Understanding the Low Photosynthetic Rates of Sun and Shade Coffee Leaves: Bridging the Gap on the Relative Roles of Hydraulic, Diffusive and Biochemical Constraints to Photosynthesis

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Abstract

It has long been held that the low photosynthetic rates (A) of coffee leaves are largely associated with diffusive constraints to photosynthesis. However, the relative limitations of the stomata and mesophyll to the overall diffusional constraints to photosynthesis, as well as the coordination of leaf hydraulics with photosynthetic limitations, remain to be fully elucidated in coffee. Whether the low actual A under ambient CO₂ concentrations is associated with the kinetic properties of Rubisco and high (photo)respiration rates also remains elusive. Here, we provide a holistic analysis to understand the causes associated with low A by measuring a variety of key anatomical/hydraulic and photosynthetic traits in sun- and shade-grown coffee plants. We demonstrate that leaf hydraulic architecture imposes a major constraint on the maximisation of the photosynthetic gas exchange of coffee leaves. Regardless of the light treatments, A was mainly limited by stomatal factors followed by similar limitations associated with the mesophyll and biochemical constraints. No evidence of an inefficient Rubisco was found; rather, we propose that coffee Rubisco is well tuned for operating at low chloroplastic CO₂ concentrations. Finally, we contend that large diffusive resistance should lead to large CO₂ drawdown from the intercellular airspaces to the sites of carboxylation, thus favouring the occurrence of relatively high photorespiration rates, which ultimately leads to further limitations to A.

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Introduction

Because CO₂ influx and water vapour efflux share a common pathway through the stomatal pores on leaf surfaces, a trade-off between transpirational costs and CO₂ assimilation is implicitly unavoidable. The coupling between stomatal conductance (gs) to CO₂ and water vapour (and the need to maintain a proper leaf water balance) has often been evidenced by the strong positive scaling between gs and the leaf hydraulic conductance per unit area, KH [1–3]. In turn, the significance of KH as a potentially limiting component of the vascular system has been further emphasised by the strong hydraulic-photosynthetic coordination observed across a large sample of diverse species [4]. Furthermore, KH is closely related to the anatomy of the leaf: KH has been shown to be positively related to both the theoretical axial conductivity of the midrib (determined from xylem conduit numbers and dimensions) and the venation density, Dv [3,5]. A unified control of hydraulic and photosynthetic traits may also be further highlighted by comparing shade and sun leaves: the former have lower rates of net CO₂ assimilation (A) and gs, therefore leading to lower demand for water and correspondingly lower KL and Dv [4,6].

In addition to stomatal limitations, A is currently known to be constrained by mesophyll conductance (gmn), which is defined as the conductance for the transfer of CO₂ from the intercellular airspaces (Ci) to the sites of carboxylation in the chloroplastic stroma (Cj) [7]. According to Flexas et al. [8], gmn limitations to photosynthesis are of similar magnitude as stomatal constraints and generally greater than biochemical limitations. Increasing evidence has shown that gmn is often intrinsically co-regulated with gs [8]. More recently, Ferrio et al. [9] showed a positive scaling between gmn and KH and proposed that water and CO₂ share an important portion of their respective diffusion pathways through the mesophyll; thus, any downregulation of leaf hydraulics may reduce not only gs but also gmn, both of which contribute to reducing A. Quantification of gmn has become important in predicting leaf photosynthetic parameters using the Farquhar- von Caemmerer-Berry (FvCB) model of leaf photosynthesis [10].

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because such a model underestimates the maximum Rubisco carboxylation rate ($V_{\text{c,max}}$) by considering $g_{\text{m}}$ as being infinite [11,12]. Furthermore, changes in $C_{\text{i}}-C_{\text{a}}$ because of variations in $g_{\text{m}}$, among species result in different $A$ values in plants with the same biochemical activity and $g_{\text{s}}$ [7].

At lower $C_{\text{i}}, A$ is limited by RuBP carboxylase activity, which in turn depends on the concentration, activation and kinetic properties of Rubisco [13]. For example, Rubisco with a higher specificity factor, $S_{c/o}$ (which determines the relative rates of carboxylation and oxygenation by Rubisco at given CO$_2$ and O$_2$ concentrations), could be regarded as conferring an advantage in minimising photosrespiration rates, $R_{\text{p}}$ [14]. Interestingly, Rubisco seems to have evolved towards higher $S_{c/o}$ under stressful conditions leading to low $C_{\text{i}}$, such as drought, salinity and high temperature [14]. Additionally, species evolved under stressful conditions with sclerophyll leaves tend to display a higher $S_{c/o}$, which may again be related to lower $C_{\text{i}}$ [14,15]. However, trade-offs between $S_{c/o}$ (or particularly the affinity for CO$_2$ ($1/K_{\text{c}}$)) and the carboxylation turnover rate ($k_{\text{cat}}$) have been described, so that there is no Rubisco in nature with both high affinity for CO$_2$ and fast activity [16]. At saturating $C_{\text{i}}, A$ becomes limited by the capacity for the regeneration of RuBP (often dominated by the electron transport capacity) but also by $S_{c/o}$ [17]. In any case, when studying the ecophysiology of plants, Rubisco kinetic parameters should not be considered a constant but rather as an active part determining the biochemical potential of plants to assimilate CO$_2$ under optimal and suboptimal conditions.

