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Article type : MS - Regular Manuscript

Age-dependent leaf physiology and consequences for crown-scale carbon uptake during the dry season in an Amazon evergreen forest

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Received: *16 September 2017*
Accepted: *8 January 2018*

Summary:

- Satellite and tower-based metrics of forest-scale photosynthesis generally increase with dry season progression across central Amazônia, but the underlying mechanisms lack consensus.
- We conducted demographic surveys of leaf age composition, and measured age-dependence of leaf physiology in broadleaf canopy trees of abundant species at a central eastern Amazon site. Using a novel leaf-to-branch scaling approach, we used this data to independently test the much-debated hypothesis – arising from satellite and tower-based observations – that leaf phenology could explain the forest-scale pattern of dry season photosynthesis.
- Stomatal conductance and biochemical parameters of photosynthesis were higher for recently mature leaves than for old leaves. Most branches had multiple leaf age categories

simultaneously present, and the number of recently mature leaves increased as the dry season progressed because old leaves were exchanged for new leaves.

- These findings provide the first direct field evidence that branch-scale photosynthetic capacity increases during the dry season, with a magnitude consistent with increases in ecosystem-scale photosynthetic capacity derived from flux towers. Interaction between leaf age-dependent physiology and shifting leaf age-demographic composition are sufficient to explain the dry season photosynthetic capacity pattern at this site, and should be considered in vegetation models of tropical evergreen forests.

Key words: drought, dry season green-up, leaf ontogeny, phenology, photosynthesis, scaling, tropical forests.

Introduction

Seasonality is a major source of natural variation in climatic variables, and is known to drive cycles of plant productivity in many ecosystems (Keeling *et al.*, 1995; Penuelas *et al.*, 2009; Richardson *et al.*, 2012). Much of the Amazon rainforest – the largest tropical forest in the world – experiences seasonality of rainfall due to convection associated with the migration of the Intertropical Convergence Zone, coastal squall lines, and other meterological systems (Horel *et al.*, 1989; Santos *et al.*, 2014; Batista da Silva Ferreira *et al.*, 2015). Evergreen forests of central Amazônia that experience wet and dry seasons show a curious pattern of dry season increases in gross primary productivity (GPP) derived from eddy covariance (EC) (Fig. 1; Saleska *et al.*, 2003; Hutyra *et al.*, 2007; Restrepo-Coupe *et al.*, 2013; Wu *et al.*, 2016), in contrast to many current land surface models (LSMs) that simulate decreasing GPP with seasonal declines in precipitation and soil water availability (Verbeeck *et al.*, 2011; Restrepo-Coupe *et al.*, 2017). Landscape-scale remote sensing studies complement site-specific EC studies and show increases in vegetation indices ('green-up') during dry seasons over much of the central Amazon basin (Huete *et al.*, 2006; Bi *et al.*, 2015). Although the magnitude of the satellite-observed dry season green-up has been questioned (Morton *et al.*, 2014), it is statistically significant (Saleska *et al.*, 2016), and it also suggests dry-season changes in photosynthetic processes. Identifying the mechanism(s) driving the dry season GPP pattern, and developing LSMs accordingly, is

important for attribution of seasonal changes to appropriate causes (Wu *et al.*, 2016) and ultimately for predictions of forest response to global climate change (Restrepo-Coupe *et al.*, 2017) as Amazônia is predicted to experience more severe dry periods in the future (Marengo *et al.*, 2012).

