A model of photosynthetic CO_2 assimilation by C_3 leaves accounting for anaplerotic flux into the Calvin-Benson cycle

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Summary

- Plants assimilate carbon primarily via the Calvin-Benson cycle. Analysing isotope abundances in sunflower leaf starch, we recently reported evidence for anaplerotic carbon flux from the plastidial oxidative pentose phosphate pathway into this cycle. Additionally, we reported isotope-derived flux rates ($v_{a,p}$) at varying atmospheric CO_2 concentration, C_a.

- To test these results by another method, we analysed gas exchange data from the same leaves and experiment by FvCB-type models considering respiration and energy recycling by the plastidial anaplerotic pathway.

- Consistent with previous results, we found $v_{a,p}$ proceeds at ≈ 46%, 25%, and 0% relative to net CO_2 assimilation at C_a ≈ 180, 280, 450 ppm, respectively. Day respiration proceeds at ≈ 58%, 32%, 5%, 8%, and 13% relative to net CO_2 assimilation at C_a ≈ 180, 280, 450, 700, and 1500 ppm, respectively. Effects of the anaplerotic pathway on metabolism are comprehensively characterised.

- The results are discussed in relation to regulatory properties of the plastidial and cytosolic anaplerotic pathway, the proposed variability of mesophyll conductance to CO_2 diffusion, and the contribution of day respiration to the A/C_i curve drop at high C_a. Overall, our work indicates the plastidial anaplerotic pathway is a central component in carbon and energy metabolism of C_3 plants.

Keywords: Calvin-Benson cycle; oxidative pentose phosphate pathway; glucose-6-phosphate shunt; Farquhar-von Caemmerer-Berry photosynthesis model; net CO_2 assimilation; day respiration; ATP-to-NADPH ratio

Introduction

Anaplerotic carbon flux into the Calvin-Benson cycle (CBC; for a list of abbreviations and symbols see Table 1) can occur by two metabolic pathways (Fig. 1). The first pathway (here denoted as cytosolic pathway) was proposed by Eicks *et al.* (2002) on discovering the xylulose 5-phosphate/phosphate translocator in *Arabidopsis thaliana*. This translocator enables exchange of pentose phosphates, and inorganic phosphate between chloroplasts and the cytosol. Screening *Arabidopsis* genome databases, Eicks *et al.* (2002) found genes encoding for the cytosolic oxidative branch of the pentose phosphate pathway (OPPP) as well as cytosolic ribulose-5-phosphate epimerase and ribose-5-phosphate isomerase. Genes encoding for cytosolic transketolase and transaldolase required to process pentose phosphates were missing. Because of the apparent lack of fate for pentose phosphates in the cytosol, Eicks *et al.* (2002) proposed their translocation into chloroplasts and injection into the CBC. Recently, two studies on *Camelina sativa* leaf metabolism reported this pathway carries high flux (≈ 5% relative to net CO_2 assimilation, A) and supplies significant amounts of cytosolic NADPH (≈ 10% relative to A) at an atmospheric CO_2 concentration (C_a) of ≈ 400 ppm (Wieloch & Sharkey, 2022; Xu *et al.*, 2022).

The second pathway (here denoted as plastidial pathway) was proposed by Sharkey and Weise (2016). It involves glucose 6-phosphate (G6P) to ribulose 5-phosphate (Ru5P) conversion by the plastidial OPPP and injection of the latter into the CBC. For *Pinus nigra*, we reported evidence consistent with flux through this pathway under drought (Wieloch *et al.*, 2018, 2022b). In sunflower, significant flux occurs when plants raised at $C_a \approx 450$ ppm are moved into low or high C_a (≈ 180, 280, 700, 1500 ppm) whereas flux at $C_a \approx 450$ ppm was not significantly greater than zero (Wieloch *et al.*, 2022a; Wieloch, 2022). By contrast, Xu *et al.* (2021) reported, in *Camelina sativa*, significant flux occurs at $C_a \approx 400$ ppm corresponding to the conditions the plants were raised in. However, several assumptions of this study were criticised (Wieloch, 2021) and, after implementing amendments, the result was not confirmed (Xu *et al.*, 2022).

In sunflower, we estimated flux through the plastidial anaplerotic pathway based on a hydrogen isotope signal in leaf starch (the term isotope signal denotes systematic variability in relative isotope abundance) (Wieloch *et al.*, 2022a; Wieloch, 2022). Assuming 50% of the net assimilated carbon becomes leaf starch (Sharkey *et al.*, 1985), anaplerotic flux proceeds at > 7%, > 5%, 0%, ≈ 2%, and ≈ 5% relative to A at $C_a \approx 180$, 280, 450, 700, and 1500 ppm, respectively (Wieloch, 2022). However, flux at 180 and 280 ppm is probably strongly

underestimated since, under these conditions, much of the hydrogen isotope signal can be expected to not arrive in starch (Wieloch *et al.*, 2022a). Thus, the present study aims at estimating flux through the plastidial anaplerotic pathway at low C_a using another technique.

For each molecule Ru5P synthesised anaplerotically, one molecule CO_2 is released from metabolism while two molecules $NADP^+$ are reduced (Fig. 1). Thus, we hypothesise anaplerotic flux affects leaf gas exchange and can be traced by gas exchange modelling. To test this, we expand Farquhar-von Caemmerer-Berry (FvCB) photosynthesis models by terms accounting for anaplerotic respiration and energy recycling. Using gas exchange data of sunflower, we estimate anaplerotic flux rates and associated effects on leaf carbon and energy metabolism. These data are unique in that they were collected during the same experiment and from the same plants that previously provided isotope evidence for flux through the plastidial anaplerotic pathway (Wieloch *et al.*, 2022a; Wieloch, 2022). The results are discussed (*inter alia*) in relation to (i) previously reported flux estimates, (ii) regulatory properties of the plastidial and cytosolic anaplerotic pathway, (iii) the proposed variability of mesophyll conductance to CO_2 diffusion (g_m) with C_a and during photosynthesis induction, and (iv) the contribution of day respiration to the often-seen A/C_i curve drop at high C_a. We conclude with a paragraph on model criticism.

Theory

Photosynthesis models accounting for anaplerotic flux into the Calvin-Benson cycle

In the FvCB photosynthesis model (Farquhar *et al.*, 1980), net CO_2 assimilation is given as

$$A = V_c - 0.5V_o - R_d = V_c(1 - 0.5\,V_o/V_c) - R_d = V_c(1 - 0.5\Phi) - R_d \qquad \text{Eqn 1}$$

where R_d denotes day respiration, V_o and V_c denote the oxygenation and carboxylation rates of ribulose 1,5-bisphosphate (RuBP), respectively, and Φ denotes the V_o/V_c ratio. The model assumes photorespiration is a closed cycle releasing 1 molecule CO_2 per 2 oxygenations.

Making day respiration by the plastidial anaplerotic pathway ($R_{a,p}$) explicit yields

$$A = V_c(1 - 0.5\Phi) - R_{a,p} - R_x \qquad \text{Eqn 2}$$

where R_x denotes day respiration by pathways other than the plastidial anaplerotic pathway.

Different biochemical processes may exert control over A. RuBP-regeneration-limited CO_2 assimilation (A_j) presumably results from a shortage of incoming electrons supplied to the CBC and photorespiration as NADPH and ATP (Farquhar *et al.*, 1980). Here, we assume NADPH rather than ATP supply is limiting. Electrons for $NADP^+$ reduction come from the linear electron transport pathway (1 mol NADPH requires 2 mol electrons). Additionally, per 1 mol CO_2 respired by anaplerotic pathways, 2 mol NADPH (4 mol electrons) are recycled (Fig. 1). However, only NADPH from the plastidial pathway can be recycled into the CBC and photorespiration. NADPH from the cytosolic pathway cannot enter chloroplasts (Wieloch & Sharkey, 2022). Thus, overall electron supply is given as

$$e^{-}supply\ rate = J + J_{\mathrm{a,p}} = J + 4R_{\mathrm{a,p}} \qquad \text{Eqn 3}$$

where J denotes the rate of linear electron transport, and $J_{\mathrm{a,p}}$ denotes the rate of electron recycling by the plastidial anaplerotic pathway.

According to Farquhar *et al.* (1980), the rate of electron consumption by the CBC and photorespiration is given as

$$e^{-}consumption\ rate = V_{\mathrm{c}}(4 + 4\Phi) \qquad \text{Eqn 4}$$

Balancing electron supply (Eqn. 3) with electron consumption (Eqn. 4) yields

$$J + 4R_{\mathrm{a,p}} = V_{\mathrm{c}}(4 + 4\Phi) \qquad \text{Eqn 5}$$

Solving equation 5 for V_{c} yields

$$V_{\mathrm{c}} = \frac{J + 4R_{\mathrm{a,p}}}{4 + 4\Phi} \qquad \text{Eqn 6}$$

Using equation 6 to remove V_{c} from equation 2 yields

$$A_{\mathrm{j}} = \frac{J + 4R_{\mathrm{a,p}}}{4 + 4\Phi}(1 - 0.5\Phi) - R_{\mathrm{a,p}} - R_{\mathrm{x}} \qquad \text{Eqn 7}$$

Net CO_2 assimilation with electrons recovered by the plastidial anaplerotic pathway in the form of NADPH (Fig. 1) is given as

$$A_{\mathrm{a,p}} = \frac{4R_{\mathrm{a,p}}}{4 + 4\Phi}(1 - 0.5\Phi) \qquad \text{Eqn 8}$$

According to Farquhar and von Caemmerer (1982), Φ is related to the CO_2-compensation point in the absence of day respiration (Γ_*) and the CO_2 partial pressure at the active site of rubisco (C_c) as

$$\Phi = \frac{2\Gamma_*}{C_c} \qquad \text{Eqn 9}$$

Using equation 9 to remove Φ from equation 7 yields

$$A_{\mathrm{j}} = \frac{J + 4R_{\mathrm{a,p}}}{4 + 8\,\Gamma_*/C_c}(1 - \Gamma_*/C_c) - R_{\mathrm{a,p}} - R_{\mathrm{x}} \qquad \text{Eqn 10}$$