Coffee is an evergreen perennial shrub with hypostomatous leaves that evolved in the African forest understoreys and is thus considered a shade-demanding species. However, in many situations, modern coffee cultivars grow well without shade and even out-yield shaded coffee [18,19]. At atmospheric CO$_2$ concentrations and saturating light, coffee displays low $A$, typically in the range of 4–11 μmol CO$_2$ m$^{-2}$ s$^{-1}$ [20,21], which is in the lower range recorded for trees [22]. However, the maximum $A$ values obtained in common species (15–20 μmol CO$_2$ m$^{-2}$ s$^{-1}$), whereas the photosynthetic capacity, determined under true CO$_2$ saturation (350 μmol CO$_2$ mol$^{-1}$ air), reaches values exceeding 30 μmol O$_2$ m$^{-2}$ s$^{-1}$ [21]. Taking the above information together, the low $A$ in coffee might largely result from diffusive constraints to photosynthesis [24]. However, the relative contributions of the stomata and mesophyll to the overall diffusional limitations to photosynthesis, as well as the coordination of leaf hydraulics with photosynthetic limitations, remain to be fully elucidated in coffee. Furthermore, the anticipated low $g_{\text{s}}$ and $g_{\text{m}}$ would compromise the transference of CO$_2$ through stomata and leaf mesophyll to Rubisco sites, therefore decreasing $C_{\text{i}}$ and ultimately favouring RuBP oxygenation in relation to carboxylation [25]. Under these conditions, Rubisco kinetic properties with a high affinity for CO$_2$ may be critical to secure optimal carbon balance and yield.

Although the general descriptors of the ecophysiology of sun-shade plants are well-known, an integrative analysis of these parameters should not be considered a constant but rather as an active part determining the biochemical potential of plants to assimilate CO$_2$ under optimal and suboptimal conditions.

Photosynthesis Rates in Coffee Leaves

Materials and Methods

Plant Material, Growth Conditions and Experimental Design

The experiment was conducted in Viçosa (20°45’S, 42°54’W, altitude 650 m) in southeastern Brazil. Uniform seedlings of Coffea arabica L. cv ‘Catuaí Vermelho IAC 44’ obtained from seeds were grown in 12 L pots containing a mixture of soil, sand and composted manure (4:1:1, v/v/v). Plants were grown either under full sunlight conditions (100% light) or under low light in a shade environment (10% full sunlight). The shade enclosure was constructed using neutral-density black nylon netting, and the plants were kept in these conditions for 12 months before measurements. Throughout the experiment, the plants were grown under naturally fluctuating conditions of temperature and relative air humidity and were fertilised and irrigated as necessary. The pots were randomised periodically to minimise any variation within each light environment. For all samplings and measurements, the youngest fully expanded leaves, corresponding to the third or fourth pair from the apex of the plagiotropic branches, were used. The experiment was arranged in a completely randomised design, with six plants in individual pots per treatment as replicates. The experimental plot included one plant per container.

Morpho-anatomical Features and Related Hydraulic and Diffusive Traits

The specific leaf area (SLA) was computed using the dry mass of 20 leaf discs (1.13 cm diameter each). For anatomical measurements, leaves were collected and fixed in FAA$_70$ for 48 h, followed by storage in 70% (v/v) aqueous ethanol. Samples of the mean region of each leaf blade were embedded in methacrylate (Historesin–Leica Microsystems Nussloch, Heidelberg, Germany) according to the manufacturer. Transverse sections (7 μm thickness), obtained using a rotary microtome (model RM2155, Leica Microsystems Inc., Deerfield, USA), were stained with toluidine blue at pH 4.0 and mounted in synthetic resin (Permout). Anatomical data were quantified using an image analysis program (Image Pro-Plus, version 4.5, Media Cybernetics, Silver Spring, USA). Video images were acquired using a video
camera attached to an Olympus microscope (AX70TRF, Olympus Optical, Tokyo, Japan). The following anatomical data were then assessed: (i) total leaf thickness; (ii) palisade and spongy mesophyll thickness; (iii) upper and lower epidermis thickness; (iv) guard cell length \( (L_g) \); (v) vertical distance from the vein to stomatal epidermis, \( D_v \); (vi) stomatal density (SD, according to DaMatta et al. [29]); (vii) stomatal index that was estimated as \( (S/(E+S)) \times 100 \), where \( S \) and \( E \) are the number of stomata and epidermal cells per unit leaf area, respectively [29]; and (viii) stomatal pore index based on guard cell length \( \text{SPI}_{gcl} \) that was estimated as \( \text{SPI}_{gcl} = SD \times L^2 \). For determining venation traits, samples of leaf lamina were cut from two leaves per plant and cleared as described by Scofield et al. [30]. Regions of approximately 6 mm \(^2\) were imaged at 30X, and \( D_v \) was calculated as the sum of the vein lengths divided by the total image area using the image analysis program described above. Leaf size was estimated, using the maximum leaf widths and lengths, with the equations described by Antunes et al. [31]. Stomatal size and epidermal cell size, as well as the level of coordination between anatomical traits and leaf size (quantified as the deviation from the expected proportional relationship to each other), were all determined following Carins Murphy et al. [6].

Midrib xylem conduits were measured to determine the theoretical midrib axial hydraulic conductance \( (K_h) \), where the conduits were treated as ellipses to calculate \( K_h \) as

\[
K_h = \sum [\pi a^3 b^3 / 644 (a^2 b^2)]
\]

where \( a \) and \( b \) are the long and short internal vessel diameters, and \( \eta \) is the viscosity of water at 25°C [32], further normalising by leaf length and area.

