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*Changes in leaf functional traits with leaf age: When do leaves decrease their photosynthetic capacity in Amazonian trees?*

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## **Abstract**

Most leaf functional trait studies in the Amazon basin do not consider ontogenetic variations (leaf age), which may influence ecosystem productivity throughout the year. When leaf age is taken into account, it is generally considered discontinuous, and leaves are classified into age categories based on qualitative observations. Here, we quantified age-dependent changes in leaf functional traits such as the maximum carboxylation rate of Rubisco ( $V_{cmax}$ ), stomatal control ( $C_{gs}\%$ ), leaf dry mass per area (LMA) and leaf macronutrient concentrations for nine naturally growing Amazon tropical trees with variable phenological strategies. Leaf ages were assessed by monthly censuses of branch-level leaf demography; we also performed leaf trait measurements accounting for leaf chronological age based on days elapsed since the first inclusion in the leaf demography, not predetermined age classes. At the tree community scale, a nonlinear relationship between  $V_{cmax}$  and leaf age existed: young, developing leaves showed the lowest mean photosynthetic capacity, increasing to a maximum at 45 days and then decreasing gradually with age in both continuous and categorical age-group analyses.

Maturation times among species and phenological habits differed substantially, from  $8 \pm 30$  to  $238 \pm 30$  days, and the rate of decline of  $V_{max}$  varied from  $-0.003$  to  $-0.065 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ day}^{-1}$ . Stomatal control increased significantly in young leaves but remained constant after peaking. Mass-based phosphorus and potassium concentrations displayed negative relationships with leaf age, while nitrogen did not vary temporally. Differences in life strategies, leaf nutrient concentrations, and phenological types, not the leaf age effect alone, may thus be important factors for understanding observed photosynthesis seasonality in Amazonian forests. Furthermore, assigning leaf age categories in diverse tree communities may not be the recommended method for studying carbon uptake seasonality in the Amazon, since the relationship between  $V_{max}$  and leaf age could not be confirmed for all trees.

**Keywords:** leaf ontogeny,  $V_{max}$ , leaf nutrients, stomatal control, leaf demography, phenological type, tropical trees, Amazon forest.

## Introduction

The Amazon rainforest is an important global carbon sink, currently offsetting the carbon emissions from fossil fuel combustion and land-use changes of Amazonian nations (Pan et al. 2011, Phillips and Brienen 2017). Approximately one-third of the assimilated carbon in Amazonian forest ecosystems is allocated into a dynamic carbon pool mainly composed of photosynthetic and reproductive organs (Chave et al. 2010). Although the total leaf stock (leaf area index — LAI) changes only moderately across seasons (Doughty and Goulden 2008, Brando et al. 2010, Wu et al. 2016), ecosystems in the Amazon basin display canopy seasonality with higher leaf production and litterfall during dry seasons (Myneni et al. 2007, Chave et al. 2010). Such dynamics, together with variations in leaf maximum



carboxylation rates ( $V_{\text{cmax}}$ ) within age classes, have been considered the primary cause of carbon sink capacity seasonality and the dry-season increase in gross primary productivity (GPP) observed in the central Amazon (Carswell et al. 2002, Doughty and Goulden 2008, Restrepo-Coupe et al. 2013, 2017, Wu et al. 2016, Albert et al. 2018).

Leaf demography and seasonal differences in leaf age compositions within tropical forest canopy layers are the basis of the "Leaf Demography-Ontogeny (LDO)" hypothesis proposed by Wu et al. (2016). More precisely, the LDO hypothesis was formulated as a mechanism to explain the higher GPP calculated during the Amazonian dry season resulting from the interplay of LAI proportions among age classes (young leaves: 1–2 months, mature: 3–5 months, and old:  $\geq 6$  months) and the associated mean  $V_{\text{cmax}}$  of each class (Wu et al. 2016, Albert et al. 2018). The leaf ontogeny prediction of the hypothesis proposes that the  $V_{\text{cmax}}$  values of mature leaves are higher than those of young and older leaves because of incomplete maturation processes and senescence-related physiological mechanisms that occur in leaves over time. The LDO hypothesis has been tested in the Amazon forest using ecosystem photosynthetic capacity estimates (Restrepo-Coupe et al. 2013, 2017, Wu et al. 2016, 2017b), satellite-based (EVI-MAIAC) measurements (Huete et al. 2006, Brando et al. 2010, Bi et al. 2015, de Moura et al. 2017), and spectroscopy (Chavana-Bryant et al. 2017, 2019, Wu et al. 2017a, 2019) and directly by leaf and branch-level photosynthesis (Albert et al. 2018).

Age-dependent declines in leaf assimilation rates have been studied since the middle of the 20<sup>th</sup> century in temperate woody species (Freeland 1952, Field and Mooney 1983, Koike 1990, Wilson et al. 2001, Escudero and Mediavilla 2003, Ethier et al. 2006, Warren 2006) and, more recently, in tropical species (Zotz and Winter 1994, Ackerly and Bazzaz 1995, Kitajima et al. 1997a, 2002, 2005, Ishida et al. 1999, Reich et al. 2009a, Albert et al. 2018). Some photosynthetic decline patterns occurring with leaf age after maturity are often

characterized as monotonic and linear (Thomas and Stoddart 1980, Kitajima et al. 1997a, 2002, Krieger-Liszay et al. 2019).

Such age-dependent declines in photosynthesis have been hypothesized as being associated with changes in leaf chemistry over time (i.e., leaf nutrients) (Reich et al. 1991). Leaf nitrogen (N) is generally correlated with  $V_{\text{cmax}}$  (i.e., activity of ribulose-1,5-bisphosphate carboxylase/oxygenase — Rubisco) (Meir et al. 2002, Xu and Baldocchi 2003, Evans and Clarke 2019), and its concentration often decreases as leaves age (Kitajima et al. 1997, Wright et al. 2006, Szymura 2009, Fajardo and Siefert 2016, Chavana-Bryant et al. 2019), with some exceptions (Niinemets et al. 2004, 2006, Wright et al. 2006, Fajardo and Siefert 2016, Chavana-Bryant et al. 2017). Leaf phosphorus (P) is involved in several metabolic processes and has a number of functions in leaves (Reich et al. 2009b), such as energy transfer and ribulose 1,5-bisphosphate (RuBP) regeneration during the photosynthetic process (Walker et al. 2014). Leaf P displays divergent patterns, either increasing (Mediavilla and Escudero 2003, Niinemets et al. 2004, Fajardo and Siefert 2016) or reducing in concentration with leaf age (Mediavilla and Escudero 2003, Wright et al. 2006, Szymura 2009, Mediavilla et al. 2011, Chavana-Bryant et al. 2017, 2019). Age-dependent decreases in mass-based leaf potassium (K) concentrations are commonly found (Niinemets et al. 2004, Mediavilla et al. 2011). Inorganic  $\text{K}^+$  is a main ion among the key solutes transported across plasma membranes and has an important influence on osmoregulatory functioning in guard cells and hence stomatal conductance (Talbot and Zeiger 1996, Roelfsema and Hedrich 2005, Pettigrew 2008), although it is recognized that stomatal responses to environmental cues are also determined by the transport of other solutes (Lawson and Blatt 2014).

Likewise, age-dependent decreases in stomatal conductance and stomatal control have been described for both evergreen and deciduous tropical trees (Reich and Borchert 1988) and may affect photosynthesis (Wilson et al. 2000). Stomatal control (i.e., stomatal opening and

closing) resulting from changes in leaf-to-air water vapor pressure deficits ( $VPD_L$ ) may either deteriorate or not change with increasing leaf age in non-senescent leaves (Ethier et al. 2006).

Leaf dry mass per unit area (LMA) may increase with leaf age (Xu and Baldocchi 2003, Niinemets et al. 2005, 2006, Ethier et al. 2006, Mediavilla et al. 2011, Noda et al. 2014, Chavana-Bryant et al. 2017, 2019) as a result of the ontogenetic properties of carbon-based compounds accumulating in the mesophyll or cell wall thickening during the structural differentiation and development of leaves. These temporal changes in LMA may influence mesophyll conductance by reducing the rate of  $CO_2$  diffusion inside leaves and therefore limiting the photosynthetic rate and capacity (e.g.,  $V_{cmax}$  and  $J_{max}$ ) (Mediavilla and Escudero 2003, Niinemets et al. 2004, 2005). Despite these observations, physiological data on the relationships among photosynthetic capacity, leaf traits and biochemistry with leaf age in tropical forests are rare and geographically sparse, especially at fine scales and high temporal resolutions.

Here, we monitored *in situ* leaf age and demography combined with leaf-level  $V_{cmax}$ , stomatal control, and leaf chemistry measurements along a continuous leaf age gradient in nine tree species over 620 days. We directly tested the leaf ontogeny component of the LDO hypothesis, which was proposed based on discrete leaf age classes in the eastern Amazon (Wu et al. 2016, Albert et al. 2018); we also investigated the possible mechanisms involved in leaf age-related changes in leaf productivity. Our main goals were (i) to investigate the effects of leaf aging on  $V_{cmax}$  at the tree community and species-specific levels, (ii) to examine whether  $V_{cmax}$ , integrated across leaf age, would vary differently with leaf age when age is represented continuously or categorically, and (iii) to investigate the effects of leaf aging on stomatal control and leaf chemistry. We hypothesize that trees would strongly differ in the rate and timing of their  $V_{cmax}$  changes due to the high diversity of phenological behaviors, stomatal control, and leaf nutrient contents among central Amazonian tree species.