To quantify flux through the plastidial anaplerotic pathway ($v_{\mathrm{a,p}}$) and associated respiration, equation 10 is solved for $R_{\mathrm{a,p}}$ as

$$v_{\mathrm{a,p}} = R_{\mathrm{a,p}} = -\frac{(A_{\mathrm{j}} + R_{\mathrm{x}})(1 + 2\,\Gamma_*/C_c) - 0.25J(1 - \Gamma_*/C_c)}{3\,\Gamma_*/C_c} \qquad \text{Eqn 11}$$

To calculate V_c, Φ is removed from equation 6 using equation 9 as

$$V_c = \frac{J + 4R_{\mathrm{a,p}}}{4 + 8\,\Gamma_*/C_c} \qquad \text{Eqn 12}$$

Using Φ (Eqn. 9), V_o is calculated as

$$V_o = \Phi * V_c = \frac{2\Gamma_* V_c}{C_c} \qquad \text{Eqn 13}$$

Rubisco-limited CO_2 assimilation (A_c) denotes assimilation limited by CO_2 supply. Under these conditions, rubisco carboxylation rate is given as

$$V_c = \frac{V_{cmax} C_c}{C_c + K_c(1 + O_c/K_o)} \qquad \text{Eqn 14}$$

where V_{cmax} denotes the CO_2-saturated maximum rate of rubisco carboxylation, O_c denotes the oxygen partial pressure at the active site of rubisco, and K_c and K_o denote the Michaelis-Menten constants of rubisco carboxylation and oxygenation, respectively (Farquhar *et al.*, 1980).

Using equations 14 and 9 to respectively remove V_c and Φ from equation 2 yields

$$A_c = \frac{V_{cmax}(C_c - \Gamma_*)}{C_c + K_c(1 + O_c/K_o)} - R_{a,p} - R_x \qquad \text{Eqn 15}$$

Effect of respiration by the plastidial anaplerotic pathway on A/C_i curves

RuBP-regeneration-limited case: Anaplerotic flux of 1 mol is associated with the release of 1 mol CO_2 and the supply of 2 mol NADPH (Fig. 1). If this NADPH is used quantitatively by the CBC (requires 2 NADPH per V_c), then all CO_2 is reassimilated. In this theoretical scenario, A_j does not change in response to $R_{a,p}$. That is, gas exchange data do not convey any information on $R_{a,p}$ no matter how large $R_{a,p}$ is. By contrast, photorespiratory use of NADPH from the anaplerotic pathway (requires 2 NADPH per V_o) involves the release of 0.5 mol CO_2 by photorespiration per 1 mol CO_2 from the anaplerotic pathway (i.e., a total of 1.5 mol CO_2 is released). Thus, in the RuBP-regeneration-limited case, $R_{a,p}$ affects A/C_i curves because of photorespiration (C_i denotes the CO_2 concentration in intercellular air spaces). The absolute effect of flux through the plastidial anaplerotic pathway on A/C_i curves (ΔA_j) is given as

$$\Delta A_j = 1.5 R_{a,p} \frac{V_o}{V_c + V_o} = \frac{1.5 R_{a,p} \Phi}{1 + \Phi} = \frac{3 R_{a,p}}{2 + C_c/\Gamma_*} \qquad \text{Eqn 16}$$

To calculate $R_{a,p}$ from observed A_j offsets, equation 16 is solved for $R_{a,p}$ as

$$R_{a,p} = \frac{\Delta A_j (1 + \Phi)}{1.5\Phi} = \frac{\Delta A_j (2 + C_c/\Gamma_*)}{3} \qquad \text{Eqn 17}$$

The relative effect of respiration by the plastidial anaplerotic pathway on A/C_i curves is given as

$$\frac{\Delta A_\mathrm{j}}{R_\mathrm{a,p}} = \frac{1.5\Phi}{1+\Phi} = \frac{3}{2 + C_\mathrm{c}/\Gamma_*} \qquad \text{Eqn 18}$$

The larger Φ, the larger the ΔA_j / $R_\mathrm{a,p}$ ratio (Fig. 2, black line). That is, the higher photorespiration, the larger the effect of respiration by the plastidial anaplerotic pathway on A/C_i curves. At $\Phi = 2$ (i.e., $C_\mathrm{c} = \Gamma_*$, see Eqn. 9), $\Delta A_\mathrm{j} = R_\mathrm{a,p}$ (i.e., $\Delta A_\mathrm{j} / R_\mathrm{a,p} = 1$). If $\Phi > 2$, then $\Delta A_\mathrm{j} > R_\mathrm{a,p}$. If $\Phi < 2$, then $\Delta A_\mathrm{j} < R_\mathrm{a,p}$.

Under rubisco-limited CO_2 assimilation, RuBP concentrations are above saturation level. Hence, NADPH from the anaplerotic pathway can be expected to exert no control on A_c. Shifts in $R_\mathrm{a,p}$ will cause A_c shifts of the same size.

NADPH and ATP demand of plastidial carbon metabolism

NADPH demand by the CBC and photorespiration is given as (Farquhar *et al.*, 1980)

$$NADPH\ demand = 2V_\mathrm{c} + 2V_\mathrm{o} = V_\mathrm{c}(2 + 2\Phi) \qquad \text{Eqn 19}$$

This demand is met by NADPH supply from the light reactions. In the presence of flux through the plastidial anaplerotic pathway, NADPH demand from the light reactions is reduced as

$$NADPH\ demand = 2V_\mathrm{c} + 2V_\mathrm{o} - 2\nu_\mathrm{a,p} = V_\mathrm{c}(2 + 2\Phi) - 2\nu_\mathrm{a,p} \qquad \text{Eqn 20}$$

because anaplerotic flux of 1 mol is associated with the supply of 2 mol NADPH (Fig. 1).

Upon carboxylation of 1 mol RuBP, the CBC requires 3 mol ATP (Farquhar & von Caemmerer, 1982). Upon oxygenation of 1 mol RuBP, photorespiration requires 3 mol ATP in chloroplasts (1.5 mol by phosphoglycerate kinase, 1 mol by phosphoribulokinase, and 0.5 mol by glycerate kinase). This accounting assumes that potentially harmful ammonium released by the mitochondrial glycine decarboxylase complex is immediately recapture by mitochondrial (not plastidial) glutamine synthetase (Taira *et al.*, 2004; Buchanan *et al.*, 2015). ATP required by

mitochondrial glutamine synthetase can be expected to come from mitochondrial oxidative phosphorylation. Hence, ATP demand by the CBC and photorespiration from the light reactions is given as

$$ATP\ demand = 3V_{\mathrm{c}} + 3V_{\mathrm{o}} = V_{\mathrm{c}}(3 + 3\Phi) \qquad \text{Eqn 21}$$

Combining equations 20 and 21, the ratio at which the CBC and photorespiration require ATP and NADPH is given as

$$\frac{ATP\ demand}{NADPH\ demand} = \frac{3V_{\mathrm{c}} + 3V_{\mathrm{o}}}{2V_{\mathrm{c}} + 2V_{\mathrm{o}} - 2v_{\mathrm{a,p}}} = \frac{V_{\mathrm{c}}(3 + 3\Phi)}{V_{\mathrm{c}}(2 + 2\Phi) - 2v_{\mathrm{a,p}}} \qquad \text{Eqn 22}$$

Evidently, the CBC and photorespiration have the same plastidial cofactor demands with a plastidial ATP-to-NADPH demand ratio of 1.5. The ratio is independent of Φ. However, NADPH demands from the light reactions vary with $v_{\mathrm{a,p}}$ resulting in varying ATP-to-NADPH demands from the light reactions.

Materials and Methods

Samples and leaf gas exchange measurements

The sunflower (*Helianthus annuus*) samples were described previously (Wieloch *et al.*, 2022a). In brief, the plants were raised in a greenhouse at $C_{\mathrm{a}} \approx 450$ ppm and a light intensity of 300-400 µmol photons m^{-2} s^{-1} (16 h photoperiod). After 7-8 weeks, they were transferred to a growth chamber (T = 22 °C : 18 °C, rH = 60% : 70%, day : night), kept in darkness for 1 day to drain the starch reserves (see Notes S3 in Wieloch *et al.*, 2022a), and subsequently grown for 2 days in groups of 8 at C_{a} of either ≈ 180, 280, 450, 700, or 1500 ppm (300-400 µmol photons m^{-2} s^{-1}, 16 h photoperiod).

Leaf gas exchange measurements were described previously (see Notes S1 in Wieloch *et al.*, 2022a). In brief, the measurements were performed with a previously described system (Laisk & Edwards, 1997) under conditions similar to those in the growth chamber (C_{a} = 173, 269, 433, 675, 1431 ppm; T = 22 ± 0.5 °C; 300 µmol photons m^{-2} s^{-1}). C_{i} values obtained from gas exchange measurements (136, 202, 304, 516, 1282 ppm) are similar to C_{i} values estimated for the 2-day growth chamber experiments (140, 206, 328, 531, and 1365 ppm) (see Notes S2 in Wieloch *et al.*, 2022a). To constrain V_{cmax}, we additionally measured gas exchange at 900 µmol

photons m^{-2} s^{-1}, C_a = 173, 269, 432, 674, 1430 ppm corresponding to C_i = 126, 188, 264, 450, 1204 ppm.