Hypothesized mechanisms for the late dry season GPP increase include: (1) increases in photosynthesis due to environmental changes (e.g. increased light availability; Tian *et al.*, 2000; Goulden *et al.*, 2004; Ichii *et al.*, 2005); (2) increases in the quantity of leaves (leaf area index, LAI) throughout the dry season (Goulden *et al.*, 2004; Myneni *et al.*, 2007); and (3) an increase in average leaf-level photosynthetic capacity (PC) (Goulden *et al.*, 2004; Doughty & Goulden, 2008; De Weirdt *et al.*, 2012; Kim *et al.*, 2012; Wu *et al.*, 2016). While these hypotheses (Fig. 2) are not mutually exclusive, they have implications for the corrective features required to re-structure LSMs and the type of data required for large-scale parameterization. Seasonal changes in the environment demand greater understanding of the microclimate of complex canopies through time; shifts in LAI demand an understanding of when and where leaf birth or death dynamics are altering structure; and changes in leaf-level photosynthesis require an understanding of how the seasonality of this physiological process relates to plant strategy and the environment.

Previous studies comparing the hypothesized drivers of GPP seasonality provide evidence that seasonal changes in environment or leaf quantity are insufficient to explain observed dry season increases in EC-derived GPP (Doughty & Goulden, 2008; Wu *et al.*, 2016, 2017b). Ecosystem-scale PC, a metric of ecosystem carbon uptake per unit incident light that is derived from GPP under fixed environmental conditions (photosynthetically active radiation, vapor pressure deficit, air temperature, and cloudiness; Restrepo-Coupe *et al.*, 2013; Wu *et al.*, 2016), also increases as the dry season progresses (Fig. 1) suggesting that dry season increases in photosynthesis are not simply due to environmental factors, but to biotic factors such as leaf quantity (Wu *et al.*, 2016). However, changes in leaf quantity alone are also insufficient to account for GPP or PC seasonality according to modeling assessments and ground-based estimates of LAI that show only modest seasonal variation (Doughty & Goulden, 2008; Brando *et al.*, 2010; Wu *et al.*, 2016).

A growing number of studies support the third hypothesis, seasonal increases in leaf-level PC, with leaf phenology (the timing of leaf production and abscission) mediating the leaf-level increase (Doughty & Goulden, 2008; Restrepo-Coupe *et al.*, 2013; Wu *et al.*, 2016). Leaves undergo structural and biochemical changes during development, aging, and senescence (Niinemets *et al.*, 2012; Pantin *et al.*, 2012). Any degree of synchronization of leaf phenology across crowns during the dry season would produce canopy-scale shifts in mean leaf age, which could explain observed seasonal changes in both EC-derived GPP (Doughty & Goulden, 2008; Wu *et al.*, 2016) and also reflectance (Chavana-Bryant *et al.*, 2017; Wu *et al.*, 2017a) during the dry season.

However, the hypothesis that leaf phenology increases mean leaf-level PC, and thus explains dry season GPP increases in central Amazon forests (the 'leaf demography-ontogeny hypothesis') lacks consensus, in part due to the uncertainties and limitations inherent in large-scale estimations of photosynthesis. Remote sensing of humid equatorial forests with dense canopies is challenging due to cloudiness (Asner, 2001; Samanta *et al.*, 2010), signal saturation (Myneni *et al.*, 2007), and sun-sensor geometry artifacts (Morton *et al.*, 2014; Saleska *et al.*, 2016). Partitioning EC-measured net ecosystem exchange into GPP and respiratory components assumes that unobserved daytime ecosystem respiration behaves the same as nighttime respiration (Reichstein *et al.*, 2005; Lasslop *et al.*, 2009), an assumption that does not always hold (Wehr *et al.*, 2016; Oikawa *et al.*, 2017). Since the main support for the leaf demography-ontogeny hypothesis relies upon estimates of PC that are themselves derived from EC-GPP (Wu *et al*., 2016), independent tests are needed.