Given the importance of the Amazon forest at regional and global scales, these results contribute to a variety of research areas that aim to understand tropical forest functional ecology and its role in the Earth system.

## Materials and Methods

### *Study site*

Measurements were carried out in two experimental plots of the AmazonFACE program (Free-Air CO<sub>2</sub> Enrichment in the Amazon) (2° 35' 42.9" S, 60° 12' 28.7" W and 2° 35' 46.4" S, 60° 12' 27.6" W) (Cordeiro et al. 2020, Pereira et al. 2019) distributed along the north-south transect of the permanent plots of the Jacaranda Project (National Institute of Amazonian Research — INPA, and *Japan International Cooperation Agency* — JICA) and located 100 m apart from each other. The survey was performed during the pre-experimental phase of the AmazonFACE Program (i.e., no CO<sub>2</sub> fertilization). This site is located in a central Amazonian evergreen tropical upland forest at the Cuieiras Biological Reserve experimental site, 60 km northwest of Manaus, Brazil (Lapola et al. 2012). The local climate is defined by high annual precipitation (varying interannually from 1990 to 2500 mm), with little variation in monthly air temperatures throughout the year (from 24.6 to 26.9 °C) and high annual relative air humidity, ranging from 75 to 92% (Ferreira et al. 2005) for the driest (June to October, mean cumulative water deficit lower than 100 mm) and wettest months (November to May), respectively (Tanaka et al. 2014).

The vegetation of this site is classified as mature, moist, *terra firme* tropical rainforest characterized by high woody and herbaceous species diversities (Higuchi et al. 1997), high tree density and high standing live biomass (Vieira et al. 2004). The canopy height is, on

average, 30 m, with canopy tree crowns in close proximity to each other (Pereira et al. 2019), resulting in low light transmittance from the upper to lower forest levels.

### *Sampling design*

At the center of the selected plots, there are two 40-m scaffolding towers granting access to the crowns of 21 individual trees (Table 1), with 15 trees neighboring tower 1 and 7 trees around tower 2. To monitor the leaf-level demography, all trees surrounding the two towers were sampled. The study comprised trees with at least 5-cm diameters at breast height (DBH  $\geq$  5 cm), and the sampled trees were of different species and light environments, from understory shaded trees (3.5–7.0 m) and mid-canopy trees (10.3–15.8 m) to upper sunlit canopy trees (17.7–28.6 m). For the collection of gas exchange measurements, nine species were selected out of the 21 trees (Table 1).

### *Leaf-level demography*

Leaf-level demography was monitored monthly from August 2016 to May 2019 by assessing the leaf flushing and shedding dynamics of labeled branches according to the method described in Reich et al. (2004) and adapted by G. Martins (personal communication, 2016). Since the leaf demography censuses were performed monthly, the leaf age data had a margin error of  $\pm$  30 days. To standardize the methodology and avoid any bias, new leaves were included in the leaf demographic census only if they satisfied all adopted criteria for leaves that were close to reaching full leaf blade expansion; that is, the leaves could not (i) be curled, (ii) be smaller than the mean leaf size compared to other leaves in the canopy, (iii) lack any sign of leaf rigidity, or (iv) contain apparent high concentrations of carotenoids or

low concentrations of chlorophyll. Considering that all leaves were standardized under these inclusion criteria, the leaf demography represents the leaf age after each leaf reaches this first developmental stage. The leaf demography and ages of all individual leaves on each branch were tracked on 7 to 20 branches per tree, depending on the access from the tower and the total number of branches available (Table 1). Branches in sunlit and shaded environments were sampled in the upper canopy trees, while for the mid-canopy and understory trees, the selection of branch positions was randomized since all were partially or completely shaded. Compound leaves were considered as a whole, from the petiole to the apex, not as individual leaflets. A leaf was considered dead when the petiole had abscised. The longevity of individual leaves was calculated as the time difference, in days, between the leaf flush and leaf fall, measured within a monthly interval ( $\pm 30$  days).

#### *Phenological habit*

All trees were classified into four phenological types according to Martins et al. (in preparation) (Table 1). The first type (“successive”) comprises trees that exchange leaves continuously throughout the year (only *Pourouma tomentosa* Mart. ex Miq.). The second type (“brevideciduous”) comprises trees that shed all leaves during a specific time of the year, remain leafless for a short period and then flush new leaves at once (only *Pterandra arborea* Ducke). The third type (“semi-flush”) consists of trees that flush a partial set of new leaves twice a year but shed leaves continuously year-round (only *Swartzia arborescens* (Aubl.) Pittier). The fourth type (“flush”) includes trees that both flush and shed a great number of leaves over a short period every one or two years; this is the dominant group at this site, comprising 75% of the trees considered in this study. Additionally, there is another type of phenological habit represented by trees that do not fit in any of the types described

previously or do not display consistent behavior across multiple years. Therefore, these trees were classified as “irregular” (only *Mabea angularis* Hollander). This species has a unique pattern of flushing new leaves, since the new leaves come from a new cohort of branches and not from within the same branches over time. Additionally, few old leaves from previous cohorts are shed over the course of many years, so we classified this species as irregular and not part of the “flush” type.

### *Leaf-level gas exchange*

Measurements of the leaf-level gas exchange of light-saturated CO<sub>2</sub> assimilation (using A–C<sub>i</sub> response curves,  $n = 135$  and the One-Point method,  $n = 78$ , for a total  $n = 213$ ) and the responses of stomatal conductance to changes in the leaf-to-air water vapor pressure deficit ( $g_s$ –VPD<sub>L</sub> response curves,  $n = 128$ ) were taken from attached leaves of different leaf ages of nine species out of the 21 studied trees (Table 1). For these measurements, two portable infrared gas analyzer systems (LI-6400 XT and LI-6800 F, LI-COR Inc., Lincoln, Nebraska, USA) were used during four campaigns in July 2017, September 2017, November 2017, and March 2018. The age of each leaf age was recorded by counting the number of consecutive days that had passed since the inclusion of that leaf in the leaf demography census. The sampled trees, as indicated by the asterisk (\*) in Table 1, consisted of four upper-canopy trees, four mid-canopy trees, and one understory tree. Although an understory tree was included, its leaf longevity was within the range of the upper- and mid-canopy trees. The nine trees that were selected for the gas exchange measurements were the only trees that had flushed new leaves by the time of the first campaign in July.

Two sampling approaches were applied to investigate age-dependent changes in photosynthetic parameters: (i) Repeated measurements and (ii) Chronosequence (see Osada

et al. 2015 for more details). In both approaches, the leaves were acclimated for at least 15 minutes before measurements to ensure the complete stability of both the assimilation and stomatal conductance values by carefully monitoring the automatic graphs provided by the LICOR 6400 and 6800 portable photosynthesis systems. Two different methods were applied in the first approach: complete A–C<sub>i</sub> curves and One-point measurements. In the Repeated A–C<sub>i</sub> method ( $n = 51$ ), three target leaves of different branches were monitored and measured repeatedly after flushing to determine the age-dependent physiological change patterns of each individual leaf. In this method, measurements of A–C<sub>i</sub> and the  $g_s$ –VPD<sub>L</sub> curves were carried out on the same leaves in three campaigns throughout 2017, with a sampling interval of two months. The first campaign started in the beginning of July, 5 days after the first main flush was observed in June’s demographic census, meaning that the leaves emerged anytime between the end of May and the end of June. In the Repeated One-point method ( $n = 78$ ), three leaves per tree of the same age (also monitored by leaf demography) and under the same light regime were selected. In these leaves, one-point light-saturated photosynthesis measurements with [CO<sub>2</sub>] = 400  $\mu\text{mol mol}^{-1}$  (hereafter A<sub>400</sub>) were carried out during the July 2017 and September 2017 field campaigns. To ensure high-quality data, we performed an analysis comparing the estimates of  $V_{cmax}$  from the CO<sub>2</sub> response curves with the estimates of  $V_{cmax}$  calculated by the One-point method on the same leaves. The  $V_{cmax}$  estimates calculated by the One-point method were only 1.2% underestimated (systematic error, calculated according to Pereira et al. 2019) compared to the  $V_{cmax}$  values calculated by fitting the full A–C<sub>i</sub> curve. Thus, the photosynthesis metrics obtained in the Repeated A–C<sub>i</sub> and One-point methods could be compared statistically to examine whether destructive sampling and analysis data could be ascribed to leaves of the former method.

The second approach to measuring age-related changes in  $V_{cmax}$  consisted of monitoring and measuring the leaf-level gas exchanges of leaves of different ages present on



the same branch or crown at a given time (i.e., Chronosequence — Kitajima et al. 2002, Osada et al. 2015). In contrast to Kitajima et al. (2002), the leaf sampling in our study determined the chronological leaf ages in all cases using leaf demographic censuses rather than the leaf positions in the branches. In Osada et al. (2015), the authors describe their method as comprising both approaches, according to the “position of individual leaves or known age in marked leaves within a shoot”. In the Chronosequence A–C<sub>i</sub> method ( $n = 84$ ), the medium-aged (~240 days) and older leaves (above 500 days) that were still available on the selected branches were measured in the September 2017, November 2017 and February/March 2018 field campaigns.