Parameterisation of photosynthesis models

C_c was calculated according to Fick's first law as

$$C_c = C_i - \frac{A}{g_m} \qquad \text{Eqn 23}$$

using g_m = 0.45 mol CO_2 m^{-2} s^{-1} reported for sunflower at 20 °C, 600 µmol photons m^{-2} s^{-1} (Schäufele *et al.*, 2011). For rubisco extracted from sunflower, Genkov *et al.* (2010) reported K_c = 19 µM CO_2, K_o = 640 µM O_2, and a relative CO_2/O_2 specificity ($S_{c/o}$) of 77 [M/M] at 25 °C. Γ_* at 25 °C was calculated based on published procedures (von Caemmerer, 2000) as

$$\Gamma_* = \frac{0.5 O_c}{S_{c/o}} \qquad \text{Eqn 24}$$

with O_c = 276 µM. To convert from concentration to partial pressure, solubilities for CO_2 of 0.0334 mol l^{-1} bar^{-1} and O_2 of 0.00126 mol l^{-1} bar^{-1} were used. Since correction factors for sunflower are unavailable, we used Q_{10} values reported in Table 2.3 of von Caemmerer (2000) and equation 2.32 in von Caemmerer (2000) to correct for leaf temperature (22 °C). This gave K_c = 447 µbar CO_2, K_o = 439 mbar O_2, and Γ_* = 48.9 µbar CO_2. To estimate V_{cmax}, equation 15 was fitted to gas exchange data obtained at 900 µmol photons m^{-2} s^{-1}, C_a = 173, 269, 432 ppm (Fig. 3). Under standard conditions (400 ppm CO_2, 21% O_2, 20-25 °C), day respiration usually proceeds at ≈ 5% relative to the rate of A (Tcherkez *et al.*, 2017; Xu *et al.*, 2022). Fixing $R_{a,p}$ + R_x at this rate (0.83 µmol CO_2 m^{-2} s^{-1}), we found V_{cmax} = 85.5 µmol m^{-2} s^{-1}. This is similar to a previously reported estimate for sunflower of 80 µmol m^{-2} s^{-1} at 20 °C (Jacob & Lawlor, 1991; Wullschleger, 1993).

Based on Farquhar and Wong (1984), J is given as

$$J = \frac{I_2 + J_{max} - \sqrt{(I_2 + J_{max})^2 - 4\theta I_2 J_{max}}}{2\theta} \qquad \text{Eqn 25}$$

where θ denotes is the empirical curvature factor of the light response of electron transport commonly set to 0.7 (Evans, 1989), J_{max} denotes the light-saturated rate of linear electron transport, and I_2 denotes useful light absorbed by photosystem II given as

$$I_2 = I * \mathrm{abs}(1 - \mathrm{f})/2 \qquad \text{Eqn 26}$$

where I denotes incident irradiance, abs denotes the absorptance of leaves commonly set to 0.85, and f denotes a correction factor for the spectral quality of light commonly set to 0.15 (Evans, 1987). Since gas exchange data collected at very high irradiance are unavailable, J_{max} was estimated based on its close relationship with V_{cmax} according to published procedures (Walker *et al.*, 2014) as

$$\ln(J_{\mathrm{max}}) = \beta_1 + \beta_2 \ln(V_{\mathrm{cmax}}) \qquad \text{Eqn 27}$$

Using parameter estimates pertaining to data reported by Wullschleger (1993) (β_1=1.425, β_2=0.837, R^2=87.2, p<.001, n=110), we estimate J_{max} = 172.2 µmol m^{-2} s^{-1}.

Results

Metabolic phases of the *A*/*C*$_i$ curve

A/*C*$_i$ curves enable identification of metabolic processes limiting net CO_2 assimilation (Farquhar *et al.*, 1980). Initial linear increases at low C_i are commonly attributed to rubisco-limited assimilation. A change point in the response of A to C_i marks the onset of RuBP-regeneration-limited assimilation. Furthermore, under high light, *A*/*C*$_i$ curves may level out at high C_i which is commonly attributed to limited triose phosphate utilisation (Sharkey, 1985).

Despite the relatively low resolution of our *A*/*C*$_i$ curve, two phases are evident (Fig. 4a). An initial linear increase (dashed line) is followed by RuBP-regeneration-limited assimilation whereas levelling out at high C_i is not evident. Hence, triose phosphate utilisation did not affect A_m (denoting measured net CO_2 assimilation). This is as expected considering the low irradiance applied during data collection (300 µmol photons m^{-2} s^{-1}).

Previously, we have shown that flux through the plastidial anaplerotic pathway is not significantly different from zero at $C_a \approx$ 450 ppm (Wieloch *et al.*, 2022a; Wieloch, 2022).

Hence, RuBP-regeneration-limited assimilation for this datapoint as derived from equation 10 is given as

$$A_{\mathrm{j}} = \frac{J}{4 + 8\,\Gamma_*/C_{\mathrm{c}}}(1 - \Gamma_*/C_{\mathrm{c}}) - R_{\mathrm{x}} \qquad \text{Eqn 28}$$

Equation 28 was fitted to A_{m} by varying R_{x} until the offset between modelled and measured ≈ 450 ppm values was zero (Fig. 4b, red line). The condition was met at R_{x} = 0.6 µmol CO_2 m^{-2} s^{-1}. Hence, at ≈ 450 ppm, day respiration proceeds at 4.9% relative to A_{m} which agrees well with previous reports. Under standard conditions (400 ppm CO_2, 21% O_2, 20-25 °C), day respiration commonly proceeds at ≈ 5% relative to A_{m} (Tcherkez *et al.*, 2017; Xu *et al.*, 2022). The agreement corroborates the validity of the model parameterisation.

Using R_{x} = 0.6 µmol CO_2 m^{-2} s^{-1} and assuming $R_{\mathrm{a,p}}$ = 0, we next modelled rubisco-limited assimilation based on equation 15 (Fig. 4b, black line). Above C_{a} ≈ 110 ppm (C_{i} ≈ 95 ppm), we found that $A_{\mathrm{c}} > A_{\mathrm{j}} \geq A_{\mathrm{m}}$ (Fig. 4b, black line versus red line versus black dots). This indicates that A_{m} at C_{a} > 110 ppm does not results from rubisco-limited but RuBP-regeneration-limited assimilation.

Effects of varying rates of day respiration on the *A*/*C*$_{\mathrm{i}}$ curve

Based on changes in hydrogen isotope fractionation in starch extracted from the leaves studied here, we previously estimated $R_{\mathrm{a,p}}$ proceeds at > 7%, > 5%, 0%, ≈ 2%, and ≈ 5% relative to A_{m} at C_{a} ≈ 180, 280, 450, 700, and 1500 ppm, respectively (Table 2) (Wieloch, 2022). That is, $R_{\mathrm{a,p}}$ exhibits a change point in response to C_{a} at C_{a} ≈ 450 ppm. Seeing that there are positive offsets between A_{m} and A_{j} both above and below C_{a} ≈ 450 ppm (Table 2; Fig. 4b, red line versus black dots), this same change point appears to be present in A_{m}, and $R_{\mathrm{a,p}}$ can be expected to contribute to the offsets.

Using equation 11, we estimate $R_{\mathrm{a,p}}$ proceeds at 46%, 25%, 0%, 9%, and 45% relative to A_{m} at C_{a} ≈ 180, 280, 450, 700, and 1500 ppm, respectively (Table 2). Estimates at low C_{a} (≈ 180, and 280 ppm) are consistent with isotope-derived estimates because, based on properties of the biochemical system, the isotope approach can be expected to underestimates $R_{\mathrm{a,p}}$ at low C_{a} (see Wieloch *et al.*, 2022a). By contrast, the isotope approach is believed to not strongly underestimate $R_{\mathrm{a,p}}$ at high C_{a} (Wieloch, 2022). However, gas-exchange estimates of $R_{\mathrm{a,p}}$ at high

C_a (≈ 700, and 1500 ppm) are considerably larger than isotope-derived estimates. This may suggest that gas exchange modelling overestimates $R_{a,p}$ at high C_a.

Overestimation of $R_{a,p}$ at high C_a may derive from variability of R_x. To follow up on this, we solve the A_j model (Eqn. 10) for R_x and substitute A_j by A_m as

$$R_x = \frac{J + 4R_{a,p}}{4 + 8\,\Gamma_*/C_c}(1 - \Gamma_*/C_c) - R_{a,p} - A_m \qquad \text{Eqn 29}$$

Using isotope-derived $R_{a,p}$ values as inputs, we estimate R_x proceeds at 5.8% and 7.8% relative to A_m at C_a ≈ 700, and 1500 ppm, respectively (Table 2). Based on regulatory properties of leaf respiratory processes, these estimates seem reasonable (see Discussion). Taken together, our results suggest A_m is a function of RuBP-regeneration-limited assimilation and variability of $R_{a,p}$ and R_x (Eqn. 10). Figure 5 summarises estimated carbon and associated NADPH fluxes.

Integration of day respiration in carbon metabolism

Based on estimations and assumptions given above, $R_{a,p}$ proceeds at ≈ 46%, 25%, 0%, 2%, and 5% relative to A_m at C_a ≈ 180, 280, 450, 700, and 1500 ppm, respectively (Fig. 6a). Thus, it governs R_d at low C_a (≈ 80% contribution at ≈ 180, and 280 ppm) and contributes substantially to R_d at high C_a (≈ 25% at ≈ 700 ppm, and ≈ 40% at ≈ 1500 ppm; Fig. 6b). Taken together, R_d proceeds at ≈ 58%, 32%, 5%, 8%, and 13% relative to A_m at C_a ≈ 180, 280, 450, 700, and 1500 ppm, respectively (Table 2). While $R_{a,p}$ exceeds photorespiratory CO_2 release at high C_a (≈ 1500 ppm), it proceeds at ≈ 50% relative to the rate of photorespiratory CO_2 release at low C_a (≈ 180, and 280 ppm; Fig. 6c). Furthermore, $R_{a,p}$ accounts for ≈ 40% of total CO_2 release at both low and high C_a (≈ 180, 280, and 1500 ppm; Fig. 6d). At low C_a (≈ 180, and 280 ppm), $R_{a,p}$ accounts for ≈ 15% of V_c (Fig. 6e) and thus causes significant futile carbon cycling involving CO_2 uptake by the CBC and release by the plastidial anaplerotic pathway. At the same C_a, ≈ 10% of all RuBP is provided by the plastidial anaplerotic pathway (Fig. 6f). When plotted against A_m, $R_{a,p}$ increases nonlinearly both below and above C_a ≈ 450 ppm (Fig. 6g). Towards very low A_m (< 5 μmol m^{-2} s^{-1}, C_a < 180 ppm), $R_{a,p}$ may approach a maximum. Above A_m = 12.3 μmol m^{-2} s^{-1} (C_a ≈ 450 ppm), $R_{a,p}$ and R_x both increase at approximately the same rate (Fig. 6g-h, ≈ 700 ppm: $R_{a,p}$ = 0.3 μmol m^{-2} s^{-1} and R_x = 0.26 μmol m^{-2} s^{-1}, ≈ 1500 ppm: $R_{a,p}$ = 0.87 μmol m^{-2} s^{-1} and R_x = 0.75 μmol m^{-2} s^{-1}).