The maximum stomatal conductance to water vapour \( (g_{w_{\text{max}}} \) was calculated according to Frankis et al. [33] as

\[
g_{w_{\text{max}}} = \text{SD} \times a / [v(t + \pi / 2 \sqrt{a/\pi})]
\]

where \( \text{SD} \) is the stomatal density, \( a \) is the diffusivity of water vapour in air, \( v \) is the molar volume of air, and \( t \) is the stomatal pore depth for fully open stomata. Values for standard constants \( a \) and \( v \) were those for 25°C \( (24.9 \times 10^{-6} \) m \(^2\) s \(^{-1}\) and \( 24.4 \times 10^{-3} \) m \(^3\) mol \(^{-1}\), respectively). \( a \) was calculated as \( \pi (p/2)^2 \), where \( p \) is the stomatal pore length, which was approximated as \( L/2 \) according to Franks and Farquhar [34]. \( L \) for fully open stomata was taken as \( L/4 \), assuming guard cells inflate to a circular cross section [33].

**Leaf Hydraulic Conductance**

The leaf hydraulic conductance \( (K_L) \) was estimated according to Brodribb and Holbrook [35] by following the kinetics of water potential \( (\Psi_o) \) relaxation in rehydrated leaf as:

\[
K_L = C \ln(\Psi_o/\Psi_t) / t
\]

where \( C \) is leaf capacitance, estimated using pressure-volume curves [36], \( \Psi_o \) is \( \Psi_o \) before rehydration, and \( \Psi_t \) is \( \Psi_t \) after rehydration for \( t \) seconds. Leaf \( \Psi_t \) was measured using a Scholander-type pressure chamber (model 1000, PMS Instruments, Albany, NY, USA).

**Gas Exchange and Fluorescence Measurements**

Leaf gas exchange and chlorophyll \( a \) fluorescence were measured simultaneously using an open-flow infrared gas-exchange analyser system equipped with a leaf chamber fluorometer (LI-6400XT, Li-Cor, Lincoln, NE, USA). Environmental conditions in the leaf chamber consisted of a leaf-to-air vapour pressure deficit between 1.2 and 2.0 kPa and a leaf temperature of 25°C.

In light-adapted leaves, the actual quantum yield of PSII \( (\Phi_{\text{PSII}}) \) was determined by measuring steady-state fluorescence \( (F_t) \) and maximum fluorescence during a light-saturating pulse of \( c. \) 8,000 \( \mu \)mol m \(^{-2}\) s \(^{-1}\) \( (F_m) \), following the procedures of Genty et al. [37]:

\[
\Phi_{\text{PSII}} = (F_m - F_t) / F_m
\]

The electron transport rate \( (J_t) \) was then calculated as

\[
J_t = \pi \Omega \text{PPFD} \Phi_{\text{PSII}}
\]

where PPFD is the photosynthetically active photon flux density, \( \pi \) is the leaf absorbance and \( \Omega \) is the PSII optical cross section. The product \( \pi \Omega \) was herein determined from the relationship between \( \Phi_{\text{PSII}} \) and \( \Phi_{\text{CO}_2} \), obtained by varying the \( \text{CO}_2 \) concentration under non-photosynthetic conditions in an atmosphere containing less than 1% \( \text{O}_2 \), as described by Valentini et al. [38].

The light respiration rate \( (R_t) \) was determined according to the ‘Laisk-method’ [39], using the \( y \) axis intersection of \( A/\text{C}_i \) (internal \( \text{CO}_2 \) concentration) curves performed at three different PPFD intensities (750, 250 and 75 \( \mu \)mol m \(^{-2}\) s \(^{-1}\)). The rate of mitochondrial respiration at darkness \( (R_0) \) was measured early in the morning in dark-adapted leaves.

The photorespiratory rate of Rubisco \( (R_p) \) was obtained according to Valentini et al. [38] using the following equation:

\[
R_p = 1/12 [J_t - 4(A + R_l)]
\]

Six \( A/\text{C}_i \) curves were obtained from different plants per treatment. In light-adapted leaves, \( A/\text{C}_i \) curves were initiated at an ambient \( \text{CO}_2 \) concentration \( (\text{C}_i) \) of 400 \( \mu \)mol mol \(^{-1}\) under a saturating PPFD of 1000 \( \mu \)mol m \(^{-2}\) s \(^{-1}\). Once steady state was reached, \( \text{C}_i \) was decreased stepwise to 50 \( \mu \)mol mol \(^{-1}\) air. Upon completion of the measurements at low \( \text{C}_i \), \( \text{C}_i \) was returned to 400 \( \mu \)mol mol \(^{-1}\) air to restore the original \( A \). Next, \( \text{C}_i \) was increased stepwise to 2,000 \( \mu \)mol mol \(^{-1}\) air. \( A/\text{C}_i \) curves consisted of 13 different \( \text{C}_i \) values.

\( \text{C}_i \) was calculated after Harley et al. [40] as

\[
\text{C}_i = (\Gamma \cdot (J_t + 8(A + R_l))) / (J_t - 4(A + R_l))
\]

where \( \Gamma \) was determined from the \textit{in vitro} Rubisco specificity factor \( (S_{a/v}) \) as

\[
\Gamma = O / S_{C/O}
\]

\( A \) was taken from gas-exchange measurements, and the \( J_t \) values were obtained from chlorophyll \( a \) fluorescence yield. After estimating \( \text{C}_i \), \( g_{w_{\text{max}}} \) was calculated following Harley et al. [40]:
\[ g_m = A/(C_i - C_c) \]

From the \( A/C_i \) and \( A/C_c \) curves, the maximum carboxylation capacity (\( V_{\text{cmax}} \)) and maximum capacity for electron transport rate (\( J_{\text{max}} \)) were calculated on a \( C_i \) and \( C_c \) basis using the kinetic parameters for coffee described in Martins et al. [27]. The FvCB model was fitted to the data by applying iterative curve fitting (minimum least square difference) using the Microsoft Excel Solver tool (Microsoft Corporation, Redmond, WA, USA). Additionally, \( g_m, V_{\text{cmax}} \) and \( J_{\text{max}} \) were estimated using the Ethier and Livingston [11] method, which is based on fitting \( A/C_i \) curves with a non-rectangular hyperbola version of the FvCB model, relying on the hypothesis that \( g_m \) reduces the curvature of the Rubisco-limited portion of an \( A/C_i \) response curve. Again, the kinetic parameters of Rubisco measured on coffee were used. Corrections for the leakage of \( CO_2 \) and water vapour into and out of the leaf chamber of the Li-6400-40 have been applied to all gas-exchange data, as described by Rodeghiero et al. [41].