To date there have been no studies that have directly tested the leaf demography-ontogeny hypothesis across individual trees with both leaf-level photosynthesis and leaf demography data from canopy species in an Amazon forest where EC-derived GPP and PC are also observed. Most studies of photosynthesis in tropical forests have focused on fully expanded leaves that were neither immature nor showing signs of senescence (e.g. Domingues *et al.*, 2014), but the rare studies of age-specific leaf traits (Chavana-Bryant *et al.*, 2017) or photosynthesis (Sobrado, 1994; Ishida *et al.*, 1999; Kitajima *et al.*, 2002; Alves *et al.*, 2014) show the effects of leaf age on physiology are significant. In evergreen forests of central Amazônia, direct observations of crowns (Brando *et al.*, 2010; Lopes *et al.*, 2016) and litterfall

(Doughty & Goulden, 2008) suggest dry-season changes in leaf demography. Missing are studies that integrate field-based leaf demographic surveys with photosynthesis measurements in the same individual trees, where such coupled measurements have the power to show whether the timing and magnitude of changes in tree PC agree with EC-derived PC. Furthermore, there are multiple limitations on photosynthesis, including biophysical limitations (e.g. stomatal conductance) and biochemical limitations (e.g. V_{cmax}, the maximum carboxylation rate of Rubisco, and J_{max}, the maximum rate of RUBP regeneration; Sharkey *et al.*, 2007), so it is important to examine multiple constraints across leaves of different ages.

Here we test the hypotheses that (1) leaf physiology, particularly PC, is affected by leaf age; and (2) the leaf age composition (leaf demography) of individual tree crowns varies during the dry season in a moist tropical forest of the Amazon. Then we examine the combined effects of leaf age-dependent physiology and leaf demography on branch-level PC. We compare the magnitude and direction of dry season changes in aggregate branch-level PC with previously reported EC-derived estimates of the canopy-level PC (Wu *et al.*, 2016; Restrepo-Coupe *et al.*, 2017) to evaluate the consistency of 'bottom-up' leaf-to-branch estimates with 'top-down' EC-derived estimates of PC.

Materials and Methods

Site

All trees studied were within the footprint of the LBA-ECO EC tower located in the Tapajós National Forest (TNF; 54°580W, 2°510S, Pará, Brazil) near kilometer 67 (K67) of BR-163 (Hutyra *et al.*, 2007). The forest is classified as moist evergreen tropical forest, receiving an average of 2022 mm y^{-1} of precipitation, and typically experiencing a 5-month dry season (months with <100 mm precipitation) from *c*. 15 July to *c*. 15 December each year (Rice *et al.*, 2004; Hutyra *et al.*, 2007). The forest is on flat terrain with a mean canopy height of *c*. 40–45 m (Hutyra *et al.*, 2007). During a typical dry season, canopy trees at this site generally maintain pre-dawn stem water potential above 2 MPa (Fig. 3a; Supporting Information Methods S1). For details about forest composition and structure see (Rice *et al.*, 2004; Vieira *et al.*, 2004), and for information about soil and water table, see Nepstad *et al*. (2002).

Tree selection and canopy access

Five canopy trees (25–44 m tall) were selected for both leaf physiology measurements and leaf demographic surveys: *Erisma uncinatum* Warm., *Manilkara elata* (Allemão ex Miq.) Monach., *Mezilaurus itauba* (Meisn.) Taub. ex Mez, *Tachigali cf. chrysophylla* (Poepp.) Zarucchi & Herend., *Chamaecrista xinguensis* (Ducke) H.S.Irwin & Barneby. Their selection was based on the criteria that they represented abundant species (Table S1), they were within the footprint of the K67 EC tower, and they could be accessed using arborist tree-climbing techniques. Additionally, one mid-canopy *Coussarea paniculata* (Vahl) Standl., a second *Manilkara elata* accessible from a walk-up tower, and a *Lecythis lurida* (Miers) S.A. Mori tree were opportunistically sampled. According to a biomass and species survey at the site (Vieira *et al.*, 2004; Pyle *et al.*, 2008) with resolved species names (Boyle *et al.*, 2013), these canopy trees belong to species accounting for *c*. 33% of basal area at the K67 site. The crowns of the canopy trees included sun and shade microenvironments, with the exception of the *T.* cf *chrysophylla*, which had an umbrella-shaped crown with few shaded branches, and the *C. paniculata*, the mid-canopy tree with all branches shaded. The *T.* cf *chrysophylla* trees and *C. xinguensis* tree had compound leaves, and all other trees had simple leaves. Leaflets from compound leaves were used for all physiological measurements and counted as leaves for the leaf demographic surveys because we observed that individual leaflets on the same compound leaf show variation in color and degree of expansion, suggesting that individual leaflets can reach maturity at different times. For sampling of leaves and branches for physiological measurements and leaf demography (see sections below), single rope access techniques were used to climb into individual crowns of canopy trees. Leaving climbing ropes in trees can compromise rope integrity, so trees were rigged and de-rigged with climbing ropes each day of sampling. Tree climbing required setting up two rope systems, one for access into the canopy, and one for movement within the canopy (branch-walking methods with a tie-in point high in the tree in combination with a lanyard). These arborist techniques allowed us to access branches that experienced sun and shade microenvironments at heights in excess of 35 m. The mid-canopy *C. paniculata* tree was accessed from above using a tie-in point from a larger canopy tree nearby or sampled with pole-pruners.