Energy recovery by the plastidial anaplerotic pathway and associated CO_2 assimilation

Initial NADPH inputs required to assimilate CO_2 respired by the plastidial anaplerotic pathway exceed NADPH outputs of this pathway (Table 3). However, relative NADPH losses decrease as Φ decreases with NADPH recovery converging one. Accordingly, CO_2 release by the anaplerotic pathway ($R_{a,p}$) exceeds CO_2 recovery with NADPH from the anaplerotic pathway ($A_{a,p}$, Table 3). Relative CO_2 recovery increases with decreasing Φ and converges one ($A_{a,p}$ / $R_{a,p}$). Hence, $R_{a,p}$ is larger than the effect that flux through the plastidial anaplerotic pathway has on A/C_i curves (ΔA_j, Table 3). With decreasing Φ, the relative effect of flux through the plastidial anaplerotic pathway on the A/C_i curve decreases and converges zero (ΔA_j / $R_{a,p}$, Table 3, black circles in Fig. 2). Taken together, anaplerotic flux is energetically wasteful and thus decreases net CO_2 assimilation but this is partly offset by NADPH recovery. Recovered NADPH is most efficiently used for CO_2 recovery when photorespiration is low since photorespiration is energetically wasteful and releases additional CO_2 (see Theory).

Effects of the plastidial anaplerotic pathway on energy metabolism

In the present study, light was maintained at 300 μmol photons m^{-2} s^{-1} across all C_a treatments. Accordingly, linear electron transport supplied a steady amount of NADPH (Fig. 5b, yellow bars). Above and below $C_a \approx 450$ ppm, the plastidial anaplerotic pathway supplied additional NADPH yet no ATP (Fig. 5b, light green bars). Balancing the ensuing NADPH surplus with the demands of the CBC and photorespiration requires additional inputs of ATP from photophosphorylation (Fig. 7, black circles). Hence, the ATP-to-NADPH demand ratio of the light reactions increases both above and below $C_a \approx 450$ ppm with increasing flux through the plastidial anaplerotic pathway (Fig. 7, red triangles).

Across all C_a treatments, ATP demands by the CBC and photorespiration (Fig. 7, black circles) exceed the estimated ATP supply related to linear electron transport including Q cycling (Table 4). ATP deficits increase with increasing flux through the plastidial anaplerotic pathway. This indicates a requirement for cyclic electron flux through photosystem I. We estimate cyclic electron flux proceeds at ≈ 20% of the rate of whole electron flux when the plastidial anaplerotic pathway is inactive but may increase to above 30% when this pathway is active. Thus, flux through the plastidial anaplerotic pathway may affect the light reactions of photosynthesis.

Discussion

Expanding FvCB photosynthesis models by terms accounting for respiration and energy recycling by the plastidial anaplerotic pathway (Eqs. 10, and 15), we estimated flux through this pathway in sunflower leaves based on gas exchange measurements. Our results suggest that the plastidial anaplerotic pathway can be an important player in plant carbon and energy metabolism affecting properties such as the rate of day respiration, net CO_2 assimilation, net carboxylation, net oxygenation (photorespiration), RuBP regeneration, NADPH availability, the ATP-to-NADPH demand ratio of plastidial carbon metabolism, and probably the contribution of the cyclic electron pathway to supplying ATP (Figs. 5-7, Tables 2-4). Commonly, initial linear increases in A/C_i curves are interpreted in terms of rubisco-limited net CO_2 assimilation (Farquhar *et al.*, 1980). In line with recent isotope evidence (Wieloch *et al.*, 2022a; Wieloch, 2022), it has been attributed here to respiration from the plastidial anaplerotic pathway (Fig. 4).

Consistent evidence in support of flux through the plastidial anaplerotic pathway at low C_a

According to the gas exchange analysis presented here, flux through the plastidial anaplerotic pathway increases with decreasing C_a below $C_a \approx 450$ ppm (Table 2, Fig. 5a). At $C_a \approx 280$ ppm and ≈ 180 ppm, flux proceeds at $\approx 25\%$ and $\approx 46\%$ relative to the rate of net CO_2 assimilation, respectively (Fig. 6a). This is consistent with both previously reported flux estimates and regulatory properties of the pathway as discussed in the following two paragraphs.

Applying another method to the same sunflower samples, we previously estimated that flux through the plastidial anaplerotic pathway proceeds at > 5% and > 7% relative to the rate of net CO_2 assimilation at $C_a \approx 280$ ppm and ≈ 180 ppm, respectively (Wieloch *et al.*, 2022a; Wieloch, 2022). These estimates rely on a hydrogen isotope signal in starch which is introduced in the starch biosynthesis pathway at the level of G6P H^1. G6P may be converted back to fructose 6-phosphate (F6P) by plastidial phosphoglucose isomerase (PGI), and F6P may leave the starch biosynthesis pathway via transketolase. Combinedly, G6P to F6P conversion and F6P use by transketolase can be expected to cause washout of the isotope signal from the starch biosynthesis pathway. As evident from an isotope signal at starch glucose H^2, the plastidial PGI reaction is strongly removed from equilibrium on the side of F6P at $C_a \geq 450$ ppm (Wieloch *et al.*, 2022a; Wieloch, 2022). This is thought to impede back conversion of G6P to F6P and concomitant signal washout. However, the PGI reaction moves progressively towards

equilibrium as C_a decreases below 450 ppm (Wieloch *et al.*, 2022a; Wieloch, 2022). This is thought to result in increasing back conversion of G6P to F6P and signal washout. Thus, increasing offsets with decreasing C_a between $v_{a.p}$ estimates from isotope and gas exchange analysis probably derive from increasing washout of the isotope signal.

Flux through the plastidial anaplerotic pathway is thought to be controlled at the level of PGI (Sharkey & Weise, 2016). Under illumination and medium to high C_a, the reaction was found to be significantly removed from equilibrium on the side of F6P resulting in low plastidial G6P concentrations (Dietz, 1985; Gerhardt *et al.*, 1987; Kruckeberg *et al.*, 1989; Schleucher *et al.*, 1999; Wieloch *et al.*, 2022a; Wieloch, 2022). Low G6P concentrations are thought to restrict the flux through the anaplerotic pathway (Sharkey & Weise, 2016). With shifts from medium to low C_a, the PGI reaction moves towards equilibrium and plastidial G6P concentrations increase (Dietz, 1985; Wieloch *et al.*, 2022a; Wieloch, 2022). This may result in increasing flux through the anaplerotic pathway by the following mechanism (Sharkey & Weise, 2016). Under illumination, the first enzyme of the plastidial anaplerotic pathway, glucose-6-phosphate dehydrogenase (G6PD), is downregulated by a thioredoxin dependent mechanism (Née et al., 2009). However, downregulation can be reversed allosterically by increasing G6P concentration (Cossar *et al.*, 1984; Preiser *et al.*, 2019).

Physiological function of flux through the plastidial anaplerotic pathway at low C_a

Flux through the anaplerotic pathway lowers net CO_2 assimilation below theoretically possible values (Fig. 4b, black dots versus red line). As a result of this flux, sunflower leaves merely achieve ≈ 89% and ≈ 77% of their theoretical assimilatory potential at $C_a \approx 280$ ppm and ≈ 180 ppm, respectively. Similarly, the anaplerotic pathway recovers only part of the energy it consumes (Fig. 5b), and energy dissipation is certainly not physiologically beneficial under RuBP-regeneration limited conditions. Thus, for the conditions and period studied here, the anaplerotic pathway appears to be detrimental for both leaf carbon and energy balances.

On the other hand, RuBP supply may be a potentially beneficial pathway property as proposed previously (Wieloch *et al.*, 2022a). Sunflowers studied here were raised at $C_a \approx 450$ ppm. Moving them into low-C_a atmospheres probably caused longer-lasting divergences from whole-plant steady state which could not be overcome within the two-day acclimation period. More specifically, the carbon demands of heterotrophic tissues had probably been adapted to the carbon inputs at $C_a \approx 450$ ppm. At $C_a < 450$ ppm, rates of carbon export from leaves to

heterotrophic tissues may then have exceeded net CO_2 assimilation rates (source limitation). Additional inputs of RuBP into the CBC and photorespiration by the anaplerotic pathway may have helped to counteract adverse effects of a carbon deficit in the regeneration part of the CBC caused by excessive carbon export from chloroplasts. It remains to be seen whether flux through the anaplerotic pathway also occurs when plants are raised and analysed at the same C_a. Our 450 ppm measurements may indicate that this is not the case. For now, we recommend raising and analysing plants at the same C_a when studying whole-plant steady state conditions.

RuBP may undergo either oxygenation or carboxylation at rubisco. With decreasing C_a, oxygenation rates become substantial (Table 3, ≈ 1 and ≈ 1.5 mol RuBP oxygenation per 2 mol RuBP carboxylation at C_a ≈ 280 ppm and ≈ 180 ppm, respectively). Thus, much of the anaplerotically supplied RuBP feeds into photorespiration. As proposed previously, maintaining photorespiration through inputs of RuBP might make sense physiologically in the context of the concept of photorespiration-linked *de novo* nitrogen assimilation (Wieloch *et al.*, 2022a). Reportedly, photorespiration rate correlates positively with the rate of *de novo* nitrate assimilation (Bloom, 2015), and it seems plausible that plants would shift metabolism towards nitrogen assimilation when carbon assimilation is impeded (at low C_a). Assimilated nitrogen may support *de novo* protein synthesis which may result in increased photosynthetic capacity at the enzyme level and help to overcome source limitations.