The chloroplastic \( CO_2 \) concentration of transition (\( C_{\text{trans}} \)), where \( C_c \) denotes the transition between the Rubisco- and RuBP regeneration-limited states, was estimated as described by Gu et al. [42]:

\[ C_{\text{trans}} = (J_{\text{max}} K_m - 8 V_{\text{cmax}} \Gamma)/\left(4 V_{\text{cmax}} - J_{\text{max}}\right) \]

where \( K_m \) is the effective Michaelis–Menten constant for \( CO_2 \) that considers the competitive inhibition by \( O_2 \) which was taken from Martins et al. [27].

The overall photosynthetic limitations were partitioned into their functional components [stomatal \( l_s \), mesophyll \( l_m \) and biochemical \( l_b \) limitations] using the values of \( g_m, g_{\text{trans}}, V_{\text{cmax}}, K_m \) and \( C_c \), following the approach proposed by Grassi and Magnani [43]:

\[ l_s = \left(g_{\text{tot}}/g_{mCO_2} \ast \partial A/\partial C_c\right)/(g_{\text{tot}} + \partial A/\partial C_c) \]

\[ l_m = \left(g_{\text{tot}}/g_m \ast \partial A/\partial C_c\right)/(g_{\text{tot}} + \partial A/\partial C_c) \]

\[ l_b = g_{\text{tot}}/(g_{\text{tot}} + \partial A/\partial C_c) \]

where \( g_{mCO_2} \) is the stomatal conductance to \( CO_2 \) \( (g_{mCO_2} = g_m/1.6) \), \( g_m \) is the mesophyll diffusion conductance according to Harlrey et al. [40] and \( g_{\text{tot}} \) is the total conductance to \( CO_2 \) from ambient air to chloroplasts \( (g_{\text{tot}} = 1/[1/g_{mCO_2} + 1/g_m]) \). \( \partial A/\partial C_c \) was calculated as:

\[ \partial A/\partial C_c = [V_{\text{cmax}}(\Gamma + K_m)]/(C_c + K_m)^2 \]

Statistical Analyses

Data are expressed as the means ± standard error. Student’s \( t \)-tests were used to compare the parameters between treatments. Additionally, one sample \( t \)-tests were performed to compare the means for shade plants with the expected values if they were proportional to the sun plants. All statistical analyses were carried out using Microsoft Excel.

Results

Sun- and shade-grown individuals differed reasonably in venation architecture and mesophyll structure (Table 1). The sun leaves, compared with shade leaves, displayed a higher leaf thickness (15%) primarily resulting from a higher thickness of palisade (43%) and spongy (14%) mesophyll, which led to a higher palisade-to-spongy parenchyma ratio (25%) and lower SLA (39%). However, the upper and lower epidermis thickness and the maximum distance from the vein to the epidermis \( (D_e) \) did not change in response to the light treatments. Sun-grown plants had 31% more midrib xylem conduits but with slightly lower mean conduit diameters (7%) than shade-grown plants. The sun plants displayed \( K_l \) and \( K_u \) values that were 160% and 58% higher, respectively, than in the shade individuals. Adjustments to the light treatments associated with the morphological characteristics of stomata were also observed, as denoted by higher SD (62%), stomatal index (27%) and SPI (62%) in sun leaves compared with the shade leaves; however, both the guard cell length and stomatal size did not change in response to the light treatments. Vein density was proportional to 1/leaf area but slightly higher (17%) than would be expected if it was directly proportional to SD (Figure 1). In line with the differences in the stomatal index, SD and epidermal cell size deviated significantly from the expected proportional relationship to leaf area: SD was 25% higher and epidermal cell size was 31% lower in shade plants than would be expected if these variables were directly proportional (Figure 1). The maximum \( g_c \) \( (g_{\text{cmax}}) \), determined by the stomatal size and density, was larger (61%) in sun leaves than in the shade leaves.

Under saturating PPFD (1,000 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\)), both \( A \) and \( g_c \) values were higher (4.55%) in sun leaves than in the shade leaves (Table 2). The values for several other photosynthetic/respiration parameters were also higher in sun leaves than in the shade leaves (Table 2): 60% for \( g_m \) (average of two independent methods), 42% for \( V_{\text{cmax}} \), 45% for \( J_{\text{max}} \) (for \( V_{\text{cmax}} \) and \( J_{\text{max}} \), the values express averages obtained on \( C_i \) and \( C_c \) bases), 140% for \( R_b \), 200% for \( R_i \), 41% for \( R_o \) and 51% for \( J_e \). In contrast, \( C_i, C_c, C_{\text{trans}} \) and the ratios \( A/g_c, g_m/g_c \) and \( J_{\text{max}}/V_{\text{cmax}} \) on both \( C_i \) and \( C_c \) bases did not differ significantly in response to the light treatments. \( V_{\text{cmax}} \) and \( J_{\text{max}} \) calculated on a \( C_i \) basis were, on average, 101% and 37% higher than \( V_{\text{cmax}} \) and \( J_{\text{max}} \), respectively, calculated on a \( C_c \) basis, whereas the \( J_{\text{max}}/V_{\text{cmax}} \) ratio was on average 32% lower when calculated on a \( C_i \) basis than on a \( C_c \) basis.

