

Comparison of steady-state and dynamic Laisk methods to study temperature effects on banana respiration

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Abstract

Daytime respiration (R_d), a crucial physiological process in plants, presents significant measurement challenges due to the difficulty of isolating it from photosynthetic processes during light conditions. The Laisk method is one of the most common ways to measure R_d , is enhanced by the updated dynamic approach of the Licor-6800, which employs advanced equations for stable measurements under changing environmental conditions. Although promising for reducing uncertainties in R_d and improving measurement efficiency, its effectiveness across diverse plant species and growth conditions remains to be thoroughly tested. We evaluated the efficacy of the steady-state and dynamic Laisk methods on banana plants (*Musa acuminata* cv. Cavendish) at four different growth temperatures. Our study indicates that the dynamic Laisk method outperforms the steady-state approach in accurately estimating internal CO_2 concentration, (C_i^*) and R_d , primarily due to its ability to use extensive data for precise curve analysis. This method also reduces stomatal limitations by minimizing exposure to low light, a significant advantage in temperature-sensitive plants like bananas, where stomatal responses can affect measurement accuracy. Our findings highlight the dynamic method's reliability and effectiveness, suggesting its broader application across different plant species and growth conditions to advance our understanding of leaf CO_2 fluxes.

Introduction

Leaves have several different gas fluxes that occur at the same time. The carbon dioxide (CO_2) diffuses into the mesophyll cells where it is converted to carbohydrates in the photosynthesis (A_n) process. On the other hand, CO_2 is produced in the leaf by mitochondrial respiration during the day (R_d) and the CO_2 is produced in mitochondria as the result of the photorespiration process (F). At nighttime, the plant continues to emit CO_2 through mitochondrial respiration (R_n) (Damesin, 2003; Silim *et al.*, 2010). The average respiration in 24 hours cycle, can be 30-50% from the gross photosynthesis (V_c) (Nobel, 2020). The ratio between R_d to R_n is typically considered to be 0.5 (Way & Yamori, 2014), however, in certain conditions, such as an increase in temperature, the ratio increases (Way *et al.*, 2015). Net photosynthetic carbon assimilation results from subtracting the CO_2 released through photorespiration and the daytime respiration (R_d) from the gross fixed CO_2 . The photorespiration could be expressed by the CO_2 compensation point in the chloroplast under conditions absent of R_d (Γ^*), this is the CO_2 concentration at which net carbon assimilation becomes zero: (Von Caemmerer & Farquhar, 1981).

The relatively small fluxes of respiration and photorespiration take on increasing significance in predicting photosynthesis parameters such as mesophyll conductance (g_m). Understanding these processes necessitates knowledge of CO_2 concentrations in the intercellular airspaces (C_i) (Hanson *et al.*, 2016). Since it is currently not possible to directly measure the CO_2 concentration inside the chloroplast (C_c), an alternative approach involves expressing Γ^* in terms of the CO_2 compensation point in the CO_2 concentration in the intercellular airspace C_i^* . According to von Caemmerer *et al.*, (1994), C_i^* is

not significantly different from Γ^* and is dependent on g_m and R_d , which are related to one another as follows: .

Measurements of R_d and C_i^* are not possible directly by using a gas exchange system and thus require indirect calculations based on photosynthesis models. As a result, several methods have been developed to evaluate these parameters. Early methods for estimating CO₂ compensation point involved placing a leaf into a closed chamber devoid of CO₂ and observing the point at which the system reached equilibrium, this equilibrium point is considered the CO₂ compensation point (Forrester' et al., 1966). The Kok method, which also assesses R_d , is based on the observation that the photosynthesis rate (A_n) tends to respond linearly to light at low irradiance levels. At the vicinity of the light compensation point, a deviation occurs, where the response curve's slope becomes significantly steeper below this point. This change is attributed to light inhibiting of dark respiration (Kok, 1948).