Photosynthetic gas exchange

Leaf-level gas exchange at the K67 site was measured with a portable infrared gas exchange measurement system (LI-6400, LI-COR Biosciences, Lincoln, NE) during dry-season fieldwork campaigns: August through December 2012, August 2013, and (for *C. paniculata* only) July 2015. Branch samples from the selected trees (Table S1) were collected via tree climbing, and included branches from both sunny and shady microenvironments if both were present. Before gas exchange measurements, branches were cut, gently lowered to the ground with ropes, and recut under water within 15 min.

For each branch, leaves were then classified into age categories based on (Chavana-Bryant *et al.*, 2017), and further informed by *in situ* leaf tagging and photo documentation carried out in these focal species to demonstrate leaf development (see Fig. S1 in Wu *et al.*, 2017a). Briefly, independently for each species, leaves were assigned age categories (young, mature, old) through visual assessment of leaf color, size, rigidity, and position in relation to other leaves and/or bud scars (see Fig. S1 for examples). 'Young' described immature leaves (>2 months old, not fully expanded and/or not fully green). 'Mature' described leaves that recently reached maturity (fully expanded, green, and 2–5 months old). 'Old' described leaves basal of young and mature leaves that were not yet senescent (fully expanded, attached below bud scars when bud scars were present, and >5 months old).

Gas exchange was typically measured for each age category present on the sampled branch. Measurement of net CO_2 assimilation rate vs calculated substomatal CO_2 concentration (A/C_i curves) were conducted with a LI-6400 under a standardized set of conditions: block temperature was 31 ± 2°C, photosynthetically active radiation was saturating (999-2001 µmol m^{-2} s^{-1}, estimated from a prior test for each tree), relative humidity was controlled between 20 and 67%. Previous temperature response curves at this site show optimal photosynthesis temperature of *c*. 31°C (Tribuzy, 2005). For all samples, leaf area for gas exchange was 6 cm^2, and stomatal ratio was assumed to be 1. Oxygen concentration was not manipulated, and was assumed to be 21%. For A/C_i curves the reference CO_2 concentrations were controlled as follows: 400, 100, 50, 100, 150, 250, 350, 550, 750 µmol mol^{-1}, and then increased by increments of between 200 to 500 to reach saturation at around 2000. We show net assimilation (A_{net}) at 350 µmol mol^{-1} over the dry season (Fig. 3b). Before curve fitting (see 'Analysis' section), quality control for gas

exchange measurements was applied to exclude values associated with instrument error and other likely outliers. Note that if initial gas exchange measurements from a candidate tree showed signs that recutting under water did not re-establish the water column, then the tree was excluded from future gas exchange measurements (this was the case for one tree, a *Lecythis lurida*). After quality control, a total of 97 A/C_i curves were available for analysis. A subset of the V_{cmax} parameters from these curves was reported in Wu *et al.* (2016). (Gas exchange data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.h83t0).