Even though we carefully sampled leaves with known ages, by monitoring leaf age in demographic censuses and not sampling age classes within a shoot, potential bias can be found in both methods, since there is variation in the leaf lifespan of each tree cohort (Table 1), and we sampled the surviving leaves on the crown (Osada et al. 2015).

Gas exchange determinations were taken from fully expanded leaves between 08:00 hours and 14:00 hours (local time). Determinations of light-saturated CO<sub>2</sub> assimilation versus intercellular CO<sub>2</sub> concentrations (A–C<sub>i</sub>) were performed with the following order 5 minutes after reaching stability for each point: 400, 300, 200, 75, 50, 400, 600, 800, 1000 and 1200  $\mu\text{mol mol}^{-1}$ ; these measurements were performed under standard chamber environmental conditions: the airflow was 400  $\mu\text{mol s}^{-1}$  (LI-6400 X) or 700  $\mu\text{mol s}^{-1}$  (LI-6800 F), the water vapor fraction was  $20 \pm 3 \text{ mmol mol}^{-1}$  (50–60% relative humidity), the leaf temperature was  $30 \pm 2 \text{ }^\circ\text{C}$  and the saturating values of the photosynthetic photon flux density (PPFD) ranged from 400  $\mu\text{mol m}^{-2} \text{ s}^{-1}$  for an understory tree species to 2000  $\mu\text{mol m}^{-2} \text{ s}^{-1}$  for an upper canopy tree species. The saturating PPFD values were determined by light response curves made prior to the A–C<sub>i</sub> curves for each species.

The light-saturated photosynthetic rates ( $A$ ) and internal  $\text{CO}_2$  substomatal concentrations ( $C_i$ ) obtained by the  $A-C_i$  response curves were used to fit the model developed by Farquhar et al. (1980), and subsequent modifications were conducted using the photosynthetic kinetic parameters proposed by Bernacchi et al. (2001). The biochemical parameters of the maximum carboxylation rate of Rubisco and the maximum electron transport rate ( $V_{cmax}$  and  $J_{max}$ , respectively;  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) were calculated with an R script that applies a curve-fitting routine based on minimum least-squares (Domingues et al. 2010). The photosynthetic capacity was defined by the  $V_{cmax}$  modeled and adjusted to the standard temperature of 25 °C (Bernacchi et al. 2001) since net assimilation rates ( $A$ ) are more susceptible to environmental changes and are therefore more liable to daily and seasonal stomatal conductance oscillations (Niinemets et al. 2004, Mendes and Marengo 2014, Mendes et al. 2017).

The photosynthetic capacities obtained from the One-point method ( $V'_{cmax}$ ) were estimated by fitting  $A_{400}$  and  $C_i$  values, as described by De Kauwe et al. (2016), using  $K_c$ ,  $K_o$ , and  $\Gamma^*$  according to Bernacchi et al. (2001). In the One-point method proposed by these authors, single light-saturated photosynthesis rate ( $A_{sat}$ ) and associated parameters are used to estimate carboxylation capacity ( $V_{cmax}$ ) from one measurement instead of fitting a full  $A-C_i$  curve using Farquhar and others' model, which commonly takes an hour to collect all the necessary data. The respiration estimates ( $R_{day}$ ) in the One-point method are assumed to be a fixed percentage of  $V_{cmax}$  (1.5%), as described in De Kauwe et al. (2016) and Collatz et al. (1991). The differences in  $V_{cmax}$  and  $R_{day}$  among leaf ages are inherent in the net assimilation rates, and the correction of the model is the same for all ages. For the  $A-C_i$  curve fitting, the respiration rates were estimated by the Farquhar et al. (1980) photosynthesis model (and its modifications); thus, respiration was not constrained in this method beyond the estimated value ascribed to all data, and variations may be found in estimates for different leaf ages.

The  $g_s$ -VPD<sub>L</sub> curves were obtained by measuring changes in relative humidity and leaf temperature in the chamber, beginning with 75% and 26 °C followed by a progressive reduction in humidity simultaneously with an increase in leaf temperature in the following order: 65% – 28 °C, 50% – 30 °C, and 35% – 32 °C, under a constant CO<sub>2</sub> concentration (400 μmol mol<sup>-1</sup>) and saturating PPFD. At each step, the leaves were allowed to acclimate for at least 15 minutes after the first sign of stability. The stomatal control ( $C_{gs}\%$ ) was calculated as the relative amplitude between the maximum and minimum stomatal conductance values obtained from the  $g_s$ -VPD<sub>L</sub> curves according to the following equation:  $\{(g_{s-max} - g_{s-min})/g_{s-max}\} * 100$ .

#### *Leaf sampling for destructive analysis*

The leaves used for gas exchange measurements (in the Repeated measurements and Chronosequence approaches) were sampled to obtain the following variables: leaf dry mass per area (LMA) and nitrogen (N), phosphorus (P), and potassium (K) concentrations (mg g<sup>-1</sup>). One to five leaf discs with known areas (1.96 cm<sup>2</sup>) were removed from the fresh leaves, stored in sealed plastic bags with silica gel, oven-dried at 65 °C for 48 hours, and then weighed. Thus, the LMA was obtained as the ratio between the dry mass (g) and the area (m<sup>2</sup>) of the leaf discs. The remaining leaf sample material was frozen and lyophilized for 72 hours and then milled to a fine powder with which to perform chemical analyses. Total nitrogen was determined by dry combustion on a C and N elemental analyzer (Carlo Elba<sup>®</sup> CHN 1110) at the Isotopic Ecology Laboratory of the Center for Nuclear Energy in Agriculture (CENA-ESALQ). For the P and K determinations, leaf extracts were produced by the nitric-perchloric digestion method (Malavolta et al. 1997). The P concentrations were determined by the colorimetric method using a spectrophotometer at 725 nm (UV-120-01,

Shimadzu, Kyoto, Japan), and the K concentrations were determined by atomic absorption spectrophotometry (AAS, 1100 B, Perkin-Elmer, Ueberlingen, Germany) (Anderson and Ingram 1993) at the Plant and Soil Thematic Laboratory of National Institute of Amazonian Research (LTSP-INPA).

### *Data analysis*

A two-tailed nonpaired t-test was applied to test whether there were differences between  $V_{cmax}$  (A-C<sub>i</sub> curves) and  $V'_{cmax}$  (one-point method). Since the relationships between leaf age and  $V_{cmax}$  strongly varied in time in all three studied levels (i.e., community, phenological habit and individual) in a nonlinear way, we applied a breakpoint and segmented regression approach to our data to test for age-dependent changes in  $V_{cmax}$  throughout the leaf age gradient for (i) the entire tree community (all trees pooled together), (ii) trees of different phenological habits, and (iii) individually for each of the nine trees sampled. Segmented regressions are particularly useful for detecting abrupt changes in patterns and determining the breakpoint in a relationship or the thresholds at which patterns change (for example, see Fyllas et al. 2009). The breakpoint is estimated iteratively by applying the least-squares method to minimize the sum of the squares of the differences and divides a series into two or more segments according to the minimum residuals. The best fit is reached when R-squared and the F-value are the highest and the mean square error (MSE) and standard error are the lowest compared to all other adjustments of the regression equations. The segmented regression analyses and creation of graphs were performed in the statistical software GraphPad version Prism 9.

Linear regression analysis was used to test the relationship between the chronological age and biophysical properties (e.g., LMA and stomatal control) of leaves, as well as for the

concentrations of leaf N, P and K, considering all nine tree species together. Finally, all variables were tested for pairwise correlations (e.g.,  $C_{gs}$  % and K).

We performed a categorical statistical analysis of the mean  $V_{cmax}$  values among the leaf age classes, defined here as young (< 60 days), mature ( $70 < x < 160$  days), and old (> 200 days), by one-way ANOVA test, according to our dataset for the tree community. The goal was to compare the results with previous studies that also considered leaf age (Wu et al. 2016, Albert et al. 2018), although there were small differences in the sampling dates between our investigation and these studies, wherein the leaf age classes were defined as young ( $30 < x < 60$  days), mature ( $90 < x < 150$  days) and old ( $\geq 180$  days). For such analysis, all estimated  $V_{cmax}$  (Repeated measurements, Chronosequence) and  $V'_{cmax}$  (One-point photosynthesis) data were used after data quality checks had been verified.

## Results

### *Comparison of $V_{cmax}$ estimation methods*

Nonpaired t-tests showed that the  $V'_{cmax}$  values of leaves with similar ages and branch positions that were detached from the trees for LMA and nutrient analyses did not differ from the  $V_{cmax}$  values of the leaves sampled for A–Ci curves and  $g_s$ –VPD<sub>L</sub> response curves in Repeated measurements approach ( $p = 0.372$ , Supplementary Material). The  $V_{cmax}$  values obtained from the A–Ci curve fitting (Domingues et al. 2010) did not show significant differences from the  $V'_{cmax}$  values calculated by the One-point measurement method (de Kauwe et al. 2016) (reduced major axis regression — RMA:  $r^2 = 0.91$ ,  $p = 0.443$ ; Supplementary Material); thus, all  $V_{cmax}$  estimates (derived from Repeated A–C<sub>i</sub> curves, and

the One-point method, and Chronosequence A–C<sub>i</sub> approaches) data were pooled together for the following analyses.