The Laisk method is used to assess both the C_i^* and R_d (Laisk, 1977). This method includes analyses of the linear part of CO₂ response curves at several PPFD levels to find the intersection of two or more linear lines. The intersection point of the CO₂ response curves represents the common C_i^* and R_d . This method operates under the assumption that R_d remains constant regardless of changes in PPFD and C_i within the specified ranges (Yin & Amthor, 2024). However, the Laisk method has its limitations. For instance, light intensities used in this method can affect the R_d rate and the C_i^* , as higher PPFD increases the C_i^* and reduces the R_d (Peisker & Apel, 2001; Tholen et al., 2012). Additionally, the measurement at low light intensities could negatively affect the stomatal aperture and the gas fluxes into and out of the leaf (Shimazaki et al., 2007) and may also affect the method reliability. Furthermore, conducting experiments across varied environmental conditions necessitates individual measurements for each condition, a process that can be time-consuming. The updated Licor-6800 introduces a dynamic method, employing enhanced equations, which alleviates the need for the system to be in a steady state for obtaining a stable measurement (Stinziano et al., 2017; Saathoff & Welles, 2021). This dynamic approach enables measurement even as environmental conditions fluctuate continuously, without affecting stability, and enabling a higher volume of measurements within a short timeframe. Therefore, non-steady state, dynamic assimilation offers the potential to reduce uncertainty in parameter estimates such as R_d and increase measurement throughput (Schmiede et al., 2023). To fully harness its potential, this method must be evaluated across a diverse range of plant species and growth conditions, ensuring its applicability and effectiveness in a broad spectrum of research scenarios.

To evaluate the efficacy of the steady-state and dynamic Laisk methods, we conducted experiment on banana plants (*Musa acuminata* cv. *Cavendish*). This herbaceous, monocotyledonous, and perennial species, native to the warm and humid regions of East Asia, is known for its thermophilic nature. It . Our study aimed to Stover in 1972) thrives in high temperatures but struggles in conditions below 14°C (assess and compare these two measurement techniques within the context of the banana leaves Furthermore, we offer comprehensive instructions for conducting non-steady-environmental adaptability.

state (dynamic) measurements with the LI-6800, ensuring researchers can accurately capture the nuanced behaviors of photosynthesis under varying environmental conditions.

Materials And Methods

Plant material

The study was conducted in a Phytotron greenhouse with four day/night temperature-controlled rooms with temperature regimes of 16/10°C, 22/16°C, 28/22°C, and 34/28°C. Tissue culture-propagated banana plantlets (*Musa acuminata* cv. *cavendish* cv. Gal) (Rahan-Meristem LTD, Rosh HaNikra, Israel) grew in 18 L pots containing 100% growth mixture, constrained of tuff, coconut, and kavul in varying ratios (Tuff Marom-Golan, Ram 6, Israel) for four weeks at 28/22°C and then transferred to the four different growth temperatures. Measurements were taken on leaf tissue that developed at the specific growth temperature, on the youngest fully developed leaf. The plants were irrigated to full pot capacity twice a day, ensuring that the soil was thoroughly moistened. The irrigation solution contained the following concentrations of specific elements: Ca 2.0 ± 0.1 mM, Mg 1.2 ± 0.2 mM, NH₄ 0.27 ± 0.04 mM, NO₃ 4.6 ± 0.1 mM, P 0.30 ± 0.02 mM and K 2.3 ± 0.2 mM (Zait *et al.*, 2017).

Gas exchange measurements

Gas exchange measurements were conducted with the LI-6800F portable photosynthesis measurement system, equipped with a 6 cm² fluorescence leaf chamber (LiCor Inc., Lincoln, NE, USA). Measurements were taken on the youngest fully expanded leaf (usually between the third and fourth from the apical shoot). To set the optimal flow rate we used "balance_flow.py" which was 550 ± 10 $\mu\text{mol s}^{-1}$, and the fan speed was set to 5000 rpm. Measurements were conducted at leaf temperatures of 16°C, 22°C, 28°C, and 34°C respectively to the room temperature. The humidity was controlled by the vapor pressure deficit in the leaf chamber (VPD_{leaf}), and was fixed on 1.5. Laisk curves were performed in three light intensities: 400, 200 and 100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$. We implemented two different Laisk methods, steady state, and dynamic method.