Metabolic origin of day respiration at high C_a

In our gas exchange analysis, respiration by the plastidial anaplerotic pathway was fixed at 0%, 2%, and 5% relative to the rate of net CO_2 assimilation at C_a ≈ 450, 700, and 1500 ppm, respectively (Fig. 5a, Table 2). These $R_{a,p}$ estimates come from a previously published isotope analysis of the samples studied here (Wieloch *et al.*, 2022a; Wieloch, 2022). Increases in $R_{a,p}$ above C_a ≈ 450 ppm are consistent with regulatory properties of the plastidial anaplerotic pathway (Wieloch, 2022). Towards high C_a, plastidial G6P concentrations increase with net CO_2 assimilation (Dietz, 1985). This can be expected to cause increased G6PD activity and flux through the plastidial anaplerotic pathway (see above). Note, in contrast to the low-C_a case (see above), $R_{a,p}$ increases at high C_a are not caused by PGI regulation (Wieloch, 2022).

Consistent with previous reports (Tcherkez *et al.*, 2017; Xu *et al.*, 2022), we estimated respiration by processes other than the plastidial anaplerotic pathway proceeds at ≈ 5% relative to the rate of net CO_2 assimilation at C_a ≈ 450 ppm (Fig. 5a, Table 2). At C_a ≈ 700 ppm and ≈

1500 ppm, R_x increases to ≈ 6% and ≈ 8% relative to the rate of net CO_2 assimilation, respectively. These increases are consistent with regulatory properties of the cytosolic OPPP which has recently been identified as a major source of day respiration (Xu *et al.*, 2022; Wieloch, 2022). Flux through the cytosolic OPPP and associated respiration is controlled at the level of the first pathway enzyme, G6PD. Under illumination, cytosolic G6PD activity increases with glucose concentration through *de novo* enzyme synthesis (Hauschild & von Schaewen, 2003). In line with this, respiration at 5 °C was shown to increase with glucose concentration and overall leaf soluble sugar concentration (Tjoelker *et al.*, 2009; Wieloch, 2022). Generally, increasing C_a results in increasing leaf soluble sugar concentration (Ainsworth & Long, 2005) and may thus cause increasing flux through and respiration by the cytosolic OPPP.

Does g_m vary with C_a?

Using the online carbon isotope discrimination method and/or the variable *J* method (Evans *et al.*, 1986; Harley *et al.*, 1992), several authors reported nonlinear relationships between g_m and C_a with similar patterns across different species including sunflower (Flexas *et al.*, 2007; Hassiotou *et al.*, 2009; Vrábl *et al.*, 2009; Xiong *et al.*, 2015). As C_a increases, g_m initially increases up to a change point beyond which it decreases again. This response is strikingly similar to the C_a-response of day respiration reported here. As C_a increases, $R_{a,p}$ initially decreases up to a change point beyond which it increases again along with R_x (Fig. 5a, Table 2). Day respiration and g_m have opposite effects on net CO_2 assimilation. That is, both processes could explain offsets between measured and theoretically possible values of net CO_2 assimilation (Fig. 4b, black dots versus red line). However, changes in day respiration estimated here based on gas exchange analysis are corroborated by results from an independent isotope analysis (Wieloch *et al.*, 2022a; Wieloch, 2022). Furthermore, underlying metabolic mechanisms seem straight forward (see above). By contrast, mechanisms underlying the C_a-response of g_m have remained unclear despite considerable research interest over the past three decades (Nadal *et al.*, 2021). Furthermore, several authors suggested that much of the observed variability of g_m may be due to methodological shortcomings (Tholen *et al.*, 2012; Gu & Sun, 2014; Nadal *et al.*, 2021). Our results corroborate this viewpoint. Therefore, we recommend expanding formula of the variable *J* method by terms accounting for variability in day respiration. Furthermore, we recommend expanding formula of the online carbon isotope discrimination method by terms accounting for variability in isotope fractionation of photorespiration and day respiration.

Respiration by the plastidial anaplerotic pathway during photosynthetic induction

Flux through the plastidial anaplerotic pathway and associated respiration depend on G6PD activity. Plastidial G6PD is inhibited by reduced thioredoxin (Née et al., 2009). However, reduction of the plastidial thioredoxin pool after illumination is not instantaneous but builds up over the course of several minutes (Scheibe, 1981). This was shown to result in a gradual activation of plastidial malate dehydrogenase (Scheibe, 1981). Similarly, gradual inhibition of plastidial G6PD can be expected. Thus, flux through the plastidial anaplerotic pathway may persist over the course of several minutes after illumination decreasing from initially higher to lower levels. This would affect day respiration, net CO_2 assimilation, NADPH supply, the isotopic composition of respired CO_2, etc. Future studies on the variability of photosynthetic parameters such as g_m during photosynthetic induction (*cf.* Kaiser *et al.*, 2017; Sakoda *et al.*, 2021; Liu *et al.*, 2022) are encouraged to consider this as a possibility.

Does day respiration explain part of the A/C_i curve drop at high C_a?

If R_d is held constant, rubisco-limited and RuBP-regeneration-limited net CO_2 assimilation modelled by the canonical FvCB equations increase continuously with increasing C_a (Farquhar *et al.*, 1980). However, especially under high light, measured A/C_i curves are frequently seen to level out or even decline at high C_a (e.g., Sharkey, 1985; Harley & Sharkey, 1991). According to our analysis, day respiration increases from ≈ 5% relative to the rate of net CO_2 assimilation at $C_a \approx 450$ ppm over ≈ 8% at $C_a \approx 700$ ppm to ≈ 13% at $C_a \approx 1500$ ppm (Fig. 5a, Table 2). This is consistent with positive responses of R_d to C_a reported by some authors (e.g., Wang *et al.*, 2001). By contrast, other authors have reported independency between R_d and C_a (e.g., Ayub *et al.*, 2011).

Increases in R_d are consistent with regulatory properties of the plastidial and cytosolic OPPP (see above). As C_a increases from medium to high values, plastidial G6P concentrations and leaf soluble sugar concentrations increase (Dietz, 1985; Ainsworth & Long, 2005). This can be expected to activate plastidial and cytosolic G6PD (Cossar *et al.*, 1984; Hauschild & von Schaewen, 2003; Preiser *et al.*, 2019), increase respiration by the plastidial and cytosolic OPPP and explain part of the A/C_i curve drop at high C_a. Furthermore, it may explain part of the lower than expected stimulation of net CO_2 assimilation by increasing C_a (Wieloch, 2022). However, high sink strengths (e.g., in vigorously growing plants) may counteract build-up of leaf soluble sugar concentrations and respiration by the plastidial and cytosolic OPPP. This may explain why some studies found R_d increases at high C_a whereas others did not.

Model criticism

Generally, modelling net CO_2 assimilation by FvCB-type models relies on numerous assumptions (Tcherkez & Limami, 2019), and the present study is no exception. For instance, our models assume that photorespiration is a closed cycle. However, some of the carbon entering photorespiration may actually be diverted into other pathways such as *de novo* nitrogen assimilation (Busch *et al.*, 2018). Similarly, in our A_j model (Eqn. 10), the CBC and photorespiration are assumed to consume all energy. However, part of the available energy can be expected to be utilised by other plastidial processes such as the reduction of nitrite to ammonia (Buchanan *et al.*, 2015) or NAD(P)H shuttling to other cell compartments (Scheibe, 2004). Furthermore, our modelling assumes constant absolute R_x at $C_a \leq 450$ ppm (Table 2). However, leaf soluble sugar concentration may decrease with decreasing C_a (Ainsworth & Long, 2005). This may cause a reduction in G6PD activity and respiration by the cytosolic OPPP (Hauschild & von Schaewen, 2003).

That said, our estimate of R_x at $C_a \approx 450$ ppm is consistent with previously reported estimates (Tcherkez *et al.*, 2017; Xu *et al.*, 2022). Furthermore, estimates of flux through the plastidial anaplerotic pathway from gas exchange analyses presented here are consistent with previously reported isotope-derived estimates (Wieloch *et al.*, 2022a; Wieloch, 2022). Lastly, all estimates of day respiration are consistent with regulatory properties of the plastidial and cytosolic anaplerotic pathway. We hope that model extensions and ideas presented here will help to advance photosynthesis modelling across scales.

Table 1 List of abbreviations and symbols

Abbreviation	Definition
CBC	Calvin-Benson cycle
F6P	fructose 6-phosphate
FvCB	Farquhar-von Caemmerer-Berry
G6P	glucose 6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
OPPP	oxidative branch of the pentose phosphate pathway
PGI	phosphoglucose isomerase
Ru5P	ribulose 5-phosphate
RuBP	ribulose 1,5-bisphosphate
Symbol	**Definition**
A	rate of net CO_2 assimilation
A_c	rate of rubisco-limited CO_2 assimilation
A_j	rate of RuBP-regeneration-limited CO_2 assimilation
A_m	measured net CO_2 assimilation
abs	absorptance of leaves
C_a	atmospheric CO_2 concentration
C_c	CO_2 partial pressure or concentration at the active site of rubisco
C_i	CO_2 partial pressure or concentration in intercellular air spaces
g_m	mesophyll conductance to CO_2 diffusion
I	incident irradiance
I_2	useful light absorbed by photosystem II
J	rate of linear electron transport
$J_{a,p}$	rate of electron recycling by the plastidial anaplerotic pathway
J_{max}	light-saturated rate of linear electron transport
K_c	Michaelis-Menten constants of rubisco carboxylation
K_o	Michaelis-Menten constants of rubisco carboxylation
O_c	oxygen partial pressure at the active site of rubisco
R_d	day respiration rate
$R_{a,p}$	rate of respiration by the plastidial anaplerotic pathway
R_x	rate of respiration by pathways other than the plastidial anaplerotic pathway
$S_{c/o}$	relative CO_2/O_2 specificity of rubisco
T	temperature
V_c	rubisco carboxylation rate
V_{cmax}	CO_2-saturated maximum rate of rubisco carboxylation
V_o	rubisco oxygenation rate
Γ_*	CO_2-compensation point in the absence of day respiration
ΔA_j	absolute effect of flux through the plastidial anaplerotic pathway on A/C_i curves
θ	empirical curvature factor of the light response of electron transport
$v_{a,p}$	rate of flux through the plastidial anaplerotic pathway
Φ	V_o/V_c ratio

Table 2 Rates of day respiration in sunflower leaves.