### *Photosynthetic capacity changes with leaf age*

At the community level, the  $V_{cmax}$  values increased in young, developing leaves until reaching a maximum mean value when the leaves were 45 days old (evidenced by the breakpoint, Fig. 1) ( $F_{1,56} = 5.0$ ,  $p = 0.03$ ,  $r^2 = 0.08$ ). After the breakpoint, a significant negative linear relationship between  $V_{cmax}$  and leaf age was detected (Fig. 1) ( $F_{1,158} = 19.8$ ,  $p < 0.001$ ,  $r^2 = 0.11$ ).

Considering the phenological habits, a similar pattern was found for the “flush” type trees (Fig. 2A); this type represents most of the community sampled (66%), and in the first slope ( $F_{1,67} = 17.2$ ,  $p < 0.001$ ,  $r^2 = 0.21$ ), the  $V_{cmax}$  values increased up to 90 days old and decreased continuously after that ( $F_{1,66} = 5.5$ ,  $p = 0.02$ ,  $r^2 = 0.08$ ) (Table 2). The “successive” type, represented by only one species, *P. tomentosa*, showed a decline in  $V_{cmax}$  since the start of the leaf age gradient (Fig. 2B) ( $F_{1,32} = 22.2$ ,  $p < 0.001$ ,  $r^2 = 0.43$ ). The phenological habits described as “brevideciduous” and “irregular”, represented in our dataset by one species each, *P. arborea* and *M. angularis*, respectively, showed similar  $V_{cmax}$  patterns to the those of the community and “flush” type trees, with  $V_{cmax}$  values rising to an age of 80 days and decreasing after that, albeit these changes were not statistically significant (Fig. 2C, Table 2).

Although only significant for the community and the “flush” phenological type, the relationships between  $V_{cmax}$  and leaf age changes were analyzed for the different studied species (Fig. 3). Individual evaluations of the nine trees sampled showed large variations in the behavior of  $V_{cmax}$  in relation to leaf maturation time, varying from  $8 \pm 30$  days for *P. tomentosa* to  $238 \pm 30$  days for *P. caimito*, and the decline rate (slope) varied from -0.003

$\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ day}^{-1}$  for *P. platyphylla* to  $-0.065 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ day}^{-1}$  for *P. tomentosa* (Table 2). At the individual species level, five trees showed a significant negative relationship between  $V_{cmax}$  and leaf age after the breakpoint: *P. tomentosa*, *P. caimito*, *D. cuspidatum*, *D. stelechantha*, and *P. guianensis* (Fig. 3, Table 2). The remaining four tree species did not show significant trends or effects of leaf age on  $V_{cmax}$ : *V. parviflora*, *P. platyphylla*, *M. angularis*, and *P. arborea* (Fig. 3, Table 2).

For the categorical analyses, the mean  $V_{cmax}$  differed significantly among the leaf age classes (Fig. 4) ( $F_{1,213} = 11.0$ ,  $p < 0.001$ ,  $r^2 = 0.09$ ). The mature age class (70–160 days,  $n = 86$ ) showed the highest mean  $V_{cmax}$  values, at  $37.4 \pm 1.4 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  (mean  $\pm$  SE), while the “young” and “old” age classes had values of  $28.3 \pm 1.8$  (5–56 days;  $n = 61$ ) and  $30.4 \pm 1.2$  (234–612 days,  $n = 66$ ), respectively. The average  $V_{cmax}$  of “mature” leaves was 32% higher than that of young leaves and 23% higher than that of old leaves. The  $V_{cmax}$  of mature leaves was comparatively higher than that of young ( $p < 0.001$ ,  $n = 61$ ) and old leaves ( $p = 0.002$ ,  $n = 66$ ).

#### *Intra- and interspecific variability in $V_{cmax}$*

Leaves in the early stage of development (up to  $5 \pm 30$  days) displayed the highest range of variation in  $V_{cmax}$  at the community level, varying from 5.8 to  $70.4 \mu\text{mol m}^{-2} \text{ s}^{-1}$  (*P. guianensis* at 14 days, *P. tomentosa* at 9 days, respectively). In fact, the variability in  $V_{cmax}$  values within the same tree species and in leaves of the same age can reach up to 146% (*V. parviflora* at 257 days — from 16.9 to  $41.7 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ). Concerning the intrinsic variability of photosynthetic behavior throughout a continuous age gradient, we note that the data exhibited two different patterns: 1) species showed a significant decrease in  $V_{cmax}$  values (*P. tomentosa*, *P. caimito*, *D. cuspidatum*, *D. stelechantha*); 2) species showed constant  $V_{cmax}$

values after a maximum was reached (i.e., *M. angularis*, *V. parviflora*, *P. platyphylla*, *P. guianensis*, and *P. arborea*). These different patterns occurred at the same time and resulted in the presence of a modest but significant correlation between the  $V_{cmax}$  values and leaf age.

### *Biochemical and biophysical parameter changes with leaf age*

Leaf dry mass per area (LMA) increased significantly with leaf age ( $p < 0.01$ ,  $r^2 = 0.13$ ,  $n = 181$ ) (Fig. 5A). The leaf nitrogen concentration remained constant ( $p = 0.13$ ,  $r^2 = 0.01$ ,  $n = 181$ ), while the phosphorus ( $p < 0.01$ ,  $r^2 = 0.25$ ,  $n = 181$ ) and potassium ( $p < 0.01$ ,  $r^2 = 0.52$ ,  $n = 181$ ) concentrations showed significant declines with increasing age (Fig. 5B, 5C and 5D, respectively).

Stomatal control ( $C_{gs}\%$ ), which is given by the amplitude of the maximum opening and closure over the  $g_s$ - $VPD_L$  curves, showed a significant increase starting from young, developing leaves up to 263 days ( $F_{1,96} = 35.4$ ,  $p < 0.001$ ,  $r^2 = 0.27$ ) and then became constant with age ( $F_{1,35} = 0.25$ ,  $p = 0.62$ ,  $r^2 = 0.01$ ) (Fig. 6).

$C_{gs}\%$  was also related to the leaf K concentration (Fig. 7), with the data demonstrating a negative relationship between these parameters ( $F_{1,124} = 48.4$ ,  $p < 0.01$ ;  $r^2 = 0.28$ ). When analyzed by leaf age class (Fig. 7B), we observed an increase in the relationship strength (steeper slope) with increasing leaf age, from young ( $F_{1,28} = 1.3$ ,  $p = 0.26$ ;  $r^2 = 0.05$ ) to old leaves ( $F_{1,50} = 16.4$ ,  $p < 0.01$ ,  $r^2 = 0.24$ ).

## **Discussion**

*Does  $V_{cmax}$  decline with leaf age in most trees?*



We observed significant changes in the  $V_{cmax}$  values of the community across the leaf age gradient in both continuous and age-categorical analyses (Fig. 1 and 4), with young, developing leaves experiencing increasing mean  $V_{cmax}$  values up to a maximum (45 days) and then decreasing values gradually over time. Our categorical analysis for the tree community (Fig. 4) found similar results as those of Wu et al. (2016) and Albert et al. (2018) using comparable age categories, giving support to the Leaf Demography-Ontogeny hypothesis. Leaves within the range of 70–160 days old showed the highest mean values of  $V_{cmax}$  compared to both younger and older leaves, although for our community-level dataset, the maximum mean  $V_{cmax}$  was reached much earlier (45 days) than the dates specified as mature by Wu et al. 2016 (90–150 days). Additionally, we found that the decrease in mean  $V_{cmax}$  that occurred from mature to old leaves was 23%, while Wu et al. (2016) and Albert et al. (2018) showed stronger declines, at 35% and 46%, respectively.

However, when the same dataset was analyzed in a species-specific approach, this relationship could not be confirmed for all trees (Fig. 3). Our study shows that the use of predefined age classes when analyzing a diverse community of tree species with different phenological habits may not be the most desirable method for studying the effect of leaf age on gas exchange capacity. We believe this is the case because, even when normalized by mean leaf longevity, a leaf that is considered old for one species may be considered mature for another species with a different lifespan, leaf emergence pattern, or rate of photosynthetic decline (Kikuzawa 1991). Therefore, current uncertainties regarding the effect of leaf aging on  $V_{cmax}$  values partly arise from the high intra- and interspecific variability in  $V_{cmax}$  values that occurs within a small range of leaf ages and the species-specific photosynthetic development patterns that occur throughout the lifespans of leaves.

Variability in photosynthetic capacity with respect to certain leaf ages was found at all levels of analysis, at the community level, species level and among the phenological habit

types, independent of the sample size, which could be explained by a series of endogenous and/or exogenous factors. Differences in the plant growth patterns, leaf phenologies, leaf morphologies, leaf longevities, crown architecture and microclimate (light, temperature and air humidity in the surroundings of the leaf) of different trees are all possible causes of this observed variability (Kitajima et al. 1997b, Kikuzawa and Lechowicz 2011).

For instance, when considering the physiological and phenological differences that occur among trees with different life strategies, *P. tomentosa*, an early-successional species, showed the fastest physiological development time and the most significant decline of all trees studied. During the demographic census, it was possible to observe that *P. tomentosa* leaves were born and completed expansion within two weeks following emergence. This behavior, coupled with its early development of high photosynthetic rates and  $V_{cmax}$ , explains why this species was characterized by a monotonic decline in  $V_{cmax}$  with age (Figure 2B). Leaves and/or branches of species with higher turnover rates (i.e., shorter mean leaf longevities) are expected to have more pronounced decline rates (Kikuzawa, 1991, Kitajima et al. 1997b, 2002), which is in accordance with the observations of branch-level leaf demography and gas exchange measurements conducted in this study. In contrast, late-successional tree species such as *P. guianensis* commonly showed lower  $V_{cmax}$  values throughout their lifespans and slower declines with leaf age than early-successional species (Kitajima et al. 2005).