For the steady-state method A/C_i curves, CO₂ concentrations were reduced from 300 to 200, 150, 100, 75, 50, and 25 $\mu\text{mol CO}_2 \text{mol}^{-1}$ air. We allowed 90 seconds between each CO₂ concentration before taking the measurement (The total time for one rep was 10.5 minutes).

To accomplish the dynamic method, prior to each measurement we applied a LI-6800 range match (matching the sample and reference IRGA readings over the full concentration range) in each temperature for all CO₂ concentrations. Next, we used the LI-6800 Utilities/Test > CO₂ test current, in order to test the best fit on the data to improve the coefficients in the dynamic measurement. The CO₂ concentrations continuously dropped from 300 to 25 $\mu\text{mol CO}_2 \text{mol}^{-1}$ air. To allow comparison between the two methods, the duration of the measurement was kept the same at 10.5 minutes.

Therefore, the drop rate was determined at $30.5 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air min}^{-1}$ using the LI-6800 CO₂ Ref ramping. Logs were monitored every 5 seconds to observe continuous curves.

For both methods, we utilized the least squares method, minimizing the sum of squared distances between actual and predicted “ ” values for each line equation, in R statistical software [version 4.3.22 (<https://cran.r-project.org>)] to calculate the common interception point of the three A/C_i curves.

Night respiration measurements were taken during the night between three hours before sunrise. The CO₂ concentrations were fixed in $420 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$. Light intensity of $0 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ was set to keep the leaves at complete dark. The other environment parameters were the same as described for the Laisk measurements.

Statistical analysis

To compare gas exchange parameters under different conditions, we employed two-sample t-tests to discern any significant differences between the methods. Additionally, to explore variations across different growth temperatures, a Tukey's HSD post hoc analysis was conducted. The statistical analyses were performed using the base R statistical software, version 4.3.22 (<https://cran.r-project.org>).

Results

Assessing the optimum thermal conditions in different growth temperature

To demonstrate the effect of growth temperature on the banana plant, we measured key physiological parameters including total biomass, net photosynthesis (A_n), and stomatal conductance (g_{sw}) at a leaf temperature of 25°C for each growth temperature. The total biomass, as measured by shoot weight, demonstrated a positive correlation with growth temperature, increasing by a factor of 2.52 from 16/10°C to an optimum at 34/28°C. The mean total biomass weights were 136, 216, 267, and 344 grams at growth temperatures of 16/10°C, 22/16°C, 28/22°C, and 34/28°C, respectively, (Fig. 1.A).

In the gas exchange measurement, the A_n increase from $10.22 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ in growth temperature of 16/10°C to an optimum growth temperature of 28/22°C, before slightly decreasing to $24.52 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ at a growth temperature of 34/28°C (Fig. 1.B). Stomatal conductance (g_{sw}) varied with growth temperature, starting at $0.1 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ at 16/10°C, rising to 0.44 at 22/16°C, peaking at 0.6 at 28/22°C, and then slightly decreasing to 0.53 at 34/28°C (Fig. 1.C). There was no significant difference in g_{sw} between the 28/22°C and 34/28°C temperatures.