C_a [ppm]	A_m [µmol m^{-2} s^{-1}]	A_j - A_m [a] [µmol m^{-2} s^{-1}]	$R_{a,p}$ = $v_{a,p}$ [b] [µmol m^{-2} s^{-1}]	$R_{a,p}$ / A_m %	$R_{a,p}$ / A_m [d] %	R_x [e] [µmol m^{-2} s^{-1}]	R_x / A_m %
≈ 180	5.13	1.55	2.37	46.3	> 7	0.60	11.8
≈ 280	8.46	1.10	2.12	25.1	> 5	0.60	7.1
≈ 450	12.29	0	0	0	0	0.60	4.9
≈ 700	14.88	0.33	1.34 [c]	9.0 [c]	≈ 2	0.86	5.8
≈ 1500	17.33	0.84	7.78 [c]	44.9 [c]	≈ 5	1.35	7.8

Plants were grown in a greenhouse at an atmospheric CO_2 concentration (C_a) of ≈ 450 ppm and then moved to growth chambers. After a day in darkness to drain the starch reserves, the plants were grown at different levels of C_a (180, 280, 450, 700, 1500 ppm) corresponding to different levels of intercellular CO_2 concentration (C_i = 140, 206, 328, 531, and 1365 ppm) for two days. During gas exchange measurements slightly lower C_i levels prevailed (136, 202, 304, 516, 1282 ppm). Symbols: A_j, modelled RuBP-regeneration-limited CO_2 assimilation; A_m, measured CO_2 assimilation; $R_{a,p}$, respiration by the plastidial anaplerotic pathway; R_x, day respiration by processes other than the plastidial anaplerotic pathway; $v_{a,p}$, flux through the plastidial anaplerotic pathway.

[a] A_j estimates based on equation 28 with R_x = 0.6 µmol CO_2 m^{-2} s^{-1}.

[b] Estimates based on gas exchange data (Eqn. 11).

[c] Probably overestimated (see text).

[d] Estimates based on previously published isotope data (Wieloch, 2022).

[e] Estimates based on equation 28 for C_a ≈ 180, 280, and 450 ppm, and equation 29 for C_a ≈ 700, and 1500 ppm.

Table 3 Energy recovery by the plastidial anaplerotic pathway and associated CO_2 assimilation in sunflower leaves.

C_a [ppm]	Φ	$R_{a,p}$ [a] [µmol m^{-2} s^{-1}]	abs. NADPH input [b] [µmol m^{-2} s^{-1}]	abs. NADPH output [c] [µmol m^{-2} s^{-1}]	rel. NADPH recovery [d]	abs. CO_2 recovery ($A_{a,p}$) [µmol m^{-2} s^{-1}]	rel. CO_2 recovery [e]	ΔA_j [µmol m^{-2} s^{-1}]	ΔA_j / $R_{a,p}$
≈ 180	0.77	2.37	8.41	4.75	0.56	0.82	0.35	1.55	0.65
≈ 280	0.53	2.12	6.49	4.25	0.66	1.03	0.48	1.10	0.52
≈ 450	0.35	0	0	0	---	0	---	0	---
≈ 700	0.20	0.30	0.71	0.60	0.83	0.22	0.75	0.07	0.25
≈ 1500	0.08	0.87	1.87	1.73	0.93	0.77	0.89	0.09	0.11

Plants were grown in a greenhouse at an atmospheric CO_2 concentration (C_a) of ≈ 450 ppm and then moved to growth chambers. After a day in darkness to drain the starch reserves, the plants were grown at different levels of C_a (180, 280, 450, 700, 1500 ppm) corresponding to different levels of intercellular CO_2 concentration (C_i = 140, 206, 328, 531, and 1365 ppm) for two days. During gas exchange measurements slightly lower C_i levels prevailed (136, 202, 304, 516, 1282 ppm). Symbols: $A_{a,p}$, net CO_2 assimilation with electrons recovered by the plastidial anaplerotic pathway in the form of NADPH; $R_{a,p}$, respiration by the plastidial anaplerotic pathway; ΔA_j, absolute effect of flux through the plastidial anaplerotic pathway on the A/C_i curve (RuBP-regeneration-limited case); Φ, oxygenation-to-carboxylation ratio of rubisco.

[a] Estimates based on equation 11 with R_x values from Table 1.

[b] = $(2+2\Phi)R_{a,p}$ (derivation of Eqn. 4: 2 electrons per NADPH)

[c] = $2R_{a,p}$ (see Fig. 1)

[d] = NADPH output / input

[e] = $A_{a,p}$ / $R_{a,p}$

Table 4 Effects of the plastidial anaplerotic pathway on electron transport in sunflower leaves.

C_a [ppm]	180	280	450	700	1500
LET [µmol m^{-2} s^{-1}]	84.2	84.2	84.2	84.2	84.2
ATP from LET [µmol m^{-2} s^{-1}]	54.1	54.1	54.1	54.1	54.1
ATP deficit [µmol m^{-2} s^{-1}]	16.1	15.4	9.0	9.9	11.6
CET [µmol m^{-2} s^{-1}]	37.7	35.9	21.0	23.1	27.1
CET/(CET+LET)	0.31	0.30	0.20	0.22	0.24

Plants were grown in a greenhouse at an atmospheric CO_2 concentration (C_a) of ≈ 450 ppm and then moved to growth chambers. After a day in darkness to drain the starch reserves, the plants were grown at different levels of C_a (180, 280, 450, 700, 1500 ppm) for two days. Gas exchange measurements were performed at a light intensity of 300 µmol photons m^{-2} s^{-1}. Estimation of linear electron transport (LET) and cyclic electron transport (CET) both including Q cycling respectively assumed transmembrane transport of 3 and 2 protons per electron and production of 3 ATP per 14 protons by ATP synthase (Seelert *et al.*, 2000). Processes not considered include the water-water cycle (affects the ATP-to-NADPH supply ratio of electron transport), GAP/3PGA cycles and the chloroplast malate valve (affect ATP-to-NADPH demand ratios of carbon metabolism).

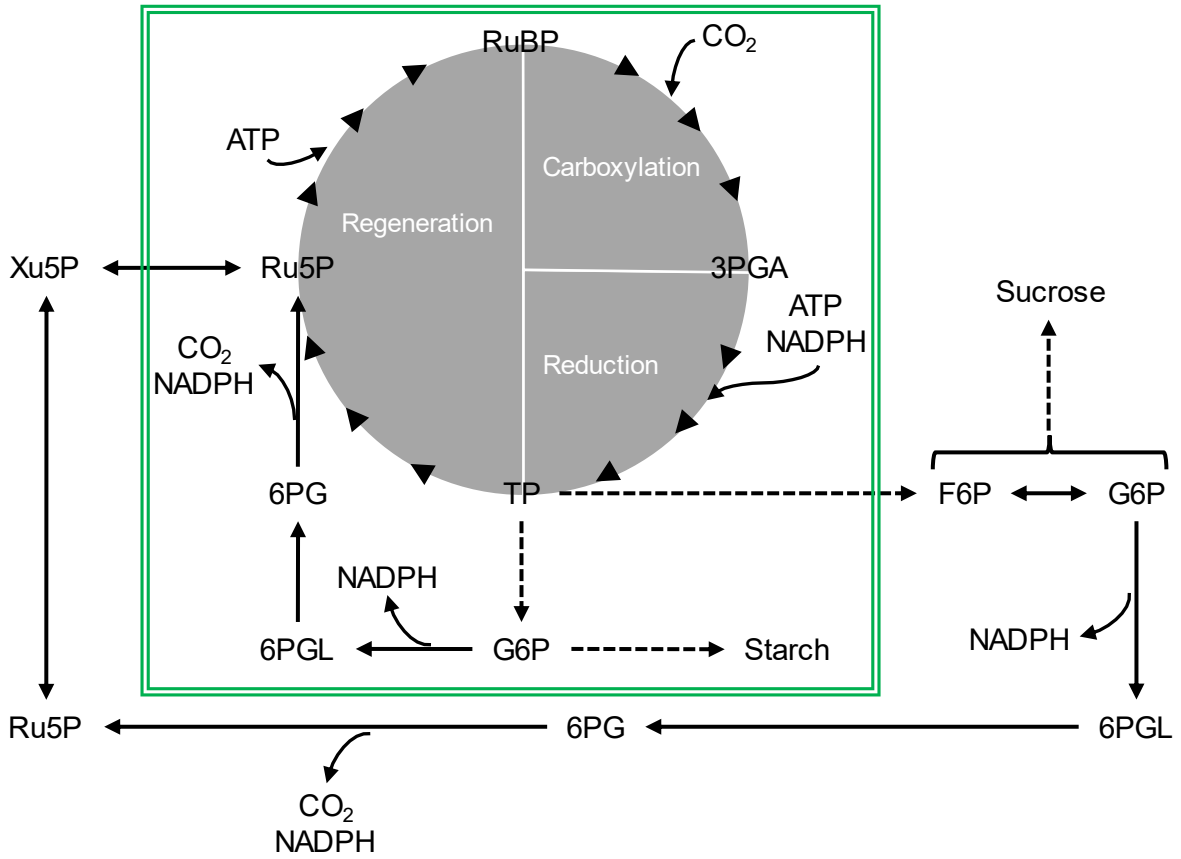

Figure 1 Anaplerotic carbon flux into the Calvin-Benson cycle. Metabolites: 3PGA, 3-phosphoglycerate; 6PG, 6-phosphogluconate; 6PGL, 6-phosphogluconolactone; ATP, adenosine triphosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; G6P, glucose 6-phosphate; NADPH, nicotinamide adenine dinucleotide phosphate; Ru5P, ribulose 5-phosphate; RuBP, ribulose 1,5-bisphosphate; TP, triose phosphate (glyceraldehyde 3-phosphate, dihydroxyacetone phosphate); Xu5P, Xylulose 5-phosphate. Chloroplast and cytosolic metabolism inside and outside the green box, respectively. Solid and dashed arrows represent transformations catalysed by a single enzyme or multiple enzymes, respectively.