The overall photosynthetic limitations were essentially similar when comparing sun- and shade-grown plants (Table 2). We found that \( A \) was mainly constrained by stomatal limitations (c. 40%), whereas mesophyll and biochemical limitations accounted similarly for the remaining limitations (c. 30% each).

The analysis of Rubisco kinetic properties are summarised in Table 3, showing that coffee presents a Rubisco with a relatively high affinity for \( CO_2 \) (low \( K_c \) and fast activity \( (k_w) \), resulting in relatively high values for \( S_{K_c} \). From the combination of \( in vivo \) \( V_{\text{cmax}} \) and \( in vitro \) data \( (k_w) \), the concentration of active Rubisco sites was estimated to be 16 and 20 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) for shade and sun plants, respectively.

Discussion

This study provided a holistic examination of the key steps that could limit the photosynthetic capacity to fix \( CO_2 \) and demonstrated that leaf hydraulic architecture imposes a major constraint on the maximisation of the photosynthetic gas exchange of coffee leaves by limiting \( g_c \). It is tempting to suggest that these constraints might to some extent explain the photosynthetic behaviour of
other important (sub)tropical crops such as cacao, citrus and tea, which have a photosynthetic performance and a slow-growth behaviour similar to that of coffee [26]. Our results suggest that improvements of the photosynthetic performance of these crops by means of selecting hydraulic traits (e.g., $D_v$ and $K_L$) that might support higher $g_s$ values (thereby decreasing stomatal limitations of photosynthesis) could be a useful strategy to facilitate the selection of promising genotypes with enhanced crop growth and production.

We have shown that adjustments in leaf hydraulics through increases in $D_v$ and $K_L$ in sun-grown individuals were coordinated with a suite of traits related to water flux and gas exchange per leaf area, such as mesophyll structure (e.g., higher mesophyll thickness and palisade-to-spongy parenchyma ratio) and stomatal attributes (increased SD and stomatal pore index). Importantly, we showed that increases in $K_L$ occurred at the expense of an increased number of midrib conduits with lower lumen in sun plants, suggesting that improvements in the hydraulic safety would not compromise the hydraulic efficiency [44]. In addition, the larger SD coupled with larger stomatal index implies a proportionally greater increase in the number of guard cells than in normal epidermal cells [45]; hence, light availability should directly and positively influence stomatal fate in coffee regardless of passive changes linked by light-induced differences in leaf blade expansion. However, differential leaf expansion was responsible for the adjustment of $D_v$ to SD, which remained nearly proportional to each other. Thus, coffee plants can balance water supply with transpirational demand through a coordination of increased initiation of stomata cells with differential expansion of epidermal cells. Such coordination reflects an optimisation of the trade-off between transpirational costs and CO$_2$ assimilation, resulting in the higher intrinsic water use efficiency observed in coffee (c. 85 μmol CO$_2$ mol$^{-1}$ H$_2$O on average), which is markedly high compared with other tropical woody species [46,47].

Our $K_L$ values, determined using the method of relaxation kinetics of $Y_l$, were remarkably lower than those found by Brodribb et al. [1] for other tropical trees using the same method (between 17 and 36 mmol m$^{-2}$ s$^{-1}$ MPa$^{-1}$). Considerably low values of $K_L$ had already been reported for $C$. arabica (c. 4.2 mmol m$^{-2}$ s$^{-1}$ MPa$^{-1}$) by Gasco et al. [48] using the high-pressure method. Consistent with the close relationship between $K_L$ and $D_v$ [4,5,49], our maximum $D_v$ values were also within the low range recorded for angiosperms [50], suggesting that the hydraulic architecture of coffee leaves imposes strong resistance for water flow which, in turn, should limit the CO$_2$ diffusion into the leaf [4]. In addition, our $K_L$ and $D_v$ values (Table 1) fit very well in the general relationship of both $K_L$ and $D_v$ with the maximum $A$ proposed by Brodribb et al. [3,51], that is, our estimated $K_L$ and $D_v$ values exactly predict our maximum measured $A$ values. Therefore we contend that the leaf hydraulic architecture ultimately should act as a prime factor limiting photosynthetic gas exchange in coffee leaves.

Figure 1. The relationships between vein and stomatal density, vein density and 1/leaf area, stomatal density and 1/leaf area, and epidermal cell area and leaf area. Data (± standard error) are shown for coffee plants grown under shade (black circles) or full sun (white circles). Asterisks denote differences (*, $P<0.05$; **, $P<0.01$) between the observed values for the shade plants and the expected values (broken line) if these were proportional to the averages for the sun plants.