Laisk measurements using both the steady-state and dynamic method

The Laisk curves demonstrate a similar pattern of intercellular CO₂ concentration C_i at the range of 0-280 $\mu\text{mol CO}_2 \text{ mol air}^{-1}$, exhibiting a non-linear behavior. In the dynamic method, when the C_i falls below 140

$\mu\text{mol CO}_2 \text{ mol air}^{-1}$, and around $90 \mu\text{mol CO}_2 \text{ mol air}^{-1}$ in the steady-state method, the response curves transition to a linear pattern. The interception point of the three curves represents the C_i^* and the R_d . The highest C_i^* , $51.71 \mu\text{mol CO}_2 \text{ mol air}^{-1}$, and R_d , $1.88 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ were found in the growth temperature of $34/28^\circ\text{C}$ with the dynamic method (Fig. 2.A). When comparing the C_i^* and R_d values between the dynamic and the steady state methods at the same growth temperature, there is no significant difference between the two methods (Fig. 3). In both the steady state and dynamic methods, it is evident that C_i^* increases with rising temperatures. However, there are notable differences between the two methods in how this increase is observed. In the steady state method, we observe a gradual rise in C_i^* as temperatures increase from $16/10^\circ\text{C}$ to $28/22^\circ\text{C}$, reaching 32.38 to $35.25 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$. Subsequently, there is a significant increase to $47.99 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$ at the warmer growth temperature of $34/28^\circ\text{C}$. Contrastingly, the dynamic method shows a more moderate increase in C_i^* . At $16/10^\circ\text{C}$, C_i^* is recorded at $27.85 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$, which then rises to $30.81 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$ at $22/16^\circ\text{C}$ and further to $35.67 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$ at $28/22^\circ\text{C}$. Like the steady state method, there is a notable increase to $51.71 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$ at $34/28^\circ\text{C}$ (Fig. 3.A). The largest difference between the methods was found in the cold growth temperature $16/10^\circ\text{C}$. At the steady state method, the rate of R_d rises with the increasing growth temperature in both methods, from $0.25 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ to $1.74 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ in growth temperature of $16/10^\circ\text{C}$ and $34/28^\circ\text{C}$ respectively. Similarly, in the dynamic method, R_d exhibits an increase from $0.17 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ at $16/10^\circ\text{C}$ to $1.88 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ at $34/28^\circ\text{C}$ (Fig. 3.B).

Assessing the relationship between daytime and nighttime respiration in different growth temperatures

The R_d and R_n in each growth temperature, measured in day/night temperature (the leaf temperature of the daytime is larger by 6°C) of each treatment. We found a clear shift in respiratory behavior as temperatures increased, with plants exhibiting a substantial rise in the R_d/R_n ratio. Starting at a growth temperature of $16/10^\circ\text{C}$, the nighttime respiration rate (R_n) stands at $0.44 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, with an R_d/R_n ratio of 0.38 . With rising temperatures, R_n increases to $0.56 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ at $22/16^\circ\text{C}$ and to $0.91 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ at $28/22^\circ\text{C}$. Concurrently, the R_d/R_n ratio grows to 0.93 at $22/16^\circ\text{C}$ and 1.37 at $28/22^\circ\text{C}$, representing a 2.45 -fold and a 3.61 -fold increase over the initial ratio, respectively. At the highest growth temperature of $34/28^\circ\text{C}$, R_n reaches $1.08 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, with an R_d/R_n ratio of 1.74 , marking a 4.58 -fold increase from the initial.

Discussion and Recommendations

The optimal temperature for banana growth is identified at 34°C , while the optimum temperature for photosynthesis is slightly lower, at 28°C (Fig. 1). Given these thermal optimums, we anticipated observing similar patterns in daytime respiration (R_d), with adjustments in respiration rates reflecting the