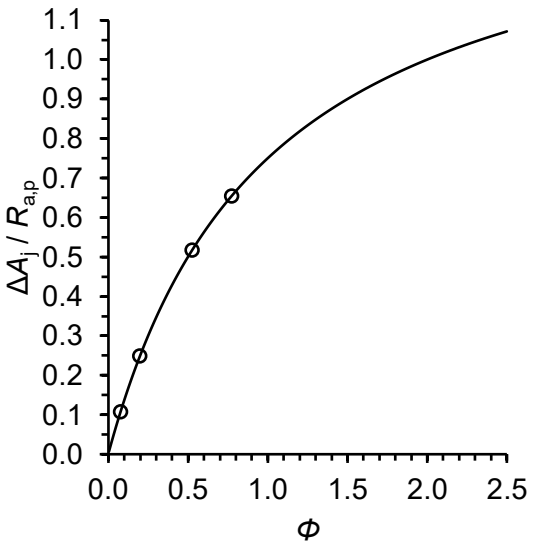

Figure 2 Relative effect of respiration by the plastidial anaplerotic pathway on A/C_i curves under RuBP-regeneration-limited conditions (ΔA_j / $R_{a,p}$) as function of the oxygenation-to-carboxylation ratio of rubisco (Φ).

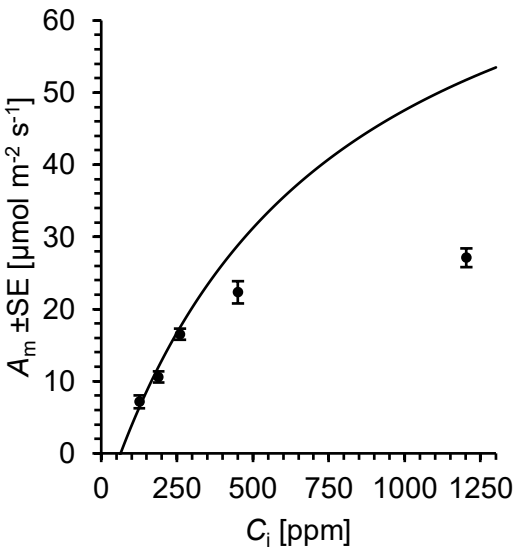

Figure 3 Measured net CO_2 assimilation (A_m) of sunflower leaves as function of intercellular CO_2 concentration (C_i). Plants were raised in a greenhouse at an atmospheric CO_2 concentration (C_a) of ≈ 450 ppm and a light intensity of 300-400 µmol photons m^{-2} s^{-1}. Gas exchange measurements were performed at 22 °C, 900 µmol photons m^{-2} s^{-1}, and atmospheric CO_2 concentrations of C_a = 173, 269, 432, 674, 1430 ppm corresponding to intercellular CO_2 concentrations of C_i = 126, 188, 264, 450, 1204 ppm. Solid line: Best fit between the data at C_a = 173, 269, 432 ppm and a model of rubisco-limited A (Eqn. 15). Model inputs: C_c = 111, 168, 226 µbar, K_c = 447 µbar CO_2, K_o = 439 mbar O_2, and Γ_* = 48.9 µbar CO_2, O_c = 206373 µbar, $R_{a,p} + R_x$ = 0.83 µmol CO_2 m^{-2} s^{-1}. Model output: V_{cmax} = 85.5 µmol m^{-2} s^{-1}.

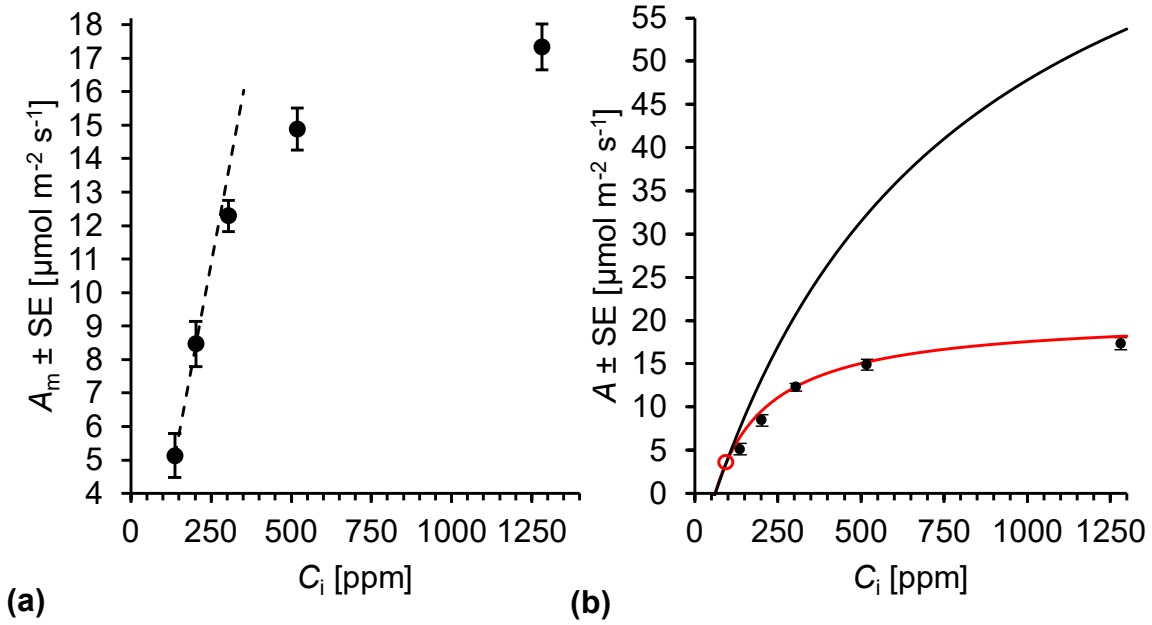

Figure 4 Net CO_2 assimilation (A) of sunflower leaves as function of intercellular CO_2 concentration (C_i). Black dots: measured values (A_m, n = 8). Dashed black line: Trendline passing through the two lowest A_m values. Solid black and red lines: modelled rubisco-limited assimilation (A_c) and RuBP-regeneration-limited assimilation (A_j), respectively (respiration by the plastidial anaplerotic pathway not considered). Red circle: intersection between A_c and A_j. Plants were grown in a greenhouse at an atmospheric CO_2 concentration (C_a) of ≈ 450 ppm and then moved to growth chambers. After a day in darkness to drain the starch reserves, plants were grown at different levels of C_a (180, 280, 450, 700, 1500 ppm) corresponding to different levels of C_i (140, 206, 328, 531, and 1365 ppm) for two days. During gas exchange measurements slightly lower C_i levels prevailed (136, 202, 304, 516, 1282 ppm). A_j-model inputs: Γ_* = 48.9 µbar CO_2, J_{max} = 172.2 µmol m^{-2} s^{-1}, θ = 0.7, I = 300 µmol photons m^{-2} s^{-1}, abs = 0.85, and f = 0.15. A_j-model output: $R_{a,p} + R_x$ = 0.6 µmol CO_2 m^{-2} s^{-1}. A_c-model inputs: K_c = 447 µbar CO_2, K_o = 439 mbar O_2, and Γ_* = 48.9 µbar CO_2, O_c = 207548 µbar, V_{cmax} = 85.5 µmol m^{-2} s^{-1}, $R_{a,p} + R_x$ = 0.6 µmol CO_2 m^{-2} s^{-1}.