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Regardless of light regimens, our actual $g_s$ values were relatively similar to the value of 108 mmol $\text{H}_2\text{O} \text{ m}^{-2} \text{s}^{-1}$ averaged over a range of studies using coffee plants grown under optimal conditions ([10], and references therein). These values are significantly lower than those of the modelled $g_{\text{max}}$, resulting in a $g_{\text{max}}/g_s$ ratio above 13, as can be calculated for sun-grown plants. By comparison, that ratio was c. 2.5 in non-water-stressed tomato [52] and 4.5 in Eucalyptus globulus [53]. The high hydraulic resistance of the coffee leaves is most likely the cause of the difference between the theoretical $g_{\text{max}}$ and the recorded $g_s$. Nevertheless, this large difference raises the following questions: why would the plant invest in a large $g_{\text{max}}$ if the maximum realisable $A$ is relatively low and constrained by the leaf hydraulic architecture? Furthermore, from an ecological point of view, what would be the advantage of having a large $g_{\text{max}}$ if, in the humid, shaded understoreys where coffee evolved, $A$ should be more constrained by light limitation than by CO$_2$ supply? Despite not having immediate responses to these queries, our results suggest a lack of coordination between the maximum capacity for stomatal aperture and carbon fixation, as also noted in saplings of Bornean rainforest tree species grown in the understorey [53]. A large $g_{\text{max}}$ may not be problematic in terms of water loss in the humid understorey, where transpiration rates are expected to be much more dependent on boundary layer resistance, and thus the importance of the stomata in optimising photosynthetic gas exchange should be reduced. In any case, considering that bigger stomata tend to close slower than smaller stomata [33,54], the relatively large stomata of coffee leaves (combined with low $K_t$) might result in excessive leaf desiccation if large stomatal apertures are realised. In this sense, the low actual $g_s$ might be a conservative strategy to maintain leaf hydration and minimise the risk of xylem embolism. This observation is in line with recent results obtained for Toona ciliata, where transpirational homeostasis to changes in vapour pressure deficit was achieved through dynamic stomatal control rather than modification of the relationship between veins and stomata [55]. Taken together, these findings lead to the interesting question of why long-term adjustments to the parameters that define $g_{\text{max}}$ have not been fixed and regulation of $g_s$ comes predominantly from short-term adjustments to environmental conditions but at the cost of inherently low $g_s$ in some species, such as coffee.

Our maximum $g_{\text{in}}$ value was c. two-fold higher than those previously reported for C. arabica seedlings [56]. In any case, our $g_{\text{in}}$ values were similar to those obtained for other evergreen woody species (e.g., [57–59]). Greater $g_{\text{in}}$ values for sun leaves, as found here, have been systematically reported and have been often explained by anatomical and morphological differences between shade and sun leaves [60]. Despite the changes in $g_{\text{in}}$ and $A$ between shade- and sun-grown coffee plants, $C_i$ and $C_c$ remained fairly similar. Thus, regardless of the light treatments, when $A$ changed, $g_{\text{in}}$ and $g_s$ scaled accordingly, and, hence, $C_c$ remained constant [61]. This proportional scaling lends support to explain why stomatal, mesophyll and biochemical limitations to photosynthesis were similar between sun and shade leaves. These findings are in agreement with other studies, which show that the approximate scaling of $g_s$ and $g_{\text{in}}$ with $A$ makes the relative limitations to photosynthesis rather conservative between sun and shade leaves, as also noted in Fagus silvatica [62].

Irrespective of the light environment, the mean drawdown from $C_i$ to $C_c$ (c. 79 mmol mol$^{-1}$) was lower than that from $C_i$ to $C_s$ (c. 153 mmol mol$^{-1}$), which is consistent with the fact that the diffusive limitations to CO$_2$ in mesophyll were lower than those in

### Table 1. Anatomical and hydraulic traits of coffee plants grown under shade or full sunlight conditions.

| Parameters                          | Treatments          | Shade               | Full sunlight      |
|-------------------------------------|---------------------|---------------------|--------------------|
| Specific leaf area (m$^2$ kg$^{-1}$) |                     | 22.9±3.5            | 14.0±2.7*          |
| Total leaf thickness (μm)           |                     | 333.9±9.1           | 384.5±17.2*        |
| Palisade thickness (μm)             |                     | 52.6±1.2            | 75.24±4.4*         |
| Spongy thickness (μm)               |                     | 220.9±6.1           | 253.1±11.5*        |
| Upper epidermis thickness (μm)      |                     | 38.8±0.2            | 37.4±1.3           |
| Lower epidermis thickness (μm)      |                     | 29.6±0.9            | 26.4±1.2           |
| $P$/$P_{\text{sat}}$               |                     | 0.24±0.01           | 0.30±0.01*         |
| Guard cell length (μm)              |                     | 28.0±0.7            | 28.9±0.4           |
| Stomatal density (mm$^2$)           |                     | 129.1±7.2           | 208.8±4.2*         |
| Stomatal index                      |                     | 20.3±1.1            | 25.7±1.0*          |
| $g_{\text{max}}$ (mol m$^{-2}$ s$^{-1}$) |                     | 1.16±0.05           | 1.82±0.02*         |
| Venation density (mm mm$^{-2}$)     |                     | 4.0±0.1             | 5.5±0.2*           |
| Midrib vessel diameter (μm)         |                     | 22.8±0.2            | 21.3±0.4*          |
| Number of midrib conduits           |                     | 117±7               | 153±11*            |
| $K_i$ (mmol MPa$^{-1}$ m$^{-2}$ s$^{-1}$) |                     | 24.5±2.5            | 63.8±1.6*          |
| $K_s$ (mmol MPa$^{-1}$ m$^{-2}$ s$^{-1}$) |                     | 6.9±0.1             | 10.9±1.4*          |
| $D_{\text{ve}}$ (μm)               |                     | 195.8±8.6           | 215.4±8.5          |

$n = 6±5$. Asterisks indicate statistically significant differences ($P<0.05$) between shade and full sun treatments. Abbreviations: $P$/$P_{\text{sat}}$, palisade-to-spongy parenchyma ratio; $P_{\text{sat}}$, stomatal pore index based on guard cell length; $g_{\text{max}}$, maximal theoretical stomatal conductance to water vapour; $K_i$, midrib conductance; $K_s$, leaf hydraulic conductance; $D_{\text{ve}}$, vertical distance from the vein to the stomatal epidermis.