plant's physiological adaptations to these optimal growth and photosynthesis temperatures. The parameters C_i^* and R_d that were estimated from both methods, steady state and dynamic, showed no significant differences when they were compared in the same growth temperature (Fig. 3). The dynamic method performed in this experiment kept in the same time frame of measurement. Now with these results, we recommend speeding up the drop rate to $50 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air min}^{-1}$ and starting the measurement from $100 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$, which is the start of the linear behavior of the curves (Fig. 2 A-H). When these recommendations are implemented, we can achieve one A/C_i curve in two minutes, allowing us to estimate C_i^* and R_d faster with the same accuracy, or to add more curves of different light intensities to increase the accuracy of the measurement. We suggest that the performance of the Laisk method is more accurate in the dynamic compared to the steady state method, due to several reasons: First, using a large number of data points increases the accuracy of estimating the linear part of the curve. Second, long measurements can be negatively affected by stomatal limitation (Busch *et al.*, 2024). The stomatal response to low light intensity is expressed in the closure stomatal aperture and reduced in stomatal conductance (Shimazaki *et al.*, 2007). In banana plants, this effect is more pronounced at low temperatures, which initially limits stomatal conductance (Fig. 1.C). Our results show that the dynamic method allows for shorter exposure times to low light, enabling to avoid stomatal limitation. Our findings differ from those reported by (Schmiede *et al.*, 2023), whose study found no significant differences between temperature treatments in terms of C_i^* and R_d values in paper birch (*Betula papyrifera*). Contrarily, in our experiments, C_i^* exhibited significant differences between the $34/28^\circ\text{C}$ treatment and other growth temperatures in both the dynamic and steady-state methods. Additionally, the R_d values in our results distinctly varied across the growth temperatures, highlighting the sensitivity of banana plants to thermal conditions in contrast to the responses observed in paper birch.

We further explored the dynamics of daytime (R_d) and nighttime (R_n) respiration rates across various growth temperatures, setting the night temperatures at 6°C below day temperature, a common scenario in tropical natural environments. Interestingly, R_n exceeded R_d only at the lower (16°C) and moderate (22°C) temperature settings, but the rates were comparable at the optimum temperature for photosynthesis (28°C). This observation indicates that, at warmer temperature regimes, banana plants prioritize growth respiration over maintenance respiration, as evidenced by a higher R_d/R_n ratio (Fig. 4). In contrast, the lower temperatures, which pose thermal stress for bananas, tend to increase the maintenance-to-growth respiration ratio. This pattern mirrors findings from previous studies on different species, where lower temperatures have been associated with an increased focus on maintenance respiration (Damesin, 2003; Kolari *et al.*, 2007; Sperlich *et al.*, 2016). However, measuring nighttime respiration (R_n) during the day, specifically in plants that have been dark-adapted for 30 minutes, is considered acceptable practice (Way & Sage, 2008; Sperlich *et al.*, 2016). Here we are stressing that the timing of measurements of R_n (midnight vs. measurement in the dark during the day) could affect the R_d/R_n ratio. Further research is warranted to explore these effects in more detail, underlining the

necessity for a deeper understanding of how measurement timing influences respiration dynamics and, consequently, interpretations of plant metabolic processes.

Declarations

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Author contributions:

Experimental design, material preparation, data collection, analysis, and the drafting of the manuscript were carried out by BBZ. Experimental design and manuscript writing was done by YZ. Manuscript editing was done by OS. All authors read and approved the final manuscript.