Thus, ecosystem-level variability in photosynthetic capacity may depend on species composition and on the assemblages and proportions of contrasting plant functional groups of forests. A secondary or intensively disturbed forest may thus have a different carbon uptake seasonality pattern than that of an old-growth primary forest with a greater proportion of evergreen late-successional species due to the higher frequency of early-successional tree species that display divergent phenological habits.

### *Biochemical and biophysical parameter changes with leaf age*

Nitrogen is an important component of several photosynthesis-related proteins and enzymes (i.e., chlorophyll and Rubisco). Although linear decreases in both N concentrations and photosynthetic performances with increasing age have been observed in many studies (Zotz and Winter 1994, Ackerly and Bazzaz 1995), we found that the N concentrations remained stable with leaf age (Fig. 5B), which could partially explain the relatively modest decline observed in the  $V_{max}$  values with age at the community level. It is also important to note that our study site and most *terra firme* undisturbed forests in Amazonia are not considered N-limited systems (Fyllas et al. 2009, Quesada et al. 2010, see also Fleischer et al. 2019 description for this site), and the proportion of total leaf N allocated to activate Rubisco in tropical rainforests may be lower than that in other biomes (Bahar et al. 2017).

The other observed leaf traits changed more notably along the leaf age gradient, including increments in LMA and stomatal control and reductions in P and K (Fig. 5). LMA increased with age, independent of changes in N, P or K concentrations, as the nutrient concentrations varied similarly with leaf age for both mass and area-based concentrations (data not shown); thus, variations in nutrient concentrations were not simply dilution effects of the increasing LMA. These factors may reflect increment of structural components and not only the thickness of leaves *per se*. It is known that there is a trade-off between investments in defense and growth during leaf development: while young leaves invest more in both photosynthetic apparatus and structure, mature leaves tend to invest more in maintenance (Jardine et al. 2016). The observed increase in LMA after the leaves reached maturity could be related to an investment in structural defenses against herbivores or unfavorable

environmental conditions and could also affect the conductivity of water and CO<sub>2</sub> inside the mesophyll (Wright et al. 2004, Poorter et al. 2009).

The highest P concentration was found in the youngest leaves sampled when metabolic processes were more intense (i.e., when each leaf is acting as a sink of carbon). Potentially affected by this, leaf respiration is one of the processes that is elevated during the early stages of leaf development and stabilizes with increasing leaf age (Xu and Baldocchi 2003, Noda et al. 2014, Cernusak 2020). As leaves age beyond the mature stage, their concentrations of mobile nutrients tend to be reduced before and during leaf senescence (Achat et al. 2018), and the remobilization efficiency of the source-sink relationship can be affected by environmental aspects, such as the soil nutrient content (Crafts-Brandner and Poneleit 1992, Killingbeck 2004, Achat et al. 2018), and ontogenetically by the leaf lifespan (Killingbeck 2004, Achat et al. 2018); both factors are negatively related to the rate of nutrient remobilization (Achat et al. 2018). During senescence, P and K are generally mobilized to active sink tissues (e.g., developing leaves and reproductive organs) as a parsimonious way to retain available P and K in the plant, particularly in soils with low concentrations of P and base cations (Stigter and Plaxton 2015, Achat et al. 2018), such as those in central Amazonia (Quesada et al. 2010). K showed the most significant age-related decline along the continuous age gradient. This element is very mobile, is found in relatively high concentrations in plants and has frequently been described as the most resorbed of all nutrients (Killingbeck 2004, Milla et al. 2005). For young photosynthetic organs such as leaves, K accumulation (Fig. 7) may facilitate growth in leaves by helping with the turgor pressure in plant cells to allow them to hang plagiotropically (flat) in the canopy (Milla et al. 2005) and contribute to osmotic control of the stomata and, consequently, metabolic functioning (Wang and Wu 2017).

The physiological parameter of stomatal control ( $C_{gs}$  %), given by the amplitude of stomatal conductance values to changes in  $VPD_L$ , was positively correlated with leaf age for the tree community during the early developmental stage, but there was no correlation with leaf age after leaf maturity (Fig. 6). We initially hypothesized that as leaves age, they gradually lose the ability to fully close their stomata under higher water deficit conditions (i.e., high VPD) due to an age-dependent deterioration of guard-cell functioning (Jones and Sutherland 1991); therefore, under this hypothesis, the observations would show lower amplitudes between the maximum and minimum stomatal conductance values with progressing leaf age. This would result in an increase in water loss, which could negatively impact photosynthesis due to reduced water use efficiency (Reich and Borchert 1988). However, our analysis showed that older leaves did not show any differences in stomatal opening, closure, or conductance (data not shown) compared to mature leaves. This response could indicate the absence of specific deterioration in stomatal functionality with age or decreased absolute  $g_s$  sensitivity to changes in  $VPD_L$  (Field and Mooney, 1983). Additionally, the lower K concentration observed in mature and older leaves compared to leaves of the youngest developmental stage (Fig. 7) may contribute to the mechanism that makes the opening or closure of stomatal apertures insensitive to changes in  $VPD_L$ , since leaves show scattered stomatal control behavior after the breakpoint.

#### *$V_{cmax}$ and leaf phenology in a heterogeneous tropical forest*

The Leaf Demography-Ontogeny hypothesis has seldom been tested, except in select studies on ecosystem estimates derived from the eddy covariance method (Carswell et al. 2002, Restrepo-Coupe et al. 2013, 2017, Wu et al. 2016, 2017b), satellite-based (EVI-MAIAC) measurements (Huete et al. 2006, Brando et al. 2010, Bi et al. 2015), and

spectroscopy (Chavana-Bryant et al. 2017, 2019, Wu et al. 2017a, 2019), in addition to limited direct leaf- and branch-level photosynthesis measurements in a different Amazon forest community with different seasonality and soil properties from those of the present study (Wu et al. 2016, Albert et al. 2018).

The decline in  $V_{cmax}$  of the ecosystem before the dry season in the central Amazon forest (Restrepo-Coupe et al. 2013) has been attributed to the occurrence of an increased leaf area with lower photosynthetic efficiency (old leaves), while the increase in GPP throughout the dry season has been ascribed to the renewal of leaves with higher photosynthetic efficiencies (Restrepo-Coupe et al. 2013, Wu et al. 2016a, 2017b). This is because most of the tree species in central Amazon forests flush a massive number of new leaves at the beginning of the dry season (Nelson et al. 2014, Guan et al. 2015, Lopes et al. 2016, Wagner et al. 2017).

For the community studied here and during our three-year leaf demographic census, at least 50% of the trees flushed new leaves during the transition to the dry season. Although our leaf demography census comprises 21 trees, the results are consistent with camera-based phenological assessments of the full-canopy dynamics of a *terra firme* forest near our study site (Nelson et al. 2014, Lopes et al. 2016), which found that 44% out of 65 living trees and 55% of the 267 individuals upper canopy crowns flushed new leaves during the five driest months. Additionally, 23% of all trees monitored by these phenocam observations in Nelson et al. (2014) were brevideciduous, spending a brief time leafless before the new flushes occurred, as was found here for *P. arborea*.

More dominant species in terms of abundance, productivity, and biomass are expected to play enforcing roles in the functionality and carbon cycling of the ecosystems in the Amazon region (ter Steege, 2013, Fauset et al. 2015). Among the species studied, two are considered hyperdominant in the Amazon (*P. caimito* and *P. guianensis*); that is, they are

listed in the 227 species that represent 50% of all stems in the basin (ter Steege, 2013). This context demonstrates the importance of these species in terms of representativeness in the Amazon basin. In contrast, other species, such as *M. angularis*, *D. stelechantha*, *D. cuspidatum* and *P. arborea*, despite being relatively common in terms of tree numbers, show low levels of biomass accumulation and low rates of productivity.

Here, we performed a highly controlled survey of gas exchange characteristics and leaf demography in the tropics, with a unique leaf-level photosynthetic capacity dataset generated by continuous fine-scale data of seasonal biochemical and biophysical changes. Improvements in the understanding of how leaf age affects physiological parameters such as gas exchange, photosynthesis, stomatal control, and leaf nutritional status should lead to more accurate photosynthetic capacity estimates and predictions of tropical forest carbon cycling. The important implications of the diversity of photosynthetic behaviors among species and of phenological habits and how these factors affect ecosystem productivity in an asynchronous way (different leaf maturation times) should be considered in future studies. This study also shows the complexity of scaling-up mechanisms in tropical rainforests; considering cryptic phenology (i.e., phenology that is difficult to distinguish and interpret based on common measurements at typical scales of phenological examination, Albert et al. 2019), it was necessary to determine the fine-scale leaf demography through a three-year demographic census in the same trees to obtain the variables of the leaf age, leaf longevity, and phenological behavior of the tree community. These variables, which are associated with the effect of leaf age on photosynthetic capacities, may help to estimate with greater precision the total carbon gain at both the leaf and whole-plant scales and the seasonal effects on community performance (Nilsen et al. 1988, Kitajima et al. 2002, Suárez 2010). These fine-scale measurements may be combined with other approaches on a broader scale by tower-

mounted phenological cameras (Lopes et al. 2016), satellite-based remote sensing indices (Wu et al. 2018) or spectroscopy (Chavana-Bryant et al. 2017, Wu et al. 2017a).