In situ stomatal conductance

Stomatal conductance (g_s) of the abaxial surface of leaves was measured *in situ* in the canopy using a steady-state leaf porometer (Decagon Devices, Pullman, WA) in automatic mode for five trees early in the dry season, before 15 October (Fig. S2) and nine trees late in the dry season, from 15 October to the end of the dry season (Fig. S3). From one to 29 leaves were selected for g_s measurement from each leaf age category present on branches at one to two locations within the crown that were accessible via climbing (see later Table S9). When the same trees were measured both early and late in the dry season, the same leaves were measured at both time periods (if a leaf was missing it was replaced with a nearby leaf of the original age category). Whenever possible, leaves were measured sequentially and repeatedly such that each leaf was measured 1-6 times between the hours of 08:20 h and 16:55 h over 1–2 d. Multiple measurements for each leaf were averaged before meta-analysis (see the Analysis subsection below). (Porometer g_s data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.h83t0).

Chlorophyll concentration

One to seven leaves from five trees (Table S1) were collected using arborist canopy access techniques on 13–15 November, 2012, wrapped individually in aluminum foil, frozen in liquid nitrogen, and kept frozen until chlorophyll was extracted (23 November, UFOPA campus). To determine Chl*a*, Chl*b*, and total (Chl*a*+*b*) content of leaves, *c*. 0.5 g (fresh weight) of each leaf was macerated in 7 ml 80% acetone, and then filtered. The filtrate volume was increased to a total of 20 ml. The supernatant was removed and absorbance was measured at 663 nm and 647

nm, for Chl*a* and Chl*b*, respectively, using a spectrophotometer (NOVA Instruments, 3300 UV) and absorbance was used to estimate chlorophyll content following Lichtenthaler (1987; see Methods S2 for equations). (Chlorophyll concentration data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.h83t0).

Leaf chemistry

A subsample of six to 95 leaves per leaf age category per tree from branch demography surveys, including almost all leaves used for gas exchange, were also analyzed for leaf chemistry (percent nitrogen and carbon-to-nitrogen ratio). Leaves were put into labeled envelopes and dried for at least 72 h at *c*. 60°C in a drying oven. Leaf carbon and nitrogen composition were determined in a combustion analyzer coupled to a mass spectrometer for carbon isotopic analysis at the Centro de Energia Nuclear na Agricultura (CENA-USP), Piracicaba, Brazil. A standard of known isotopic content was run every 11 samples. (Leaf chemistry data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.h83t0).

Leaf demography of branches

We coupled the above measurements of leaf physiology with branch-level leaf demography surveys of co-occurring leaves on the same focal trees (Table S1). Field-based surveys of the number of leaves of different ages on individual branches (leaf demography of branches) can be a useful way to estimate leaf demography of crowns (Chavana-Bryant *et al.*, 2017). Leaf demography was assessed for each tree during the dry season in 2012 (August–December), in 2013 (November), and in 2014 (March for all trees, and again in July–August for three trees). To survey leaf demography for each tree, *c*. 1-m long branches were collected from sun and shade microenvironments within the crown, depending upon the microenvironments present due to crown structure (i.e. only sun branches for the *Tachigali* cf *chrysophylla* tree). For each branch, leaves were then classified into age categories and counted. Leaves were categorized by age as described in the ‘Photosynthetic gas exchange’ section. Sometimes multiple (one to five) 1-m branches from similar microenvironments were surveyed on the same date, and averaged by combination of light environment (sun or shade) and sample date for each tree before analysis.

(Leaf demography data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.h83t0).

