, Singsaas EL. 2007.** Fitting photosynthetic carbon dioxide response curves for $C_3$ leaves. *Plant, Cell & Environment* **30**: 1035–1040.

**Stinziano JR. 2018.** *RACiR analysis (version v1.0.0).* Zenodo. [WWW document] URL http://doi.org/10.5281/zenodo.1251925

**Stinziano JR, Morgan PB, Lynch DJ, Saathoff AJ, McDermitt DK, Hanson DT. 2017.** The rapid $A$-$C_i$ response: photosynthesis in the phenomic era. *Plant, Cell & Environment* **40**: 1256–1262.

**Taylor SH, Long SP. 2019.** Phenotyping photosynthesis on the limit – a critical examination of RACiR. *New Phytologist* **221**: 621–624.

**Walker BJ, Cousins AB. 2013.** Influence of temperature on measurements of the $CO_2$ compensation point: differences between the Laisk and $O_2$-exchange methods. *Journal of Experimental Botany* **64**: 1893–1905.

**Walker BJ, Ort DR. 2015.** Improved method for measuring the apparent $CO_2$ photocompensation point resolves the impact of multiple internal conductances to $CO_2$ to net gas exchange. *Plant, Cell & Environment* **38**: 2462–2474.