## Conclusions

We tested one of the predictions of the Leaf Demography-Ontogeny hypothesis (Wu et al. 2016) by two different approaches (by assigning leaf ages on continuous and categorical scales) and downscaled the leaf demography to leaf-level assessments in the same trees. Our results partially support the hypothesis of ontogenetic and phenological changes in  $V_{cmax}$  values in a tropical rainforest tree community. The categorical age-group analysis showed a significant decline in  $V_{cmax}$  values throughout the leaf lifespan. However, as there was variation from this dominant behavior in a few species in this study, the observed relationship between  $V_{cmax}$  and leaf age for the community dataset was slightly weakened, reflecting a degree of variation in terms of the timing and amplitudes of the processes of natural leaf development among species. Most of the trees showed significant leaf-aging effects on  $V_{cmax}$  values. We also observed high intraspecific and interspecific variability in  $V_{cmax}$  values within a small range of leaf ages as well as different leaf maturation times and different intensities of the leaf age effect. Our data suggest that considering a tree community as a composite of functional groupings, each with their own specific phenological traits such as leaf longevity and phenological habits, will be valuable for reducing the uncertainty in studies quantifying the carbon cycle seasonality of tropical forests with high species diversity.

## Conflict of interest

The authors declare no conflict of interest.



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### **Author's contributions**

J.C.G.M, S.G., A.G., H.E.M.N., C.A.Q., and D.M.L designed the research; J.C.G.M., S.G., A.G., H.E.M.N., C.A.Q., B.P., and D.M.L. performed the research; J.C.G.M., S.G., A.G., H.E.M.N., T.F.D., B.K., K.F., A.R., and P.M analyzed data; J.C.G.M., S.G., T.F.D., and I.A. wrote the paper; and A.G., R.J.N., T.F.D., K.F., A.R., C.A.Q., B.K., and P.M. discussed and commented on the paper.

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## List of Figures

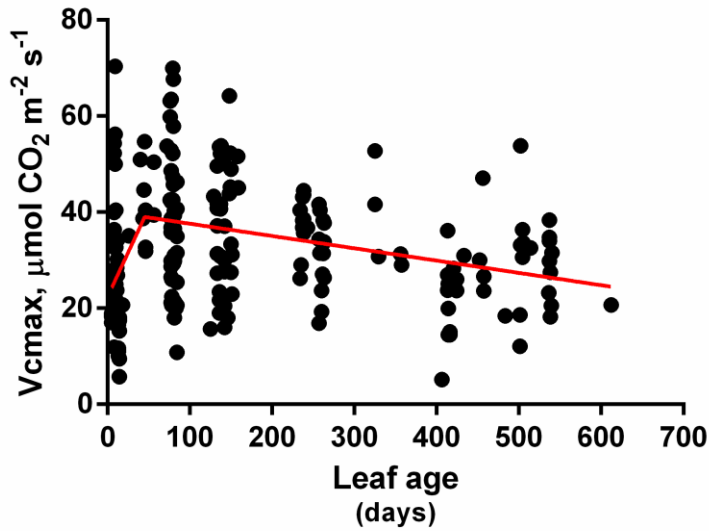


Figure 1. Relationship between  $V_{cmax}$  at 25 °C and the continuous leaf age gradient in days for all species sampled ( $n = 213$ , species = 9). The segmented linear regressions of different directions (upward:  $F_{1,59} = 5.7$ ,  $p = 0.02$ ,  $r^2 = 0.09$ , downward:  $F_{1,60} = 20.6$ ,  $p < 0.001$ ,  $r^2 = 0.12$ ) were divided by a breaking point of 45 days. Leaf age has a margin of  $\pm 30$  days.

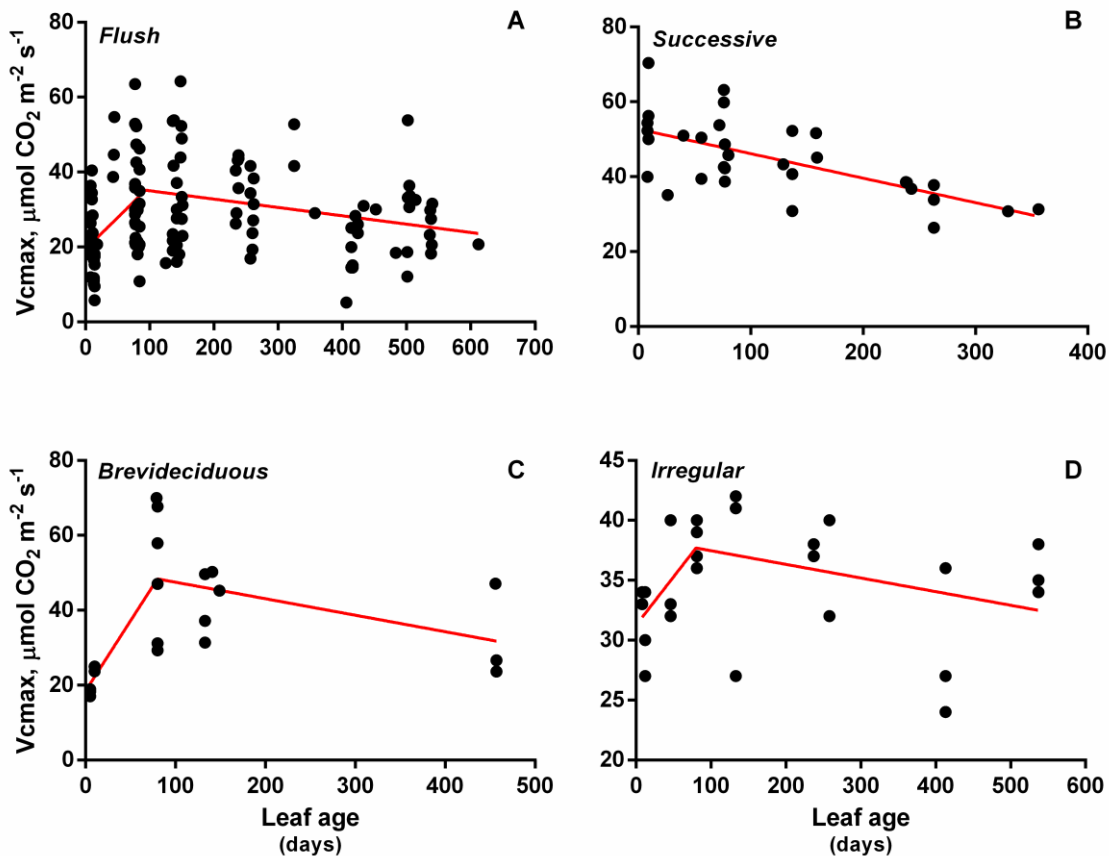


Figure 2. Relationship between  $V_{cmax}$  at 25 °C and the continuous leaf age gradient in days for the A) flush type ( $n = 133$ , species = 6), B) successive type ( $n = 32$ , species = 1), C) brevideciduous type ( $n = 20$ , species = 1), and D) irregular type ( $n = 28$ , species = 1) phenological habit classifications. Trees of the “flush” type both flush and shed large amounts of leaves over short periods every one or two years; “successive” species exchange leaves continuously; “brevideciduous” trees shed all leaves and remain leafless for short periods before flushing new leaves; “irregular” species do not fit in any of the types described previously. For more details, see the phenological habit explanation in the methods section. The linear regressions of types the “flush” (upward:  $F_{1,67} = 17.2$ ,  $p < 0.001$ ,  $r^2 = 0.21$ ; downward:  $F_{1,66} = 5.5$ ,  $p = 0.02$ ,  $r^2 = 0.08$ ), “successive” ( $F_{1,12} = 22.2$ ,  $p < 0.001$ ,  $r^2 = 0.43$ ), “brevideciduous” (upward:  $F_{1,12} = 16.0$ ,  $p = 0.003$ ,  $r^2 = 0.62$ ; downward:  $F_{1,13} = 2.3$ ,  $p = 0.16$ ,  $r^2 = 0.18$ ) and “irregular” (upward:  $F_{1,15} = 12.9$ ,  $p = 0.003$ ,  $r^2 = 0.50$ ; downward:  $F_{1,19} = 2.8$ ,  $p$



= 0.11,  $r^2 = 0.14$ ) types are shown. The flush, brevideciduous and irregular types were divided into two curves with breakpoints at days 90, 80, and 81, respectively. Leaf age has a margin of  $\pm 30$  days.