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reaching values typically ranging from 10 to 50 mmol H2O
during most of their development under light limitation, the
stomata, thus ultimately reflecting a high $g_{st}/g_c$ ratio. Indeed, by
analysing the dataset reported by Flexas et al. [63], we noted that
species operating at low $C_t$ tended clearly to display an increased
$g_{st}/g_c$ ratio (Figure S1) which contributes to an improved
performance in photosynthetic water use [63]. We believe that
such feature is essential for keeping a positive carbon balance given
that stomatal limitations may even be exacerbated in coffee trees
grown under field conditions, particularly because $g_{st}$ peaks in the
early morning and decreases progressively throughout the day,
reaching values typically ranging from 10 to 50 mmol H2O
m$^{-2}$ s$^{-1}$ from midday onwards [23,24,64–67] as a consequence of
rising vapour pressure deficit.

Under saturating PPFD, $A$ at ambient CO$_2$ was limited by
Rubisco activity regardless of the light treatment given that the
estimated $C_t$ was lower than $C_{car,trans}$ (Table 2). However, the
realised $A$ and $J_{\text{c}}$ at maximum growth irradiance of shade plants (c.
200 $\mu$mol PPFD m$^{-2}$ s$^{-1}$) are c. 60% of their saturating values
(Figure S2), indicating that, even though these plants operate
during most of their development under light limitation, the
balance between RuBP regeneration and Rubisco activity (the
$J_{\text{max}}/V_{\text{cmax}}$ ratio) was essentially similar in the shade and sun

### Table 2. Mean values for the photosynthetic and respiration parameters of coffee plants grown under shade or full sunlight conditions.

| Parameters                                      | Treatments          |
|-------------------------------------------------|---------------------|
|                                                  | Shade               | Full Sun            |
| $A$ ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$)          | 7.9±0.4             | 12.0±0.8*           |
| $g_{st}$ ($\mu$mol H$_2$O m$^{-2}$ s$^{-1}$)     | 94±9                | 146±17*             |
| $g_{m,\text{Harley}}$ (mmol CO$_2$ m$^{-2}$ s$^{-1}$) | 76±4                | 116±10*             |
| $g_{m,\text{Hue}}$ (mmol CO$_2$ m$^{-2}$ s$^{-1}$) | 65±12               | 109±5*              |
| $C_t$ (mmol CO$_2$ mol$^{-1}$ air)               | 247±11              | 246±7               |
| $C_{car,\text{Harley}}$ (mmol CO$_2$ mol$^{-1}$ air) | 143±10              | 142±9               |
| $J_{\text{c}}$ ($\mu$mol e$^{-}$ m$^{-2}$ s$^{-1}$) | 70±5                | 106±5*              |
| $V_{\text{cmax,Cl}}$ (mmol CO$_2$ m$^{-2}$ s$^{-1}$) | 26.8±1.6            | 42.1±2.2*           |
| $V_{\text{cmax,Ci}}$ (mmol CO$_2$ m$^{-2}$ s$^{-1}$) | 58.1±3.5            | 78.5±4.0*           |
| $J_{\text{max,Cl}}$ (mmol e$^{-}$ CO$_2$ m$^{-2}$ s$^{-1}$) | 71.1±4.4           | 109.5±3.1*          |
| $J_{\text{max,Ci}}$ (mmol e$^{-}$ m$^{-2}$ s$^{-1}$) | 102.7±6.3           | 142.6±7.4*          |
| $V_{\text{max}}/V_{\text{cmax,Cl}}$           | 2.6±0.04            | 2.7±0.1             |
| $J_{\text{max}}/V_{\text{cmax,Cl}}$           | 1.8-0.1             | 1.8±0.1             |
| $C_{\text{car,trans}}$ (mmol CO$_2$ mol$^{-1}$ air) | 228±17              | 249±23              |
| $R_o$ (mmol CO$_2$ m$^{-2}$ s$^{-1}$)            | 0.5±0.06            | 1.2±0.05*           |
| $R_i$ (mmol CO$_2$ m$^{-2}$ s$^{-1}$)            | 0.1±0.05            | 0.3±0.08*           |
| $A/g_c$ (mmol CO$_2$ mol$^{-1}$ H$_2$O)          | 86±6                | 84±4                |
| $g_{m,\text{Harley}}/g_c$ (mmol CO$_2$ mol$^{-1}$ CO$_2$) | 1.4±0.1             | 1.3±0.1             |
| $R_o$ (mmol CO$_2$ m$^{-2}$ s$^{-1}$)            | 3.2±0.4             | 4.5±0.1*            |
| Stomatal limitation                              | 0.4±0.04            | 0.38±0.02           |
| Mesophyll limitation                             | 0.30±0.02           | 0.30±0.01           |
| Biochemical limitation                           | 0.29±0.03           | 0.32±0.03           |

$n = 6±5SE$. The overall photosynthetic limitations associated with stomatal, mesophyll and biochemical factors are also shown. Data for $A$, $g_{st}$, $g_{m,\text{Harley}}$, $C_t$, $C_{\text{car,trans}}$, $J_{\text{c}}$, and $R_i$ were obtained under PPFD of 1000 $\mu$mol m$^{-2}$ s$^{-1}$, $C_o$ of 400 $\mu$mol mol$^{-1}$ and leaf temperature of 25°C. Asterisks indicate statistically significant differences ($P<0.05$) between shade and full sun treatments.

**Abbreviations:** $A$, net photosynthesis; $g_{st}$, stomatal conductance to water vapour; $g_{m,\text{Harley}}$, mesophyll conductance to CO$_2$; $C_t$, sub-stomatal CO$_2$ concentration; $C_{\text{car,trans}}$, chloroplastic CO$_2$ concentration; $J_{\text{c}}$, electron transport rate estimated by chlorophyll fluorescence; $V_{\text{cmax,Cl}}$, maximum carboxylation capacity; $J_{\text{max,Cl}}$, maximum capacity for electron transport rate; $C_{\text{car,trans}}$, the $C_t$ that denotes the transition between the Rubisco- and RuBP regeneration-limited state; $R_o$, dark respiration; $R_i$, light respiration; $R_c$, photosynthesis rate.