Analysis

We fitted the photosynthesis model developed by Farquhar *et al*. (1980), with triose phosphate use limitation (Harley *et al.* 1992), to the A/C_i response curve data using a curve fitting routine in the R computing environment (R Core Team, 2016) based on minimum least-squares (Domingues *et al.*, 2010). We report the following parameter estimates normalized to 25°C using the temperature dependencies summarized in Sharkey *et al*. (2007): V_{cmax} (maximum carboxylation capacity), J_{max} (maximum rate of photosynthetic electron transport), and TPU (triose phosphate use).

Because not all trees exhibited leaves of all age categories during our sampling periods, and because timing of sampling was constrained by conditions safe for tree-climbing, datasets had unequal sample sizes and/or did not include all possible combinations of time, tree, and leaf age category. Meta-analysis, often used for pooling results from multiple independent datasets (Hedges *et al.*, 1999), therefore provided a useful method of summarizing effect size of leaf age across multiple trees in our study. For each tree, we calculated the mean and variance of each physiological variable for each leaf age category (Table S2-S13), then used log-transformed response ratios (L) and associated variance (v) of photosynthetic characteristics to contrast mature leaves – the default for measurement in most ecophysiological studies – with young leaves and with old leaves for each tree (see Methods S3 for equations). We calculated L and v for the parameters from A/C_i curves, stomatal conductance, and physiological trait data (total chlorophyll, Chl*a* : *b* ratio, percent nitrogen, and carbon : nitrogen ratio), then used fixed effect models fitted by weighted least squares with the 'Metafor' package (Viechtbauer, 2010) in R (version 3.3.1). We evaluated the robustness of each meta-analysis by removing individual trees one at a time from the overall analysis to examine changes in statistical significance. We report tree sample size (N), unlogged response ratio (RR), z-score (z) and P-value.

Meta-analysis of log response ratios was also used to contrast the number of leaves in different age categories early in the dry season compared with late in the dry season. Demographic survey data (the number of leaves in each age category and the total number of

leaves) for all branches (sun and shade together) before 15 October (day of year, DOY = 288) for each tree were binned together as 'early' dry season, and leaf demographic surveys after 15 October were binned together as 'late' dry season. The wet season demographic surveys from 2014 were not included in this analysis since there was only one wet season survey date for each tree.

Upscaling to estimate branch-level V_{cmax}

To examine how leaf-level changes in carboxylation capacity could affect ecosystem PC, we examined the combined effect of leaf aging and leaf demography. We estimated V_{cmax} weighted by leaf demography (referred to as 'branch-level V_{cmax}' in this study) for sun-exposed branches, shaded branches, and all branches (sun and shade pooled) for each tree and sampling date (S4). To estimate the magnitude of the change in branch-level V_{cmax} for sun and shade branches of each tree during the dry season, we fit linear regressions of branch-level V_{cmax} vs DOY for the dry season period well-constrained by data (DOY 200–350, see later Fig. 7a,b). To estimate the magnitude of the dry season shift in branch-level V_{cmax} across our focal trees, we calculated the mean slope and intercept for sun, shade, and all branches, and tested whether the mean slope was significantly different from zero using two-sided t-tests (Methods S4).

Results

Gas exchange

Mature leaves show an A_{net} decrease from early to late in the dry season (Fig. 3b; although the significance is driven by one tree: Table S2). However, contrasting A/C_i parameters from gas exchange for leaves of different ages revealed age-dependency (Fig. 4). Sun and shade leaf A/C_i parameters showed similar age-dependency, so they were pooled for the analysis of leaf age means. Of the three age classes, mature leaves had the highest value for all A/C_i parameters. Average V_{cmax} of mature leaves was *c*. 60% greater than that of young leaves (n = 6 trees, RR=0.51, z=-7.47, P <0.01; Fig. 4a), and *c*. 46% greater than that of old leaves (n = 6 trees, RR=0.80, z=-3.44, P <0.01). J_{max} of mature leaves was on average *c*. 60% greater than young leaves (n = 6 trees, RR=0.53, z=-8.72, P <0.01; Fig. 4b), and *c*. 40% greater than old leaves (n = 6 trees, RR=0.73, z=-6.73, P <0.01). TPU of mature leaves was on average *c*. 34% greater than

TPU of young leaves (*n* = 6 trees, RR=0.61, z=-7.70, *P* <0.01; Fig. 4c), and *c*. 27% greater than TPU of old leaves (*n* = 6 trees, RR=0.76, z=-5.42, *P* <0.01).