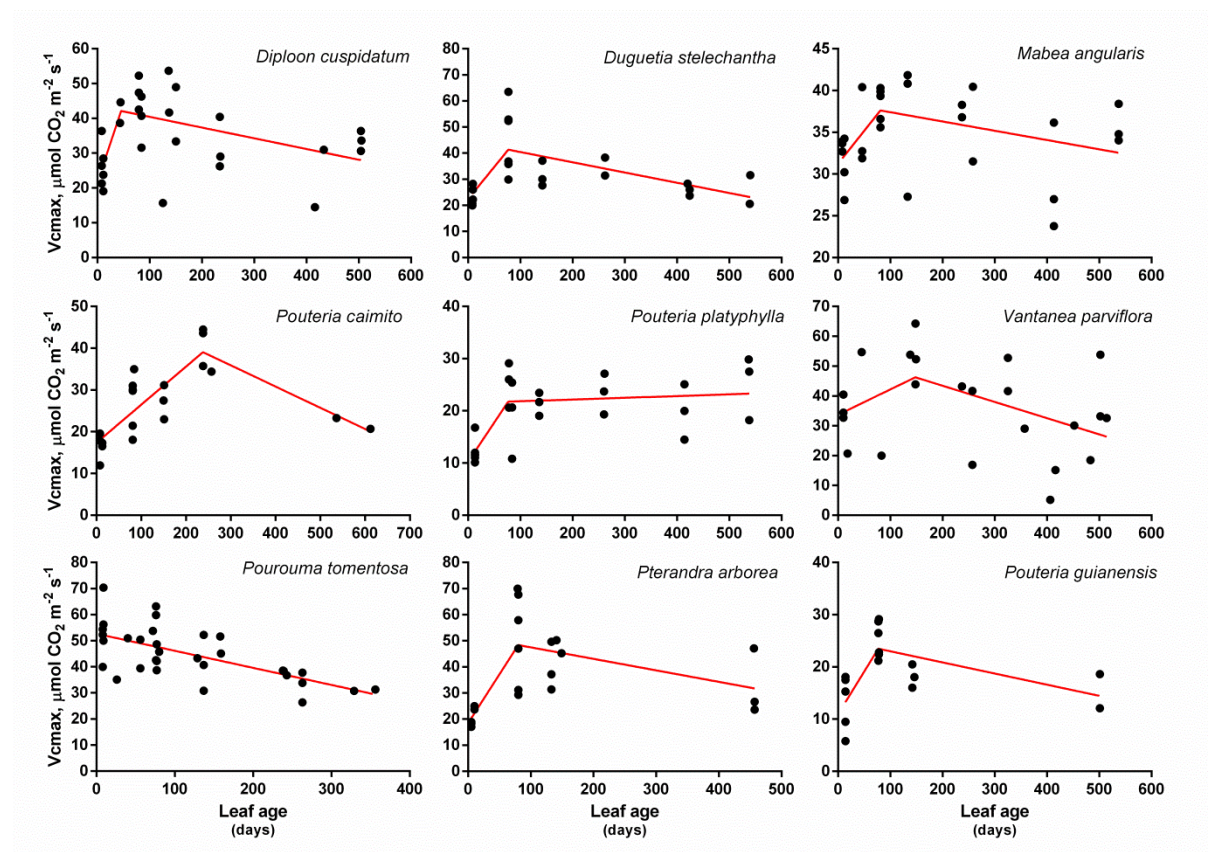


Figure 3. Relationship between  $V_{cmax}$  at 25 °C and the chronological leaf age in days for all nine trees: *Diploon cuspidatum*, *Duguetia stelechantha*, *Mabea angularis*, *Pouteria caimito*, *Pouteria platyphylla*, *Vantanea parviflora*, *Pourouma tomentosa*, *Pterandra arborea*, and *Pouteria guianensis*. Individual statistics for each species of the segmented regression are presented in Table 2. Leaf age has a margin of  $\pm 30$  days.

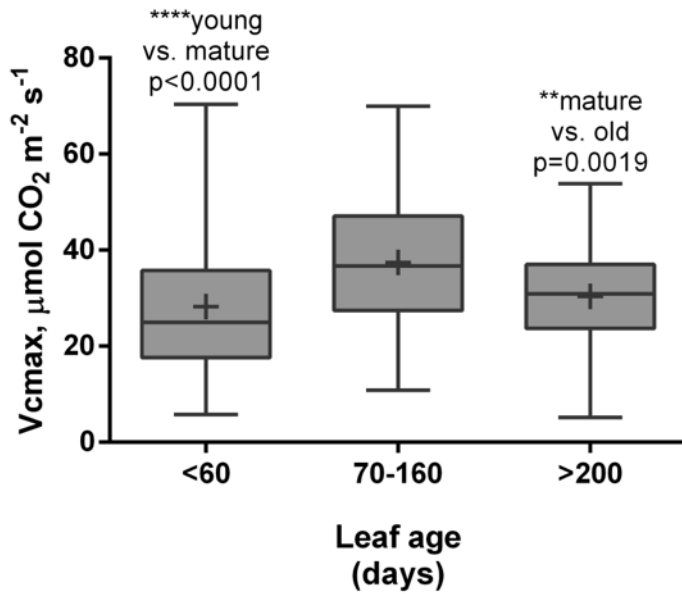


Figure 4. Leaf age effect on  $V_{cmax}$  at 25 °C, derived from a combination of the gas exchange measurements of A– $C_i$  curves and One-point measurements, among leaf age classes for the nine tree species sampled ( $n = 213$ ). Boxplots represent the age categories, which are divided into young (< 60 days,  $n = 61$ ), mature ( $70 < x < 160$  days,  $n = 86$ ), and old (> 200 days,  $n = 66$ ). The horizontal boxplot lines represent the medians, the whiskers are the mean error deviations, and the points are the outliers. Leaf age has a margin of  $\pm 30$  days.

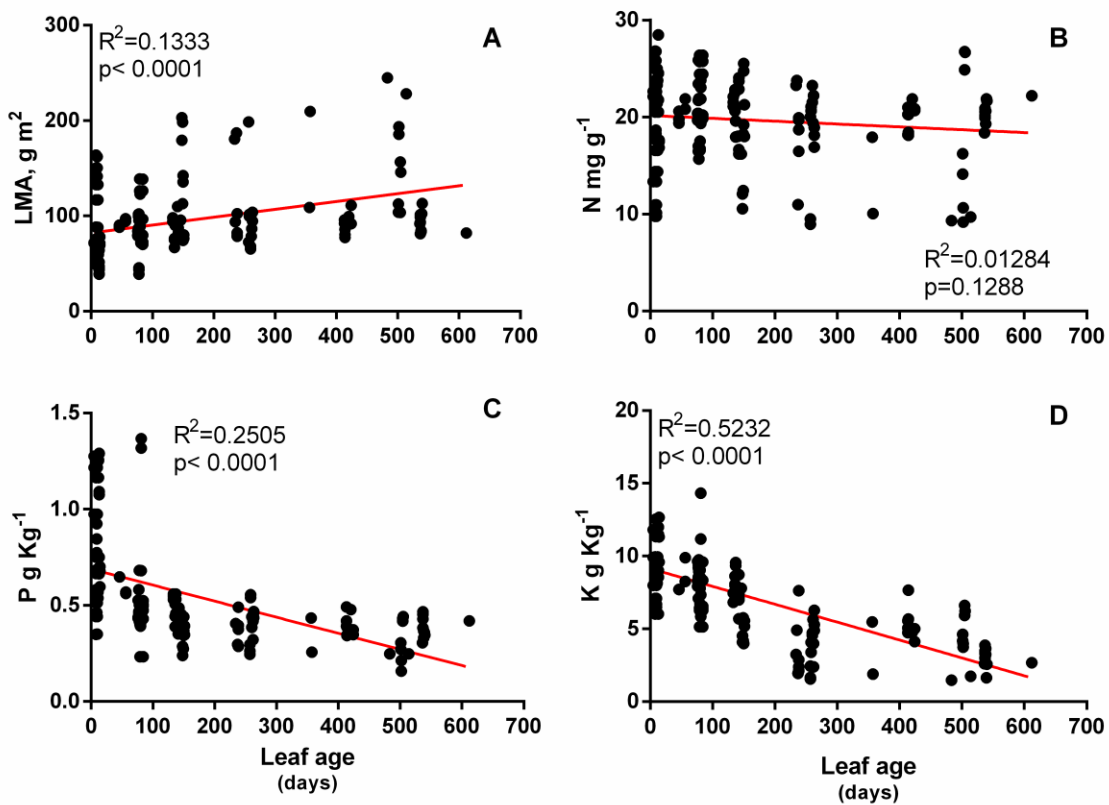


Figure 5. Relationships between A) LMA ( $\text{g m}^{-2}$ ), B) leaf nitrogen ( $\text{mg g}^{-1}$ ), C) leaf phosphorus ( $\text{mg g}^{-1}$ ), D) leaf potassium ( $\text{mg g}^{-1}$ ) and leaf chronological age in days for all nine tree species, from younger to older leaves ( $n = 181$ ). Leaf age has a margin of  $\pm 30$  days.