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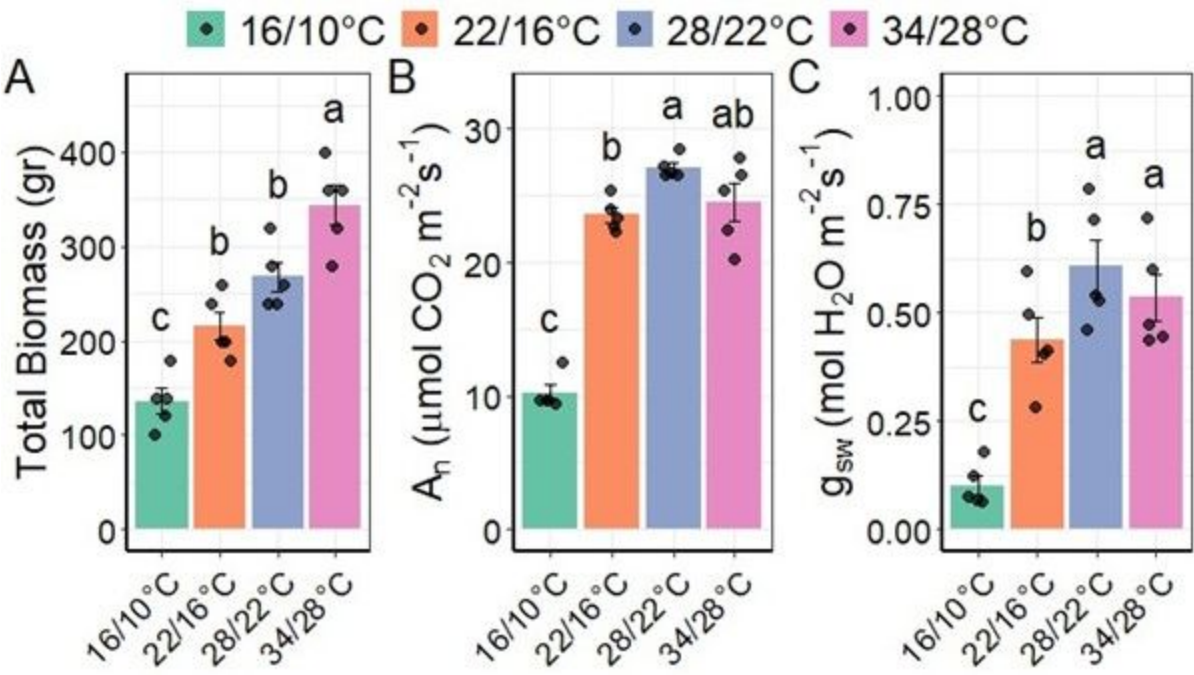
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Tables

Table 1: Summary means and ± standard error (n = 3), of C_i^* and R_d in fore growth temperatures and the two methods.

<i>Method</i>	<i>Growth temperature</i> (°C)	C_i^* mean ($\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$)	R_d mean ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
<i>Steady state</i>	34/28	47.99 ±4.30	1.74 ±0.37
	28/22	35.25 ±0.89	1.40 ±0.04
	22/16	33.30 ±2.57	0.88 ±0.28
	16/10	32.38 ±3.32	0.25 ±0.13
<i>Dynamic</i>	34/28	51.71 ±3.82	1.88 ±0.28
	28/22	35.67 ±1.97	1.25 ±0.22
	22/16	30.81 ±0.97	0.52 ±0.16
	16/10	27.85 ±2.15	0.17 ±0.03

Figures



The effect of growth temperature on the (A) total biomass, (B) net photosynthesis A_n and (C) stomatal conductance g_{sw} . Data points are shown, with bars representing mean values $\pm$ standard error (n = 5). Statistically significant differences are denoted by distinct letters at a significance level of $p < 0.01$.



Laik curves performed with two different methods: dynamic (A, C, E, G) and a steady state (B, D, F, H) in day/night growth temperature regimes of 34/28°C (A, B), 28/22°C (C, D), 22/16°C (E, F) and 16/10°C (G, H). The figures show the net CO₂ assimilation rate (A_n) response to changes in CO₂ concentration in the intracellular airspace of the leaf (C_i), under light intensities of 400 (green), 200 (orange), and 100 (purple) $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ (PPFD). The calculated linear line of the mean values is represented by the solid lines.