In situ stomatal conductance

Examination of weekly mean stomatal conductance (g_s) using tree-level means for mature leaves for seven trees qualitatively shows no clear trend with dry season progression (Fig. 3c). Yet contrasts between g_s for mature vs old leaves revealed that g_s depends on leaf age (Fig. 5a; Table S9). Late in the dry season, after 15 October, mature leaves had 34% greater g_s than old leaves (*n* = 7, RR=0.77, z=-5.50, *P* <0.01). While there were not enough contrasts of young and mature leaves within the same tree to perform a meta-analysis, qualitative examination of partial diurnal cycles for trees that had young and mature leaf ages present suggest that g_s is higher in mature leaves than young leaves in *M. itauba* and *E. uncinatum* (Figs S2, S3). Thus conductance may follow a similar pattern with respect to age as the biochemical limitations, with mature leaves exhibiting higher conductance than old or young leaves.

Chlorophyll content

Chl*a* : *b* ratio and total chlorophyll content depended upon leaf age. Chl*a* : *b* ratio was 29% higher in young leaves than mature leaves (*n* = 3 trees, RR=1.20, z=3.73, *P* <0.01; Fig. 5b), and 37% greater in mature leaves than old leaves (*n* = 3 trees, RR=0.75, z=-3.80, *P* <0.01). Total chlorophyll was 90% greater in mature leaves than young leaves (*n* = 3 trees, RR=0.55, z=-6.00, *P* <0.01; Fig. 5c), but 15% greater in old leaves than mature leaves (*n* = 3 trees, RR=1.33, z=3.01, *P* <0.01).

Leaf chemistry

Leaf chemistry showed differences between mature and old but not mature and young categories. Percent nitrogen in leaves was similar between mature and young leaves (*n* = 5, RR=1.00, z=0.09, *P* =0.92; Fig. 5d), but on average 8% higher in mature leaves than old leaves (*n* = 6, RR=0.93, z=-7.75, p<0.01). The ratio of leaf carbon to nitrogen was also similar between mature and young leaves (*n* = 5, RR=1.02, z=-0.94, *P* =0.35; Fig. 5e), but C : N of old leaves was on average 9% higher than mature leaves (*n* = 6, RR=1.09, z=8.66, *P* <0.01).

Leaf demography

Dry season leaf demography on 1-m branches showed that leaf age composition varied across the dry season as young leaves became mature leaves and old leaves senesced and abscised (Fig. 6; Tables S18–S20). The difference between 'early' (23 July–15 October) and 'late' (15 October–5 December) dry season leaf number was significant for all leaf age categories. On average, the number of young leaves decreased 79% from early to late dry season (n = five trees, RR=3.05, z=2.50, P =0.01), mature leaves increased 62% from early to late dry season (n = five trees, RR=0.58, z=-2.97, P < 0.01), and old leaves decreased 66% from early to late dry season (n = five trees, RR=2.01, z=4.79, P < 0.01). These changes in composition occurred despite relatively constant total leaf number on 1-m branches. The total number of leaves showed a subtle and insignificant 4% increase from early to late dry season (n = five trees, RR=0.85, z=-1.21, P = 0.23).