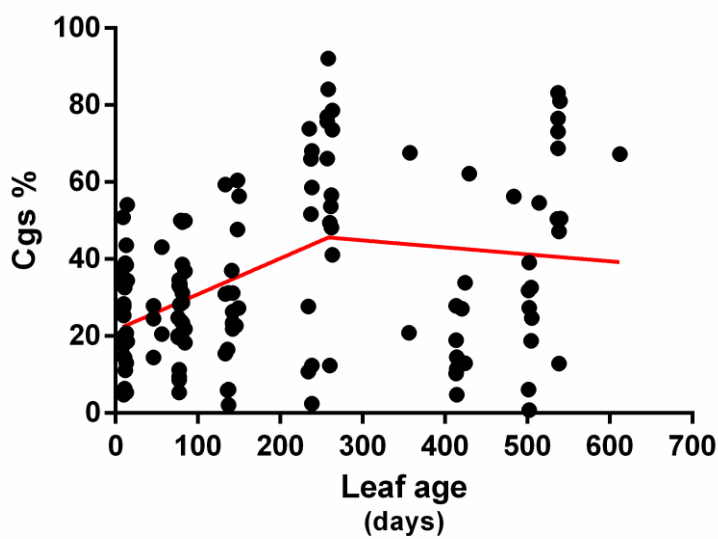


Figure 6. Relationship between stomatal control ( $C_{gs}\%$ ) and chronological leaf age in days for all tree species ( $n = 128$ , species = 9). The two linear regressions of different directions were divided with breakpoints of approximately 263 days (upward:  $F_{1,96} = 35.4$ ,  $p < 0.001$ ,  $r^2 = 0.27$ ; downward:  $F_{1,35} = 0.3$ ,  $p = 0.62$ ,  $r^2 = 0.01$ ). Leaf age has a margin of  $\pm 30$  days.

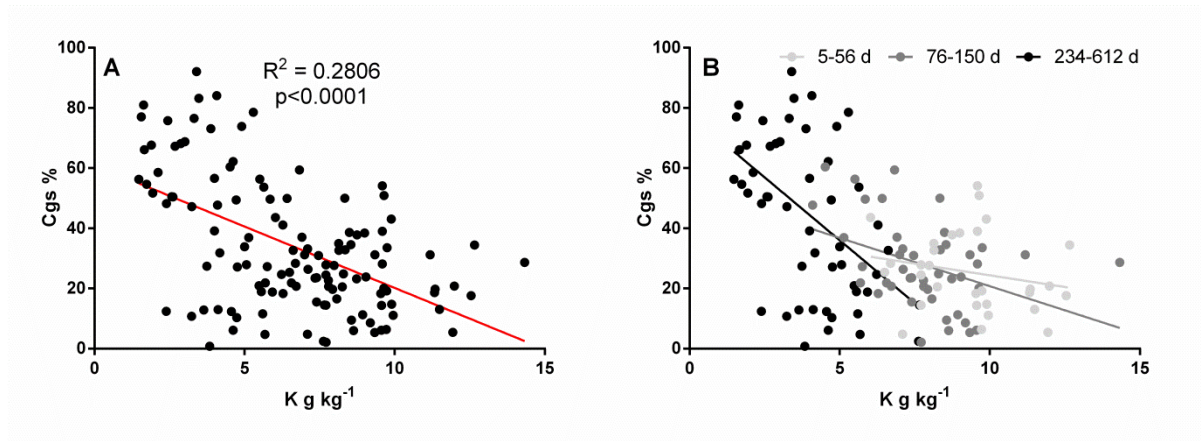


Figure 7. A) Relationship between stomatal control ( $C_{gs}\%$ ) and potassium concentration (K) for all trees, from younger to older leaves ( $n = 128$ ), and B) categorized into leaf age classes as young (5–56 days), mature (76–150 days) and old (234–612 days) leaves. Leaf age has a margin of  $\pm 30$  days.

Table 1. Characteristics of monitored trees. Species, botanical family, number of monitored branches, canopy environment (UC = upper canopy, MC = mid-canopy, US = understory), crown height (m), mean leaf longevity (days  $\pm$ SD) and their respective number of monitored leaves used in the estimation of leaf longevity, phenological habit<sup>1</sup> (described in Methods section - Phenological habit as Successive, Brevidciduous, Semi-flush, Flush or Irregular) of the 21 trees surrounding towers of the AmazonFACE program. The nine species highlighted with an asterisk were selected for the gas exchange measurements on the 2017 and 2018 field campaigns, since only these have flushed new leaves by the time of the first campaign has started in July. <sup>2</sup>Potential leaf

longevity, since the leaves remained alive until the end of demographic census (1025 days on May 2019), thus, the maximum or average leaf longevity are still unknown.

Species	Botanical Family	branches	Canopy position	Height	Mean Longevity	Number of leaves	Phenological habit <sup>1</sup>
<i>Diploon cuspidatum</i> (Hoehne) Cronq*	Sapotaceae	20	UC	28.6	265.27 ± 125.3	153	Flush
<i>Licania coriacea</i> Benth.	Chrysobalanaceae	20	UC	26.8	327.20 ± 147.61	141	Flush
<i>Matayba purgans</i> (Poepp.) Radlk.	Sapindaceae	16	UC	17.7	556.75 ± 279.66	61	Flush
<i>Ocotea cernua</i> (Nees) Mez	Lauraceae	20	UC	23.2	437.8 ± 255.02	182	Flush
<i>Pteranda arborea</i> Ducke *	Malpighiaceae	15	UC	26.8	266.5 ± 80.44	149	Brevideciduous
<i>Pourouma tomentosa</i> Mart. ex Miq. *	Urticaceae	7	UC	17.7	196.56 ± 87.94	66	Successive
<i>Swartzia tessmannii</i> Harms	Fabaceae	10	UC	25	336.37 ± 104.48	19	Flush
<i>Vantanea parviflora</i> Lam. *	Humiriaceae	20	UC	26.8	367.37 ± 215.88	138	Flush
<i>Duguetia stelechantha</i> (Diels) R.E.Fr.*	Annonaceae	17	MC	14.1	472.10 ± 266.92	41	Flush
<i>Mabea angularis</i> Hollander *	Euphorbiaceae	16	MC	14	499.87 ± 277.16	83	Irregular
<i>Mabea angularis</i> Hollander	Euphorbiaceae	10	MC	15.8	294.17 ± 197.01	6	Irregular
<i>Pouteria caminito</i> (Ruiz & Pav.) Radlk*	Sapotaceae	17	MC	12.1	733.53 ± 188.86	30	Flush
<i>Pouteria guianensis</i> Aubl.*	Sapotaceae	10	MC	11	341.38 ± 187.80	13	Flush
<i>Protium ferrugineum</i> (Engl.) Engl.	Burseraceae	10	MC	10.3	300.33 ± 257.6	9	Flush
<i>Protium hebetatum</i> D.C. Daly	Burseraceae	12	MC	11	587.37 ± 227.9	19	Flush
<i>Erythroxylum</i>	Erythroxylaceae	10	US	7	602.50 ± 253.61	4	Flush

*amplum* Benth.

<i>Eschweilera grandiflora</i> (Aubl.) Sand	Lecythidaceae	10	US	6.6	Unknown ( $>1025$ ) <sup>2</sup>	47	Flush
<i>Eschweilera wachenheimii</i> (Benoist) Sandwith	Lecythidaceae	10	US	4.8	Unknown ( $>1025$ ) <sup>2</sup>	22	Flush
<i>Papayrola grandiflora</i> Tul.	Violaceae	10	US	5	$260.83 \pm 142.8$	6	Flush
<i>Pouteria platyphylla</i> (A.C.Sm.) Baehni*	Sapotaceae	10	US	6.6	$432.00 \pm 238.28$	4	Flush
<i>Swartzia arborescens</i> (Aubl.)Pittier	Fabaceae	10	US	3.5	Unknown ( $>1025$ ) <sup>2</sup>	16	Semi-flush

Table 2. Summary of segmented linear regressions testing relationships between photosynthetic capacity ( $V_{cmax}$ ) and leaf age in continuous age gradient for individual tree species and for the community (all trees pooled together). NS:  $p > 0.05$ .

Segmented regression	Maturatio n time / breakpoin t (days)	Upward Equation	F	p	r <sup>2</sup>	n	Downward Equation	F	p	r <sup>2</sup>	n
<i>Diploon cuspidatum</i>	45	$y = 0.4508x + 21.706$	9.5	0.02	0.61	8	$y = -0.0305x + 43.399$	5.3	0.03	0.22	21
<i>Duguetia stelechanth a</i>	77	$y = 0.3221x + 20.443$	6.1	NS	0.62	12	$y = -0.0411x + 45.04$	8.85	0.01	0.39	16
<i>Mabea angularis</i>	81	$y = 0.0846x + 31.137$	12.9	0.003	0.50	15	$y = -0.011x + 38.527$	2.8	NS	0.14	19
<i>Pouteria caimito</i>	238	$y = 0.099x + 16.728$	41.9	$<0.001$	0.72	18	$y = -0.0532x + 52.489$	23.0	0.01	0.85	6
<i>Pouteria platyphylla</i>	77	$y = 0.2008x + 9.579$	37.1	$<0.001$	0.84	9	$y = 0.0033x + 21.509$	0.22	NS	0.01	18
<i>Pouteria guianensis</i>	77	$y = 0.194x + 10.517$	11.5	0.014	0.66	8	$y = -0.0213x + 25.131$	7.2	0.03	0.45	11

<i>Pourouma tomentosa</i>	-	-	-	-	-	-	$y = -0.065x + 52.09$	22.2	<0.001	0.43	32
<i>Pterandra arborea</i>	80	$y = 0.405x + 18.21$	16.0	0.003	0.62	12	$y = -0.0357x + 48.606$	2.3	NS	0.18	13
<i>Vantanea parviflora</i>	148	$y = 0.1376x + 31.213$	3.2	NS	0.32	9	$y = -0.0578x + 55.653$	3.9	NS	0.22	16
Community (all species)	45	$y = 0.4508x + 21.5$	5.0	0.03	0.08	56	$y = -0.0259x + 40.286$	19.76	<0.001	0.11	157

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