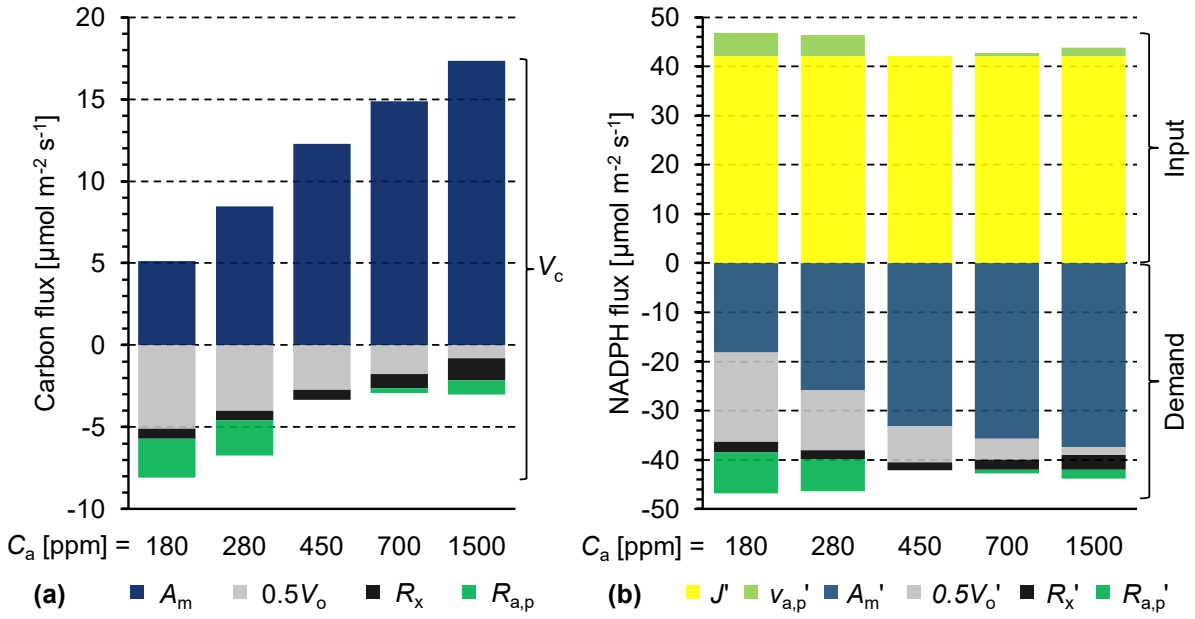

Figure 5 Carbon **(a)** and NADPH fluxes **(b)** at varying atmospheric CO_2 concentration (C_a) in sunflower leaves. Estimates come from modelling RuBP-regeneration-limited CO_2 assimilation. The model considers the Calvin-Benson cycle, the photorespiration cycle, the plastidial anaplerotic pathway, and respiration by processes other than the plastidial anaplerotic pathway. Plants were grown in a greenhouse at $C_a \approx 450$ ppm and then moved to growth chambers. After a day in darkness to drain the starch reserves, plants were grown at different levels of C_a (180, 280, 450, 700, 1500 ppm) corresponding to different levels of C_i (140, 206, 328, 531, and 1365 ppm) for two days. During gas exchange measurements slightly lower C_i levels prevailed (136, 202, 304, 516, 1282 ppm). NADPH demands of individual carbon fluxes (see Fig. 5a) were calculated as $(2 + 2\Phi)$ * flux with flux = {$-A_m$; $0.5V_o$; R_x; $R_{a,p}$}. Symbols: A_m, measured net CO_2 assimilation; A_m', NADPH demand of net CO_2 assimilation; J', NADPH supply by whole chain electron transport ($0.5J$); $R_{a,p}$, respiration by the plastidial anaplerotic pathway; $R_{a,p}$', NADPH demand for assimilation of CO_2 respired by the plastidial anaplerotic pathway; R_x, day respiration by processes other than the plastidial anaplerotic pathway; R_x', NADPH demand for assimilation of CO_2 respired by processes other than the plastidial anaplerotic pathway; V_c, rubisco carboxylation; $0.5V_o$, photorespiratory CO_2 release; $0.5V_o$', NADPH demand for assimilation of CO_2 respired by photorespiration; $v_{a,p}$', NADPH supply by the plastidial anaplerotic pathway ($2v_{a,p}$); Φ, oxygenation-to-carboxylation ratio of rubisco.

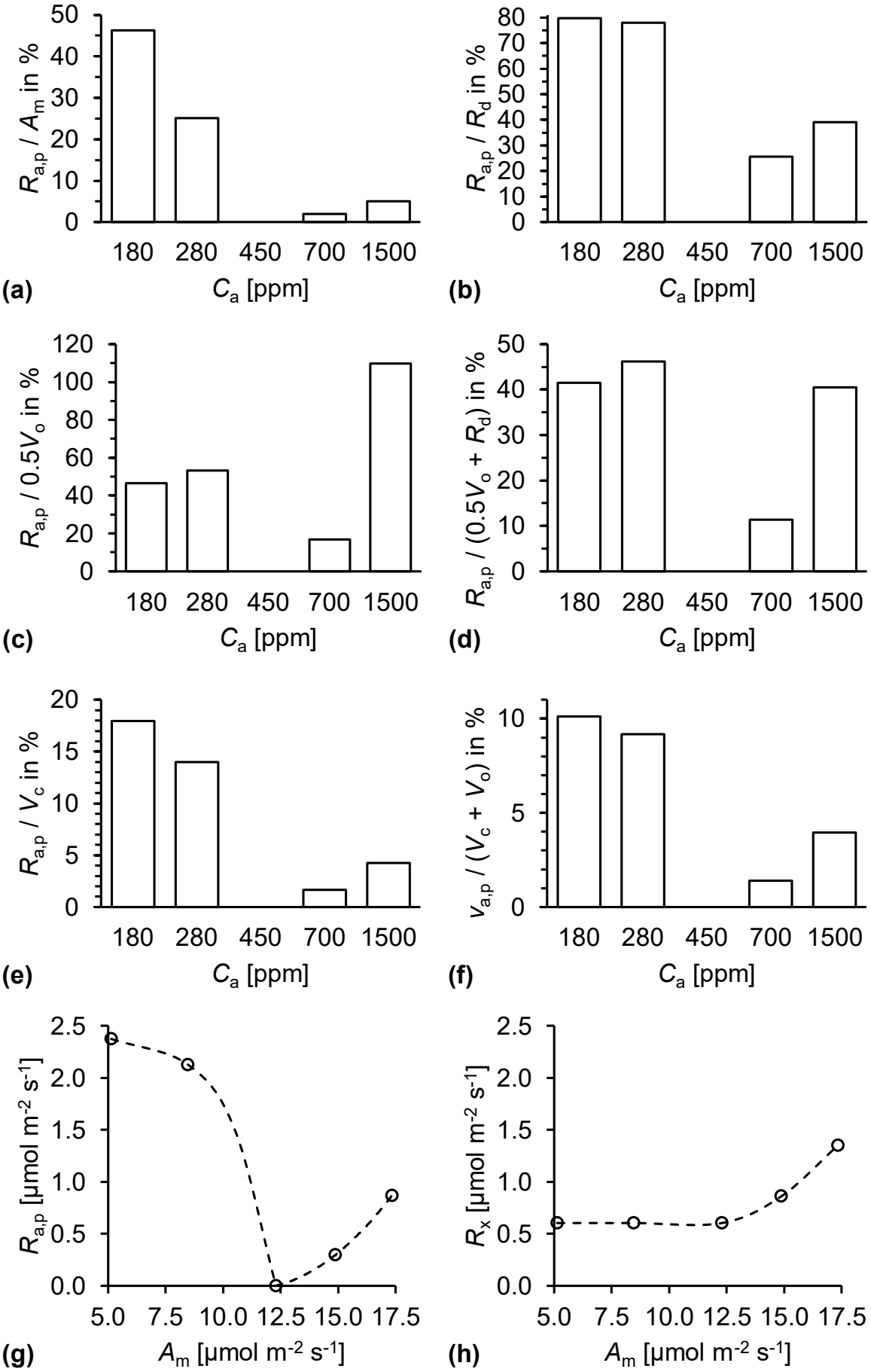
(a)
$R_{a,p}$ / A_m in %
C_a [ppm]
(b)
$R_{a,p}$ / R_d in %
C_a [ppm]
(c)
$R_{a,p}$ / 0.5V_o in %
C_a [ppm]
(d)
$R_{a,p}$ / (0.5V_o + R_d) in %
C_a [ppm]
(e)
$R_{a,p}$ / V_c in %
C_a [ppm]
(f)
$v_{a,p}$ / (V_c + V_o) in %
C_a [ppm]
(g)
$R_{a,p}$ [µmol m^{-2} s^{-1}]
A_m [µmol m^{-2} s^{-1}]
(h)
R_x [µmol m^{-2} s^{-1}]
A_m [µmol m^{-2} s^{-1}]

Figure 6 Integration of day respiration in carbon metabolism of sunflower leaves. Plants were grown in a greenhouse at $C_a \approx 450$ ppm and then moved to growth chambers. After a day in darkness to drain the starch reserves, plants were grown at different levels of C_a (180, 280, 450, 700, 1500 ppm) corresponding to different levels of C_i (140, 206, 328, 531, and 1365 ppm) for two days. During gas exchange measurements slightly lower C_i levels prevailed (136, 202, 304, 516, 1282 ppm). Symbols: A_m, measured CO_2 assimilation; C_a, atmospheric CO_2 concentration; C_i, intercellular CO_2 concentration; $R_{a,p}$, respiration by the plastidial anaplerotic pathway; R_d, total day respiration; R_x, day respiration by processes other than the plastidial anaplerotic pathway; V_c, rubisco carboxylation; V_o, rubisco oxygenation; $v_{a,p}$, flux through the plastidial anaplerotic pathway. All panels show discrete data. Dashed lines in added to guide the eye.

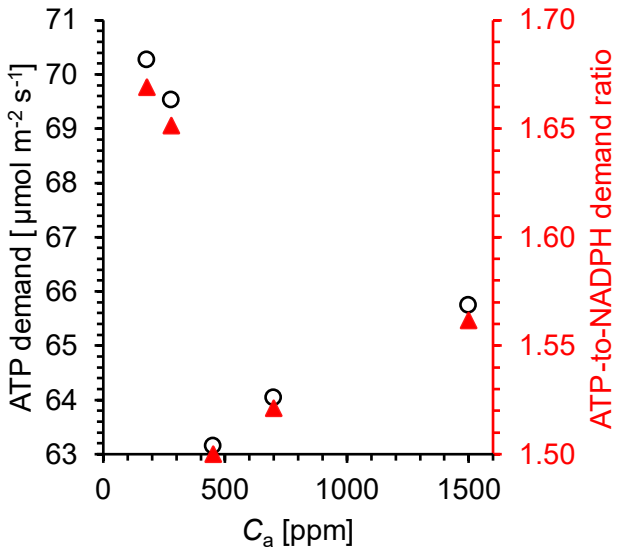

Figure 7 Energy demand from the light reactions of photosynthesis at varying atmospheric CO_2 concentration (C_a) in sunflower leaves. Black circles: ATP demand. Red triangles: ATP-to-NADPH demand ratio. Energy fluxes were estimated from carbon flux estimates from modelling RuBP-regeneration-limited CO_2 assimilation. The model considers the Calvin-Benson cycle, the photorespiration cycle, the plastidial anaplerotic pathway, and respiration by processes other than the plastidial anaplerotic pathway. Plants were grown in a greenhouse at $C_a \approx 450$ ppm and then moved to growth chambers. After a day in darkness to drain the starch reserves, plants were grown at different levels of C_a (180, 280, 450, 700, 1500 ppm) corresponding to different levels of C_i (140, 206, 328, 531, and 1365 ppm) for two days. During gas exchange measurements slightly lower C_i levels prevailed (136, 202, 304, 516, 1282 ppm).

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Author contributions

T.W. conceived the study, led the research, expanded FvCB models, and analysed the data. A.A., and J.S. prepared samples, and acquired gas exchange data. T.W. wrote the paper with input from A.A.

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