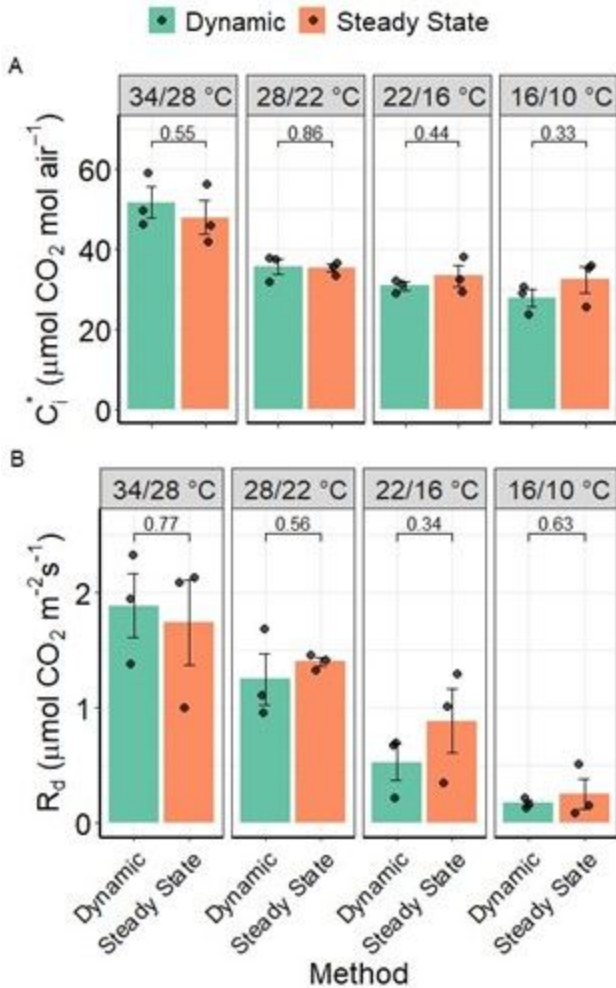


Figure 3

No differences between the two methods (Dynamic and Steady State) in the calculation of C_i^* and R_d . The compensation point of CO₂ (C_i^*) was calculated with the Dynamic (green) and the Steady State (orange) method, in four day/night growth temperature regimes of 16/10, 22/16, 28/22, and 34/28°C (A). The mitochondrial respiration (R_d) was calculated using the dynamic (green bars) and Steady State (orange bars), in four day/night growth temperature regimes of 16/10, 22/16, 28/22 and 34/28°C (B). All data points are presented (points), bars are means $\pm$ standard error (n = 3).

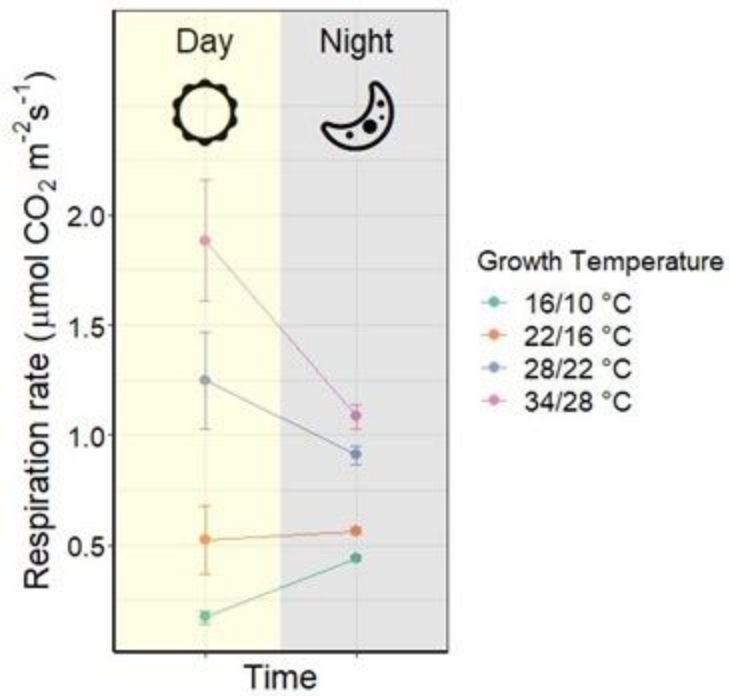


Figure 4

Respiration rate in the day and night in four different growth temperatures. Respiration rate in the daytime (yellow background) and nighttime (gray background), under temperature regimes of 16/10°C (green), 22/16°C (orange), 28/22°C (purple) and 34/28°C (pink). Mean values are presented $\pm$ standard error (n = 3).