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### Table 3. Rubisco kinetic constants measured for coffee (taken from Martins et al. [27]).

| Species  | $S_{\text{fix}}$ | $\Gamma^*$ ($\mu$bar) | $K_c$ ($\mu$M) | $K_o$ ($\mu$M) | $k_{\text{cat}}$ (s$^{-1}$) |
|----------|------------------|-----------------------|----------------|----------------|----------------------------|
| Coffee   | 98.4±4.3         | 39.6±1.7              | 10.3±1.3       | 479±113        | 3.2±0.1                    |

$n = 4±SE$.

**Abbreviations:** $S_{\text{fix}},$ Rubisco specificity factor; $\Gamma^*$, CO$_2$ compensation point in the absence of mitochondrial respiration; $K_c$ and $K_o$, the Michaelis-Menten kinetics for CO$_2$ and O$_2$, respectively; $k_{\text{cat}}$, Rubisco catalytic turnover rate for the carboxylase reaction.

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these Rubisco properties with those of several $C_3$ species (Table S1). Importantly, we found that, all else being equal, the $A$ values that would be achieved using the Rubisco kinetic properties of coffee or $Limonium$ would be quite similar and superior to those from all other species analysed (Figure 2) suggesting that coffee presents an "efficient" Rubisco well-tuned for operating at low $C_c$. Additionally, the higher $k_{cat}$ and lower $K_c$ mean that fewer Rubisco molecules are required to realise a given $A$. Nevertheless, given that lower $K_c$ leads to a reduction in $C_{c\text{-trans}}$, $A$ of coffee leaves is expected to begin to be limited by RuBP regeneration at relatively low $C_c$, which could, to some extent, reduce the revenue stream in a scenario of increasing CO$_2$ atmospheric levels.

The rates of respiration and photorespiration are other key processes influencing the plant carbon balance. In sun plants, $R_D$ and $R_P$ corresponded to 10% and 38% of $A$, respectively. However, given that $R_i$ represented only a fraction (25%) of $R_D$, which is in line with the inhibition of mitochondrial respiration in the presence of light [71,72], the realised constraint of respiration on $A$ is expected to be relatively low. In turn, $R_P$ is expected to significantly affect $A$ at midday, when the stomatal closure in coffee leaves exacerbates the drawdown from $C_g$ to $C_i$ [24], thus favouring the oxygenase activity of Rubisco.

**Conclusion**

Regardless of the light treatments, $A$ was mainly limited by stomatal factors followed by similar limitations associated with the mesophyll and biochemical constraints. Our data suggest that an increased $g_{st}/g_s$ ratio coupled with a Rubisco well-tuned for operating at low $C_c$ might be adaptations to a lower $C_i$ resulting from the low $g_s$; these adaptations might have helped the establishment of coffee plants when they were moved from the understoreys to the more stressful conditions of open fields, where high vapour pressure deficit and elevated temperatures may constrain gas exchange. Our results also suggest that the coffee plant does not properly optimise its resource allocation: on the one hand, there seems to be an over-investment in capacity for carboxylation and electron transport despite limited light availability under shade; on the other hand, an excess of electron transport capacity is to be expected under full sun. Nevertheless, this excess might be useful in attempts to increase $A$ in coffee through breeding aimed at improving hydraulic traits ($D_v$ and $K_v$).

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**Figure 2. Comparison of the CO$_2$ assimilation rate as a function of $C_c$, modelled with the kinetic parameters of Rubisco in coffee and several $C_3$ species.** The RuBP saturated rates of CO$_2$ assimilation at 25°C were calculated using the following equation [13]: $A = \left(\frac{C_c - \Gamma}{\Gamma\cdot[\text{Rubisco}] + 1}\right)\left(\frac{C_c + K_c(1 + O/K_o)}{C_c + K_c(1 + O/K_o)}\right) - R_L$. $C_c$ the chloroplastic CO$_2$ partial pressure; $O$, the intercellular O$_2$ partial pressure; $\Gamma^*$, the CO$_2$ compensation point in the absence of day respiration; [Rubisco], the catalytic site content of Rubisco. The Rubisco kinetic properties for coffee (Table 3) and the kinetic properties for the other species were retrieved from Savir et al. [69] and are summarized in Table S1. The $R_i$ and $[\text{Rubisco}]$ were set as 1.0 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$ and 25 $\mu$mol sites m$^{-2}$, respectively.

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and expectedly supporting a high $g_t$. Finally, we contend that the large diffusive resistance should lead to a large drawdown from $C_i$ to $C_a$, thus favouring the occurrence of relatively high $R_h$ despite the relatively high $S_v/c$ of coffee Rubisco, which ultimately leads to further limitations to $A$.

**Supporting Information**

**Figure S1** The relationship between sub-stomatal CO$_2$ concentration and mesophyll-to-stomatal conductance ratio in the multi-species dataset from Flexas et al. [63]. (TIF)

**Figure S2** The response of net photosynthetic rate and electron transport rate to the photosynthetic photon flux density in coffee plants developed under shade or full sun. (TIF)

**Table S1** The Rubisco kinetic constants for several C$_3$ species. (DOCX)

**Author Contributions**

Conceived and designed the experiments: SCVM JG FMD. Performed the experiments: SCVM PCC LFP MCV. Analyzed the data: SCVM JG FMD. Contributed reagents/materials/analysis tools: FMD. Wrote the paper: SCVM JG FMD.

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