Branch-level V_{cmax}

Linear regression slopes for branch-level V_{cmax} for individual trees was significantly positive or near zero during the dry season (Table S21). Aggregate branch-level V_{cmax} increased significantly during the dry season for all leaves sampled (slope = 0.056, slope SE = 0.0113, t statistic = 4.88, n = 5, P <0.01), and for sun branches (Fig. 7a, slope = 0.071, SE = 0.0101, t statistic=7.05, n = 5, P <0.01), but not for shade branches (Fig. 7b), as the shade mean slope was high (0.051) but not detectably different from zero (SE = 0.0371, t statistic = 1.38, n = 4, P =0.26)

Using the aggregate slope and intercept to calculate the percent increase in monthly average branch-level V_{cmax} between August and November yielded an increase of 24.1% for sun branches (significant) and 18.5% for shade branches (not significant). We compared branch-level V_{cmax} for sun and shade leaves to the ecosystem PC reported in Fig. 1 (from Wu *et al.*, 2016) and found a similar rate of increase as the dry season progressed (Fig. 7c).

Discussion

We investigated whether leaf physiology varies with leaf age in tropical trees, and whether leaf demography (the proportion of young, mature, and old leaves) varies during the dry season at a moist tropical site. Then we coupled field-based measurements of leaf V_{cmax} and leaf demography for the first 'bottom-up' test of the hypothesis – which arises primarily from remote observations – that the combination of leaf age-dependent physiology and leaf demography drive the dry season increase in GPP and PC in central Amazon forests (the leaf demography-ontogeny hypothesis; Wu *et al*. 2016). We found evidence that leaf physiology depends upon leaf age such that recently mature leaves have higher capacity for photosynthesis than either young or old leaves (Figs 4, 5). On average, the total number of leaves on sampled branches did not vary significantly across the dry season, consistent with observations of only minor variations in canopy-scale LAI (Fig. 6j), but the age composition of those leaves did vary, giving direct evidence to support previous inferences from simple models (Wu *et al*., 2016, 2017c) that trees exchange old leaves for young leaves via leaf turnover during the dry season (Fig. 6). Combining the age-dependence of leaf physiology results with the leaf demography results, we found that branch-level V_{cmax} of branches with sun-exposed leaves increased by 24.1% between August and November means, consistent with the 26.5% average increase independently observed over the same period in ecosystem-level PC estimated from EC (Fig. 7c). Shade leaf dynamics may also cause an increase in shade branch-level V_{cmax} (average increases were *c*. 18.5%) but high variation in shade branch-level V_{cmax} prevented us from statistically resolving the signal. We emphasize that measuring only mature leaves – the convention in most ecophysiological studies – neglects leaf demography and could lead to the conclusion that canopy photosynthesis does not increase with dry season progression (for example A_{net} time series of mature leaves only; Fig. 3b).

Although the five trees sampled represent a small fraction of the taxonomic diversity in this tropical evergreen forest, our samples represented five species accounting for a substantial portion (*c*. 33%) of tree basal area (Table S1). Our results suggest that leaf phenology impacts ecosystem-level carbon exchange at this evergreen forest. This implies that LAI alone should not be used as an indicator of leaf phenology in tropical evergreen forests (Fig. 6j), LSMs seeking to incorporate leaf phenology in tropical evergreen forests should include the age-dependency of limitations on photosynthesis that they represent, such as V_{cmax}, J_{max}, TPU, and g_s, and the

replacement of old leaves with new leaves early in the dry season by tropical evergreen trees could represent a strategy to optimize carbon gain by aligning peak PC of the crown with the period of high light during the dry season, a hypothesis warranting future investigation.

Leaf physiology depends upon leaf age

Our finding of age-dependent development of leaves in tropical trees is consistent with the general paradigm of leaf ontogeny seen also in temperate zones (Reich & Walters, 1991; Wilson *et al.*, 2001; Niinemets *et al.*, 2012; Pantin *et al.*, 2012), and also with the limited studies from trees of the Amazon basin of how leaf age affects photosynthesis (Doughty & Goulden, 2008; Alves *et al.*, 2014) and functional traits (Chavana-Bryant *et al.*, 2017; Wu *et al.*, 2017a). In this paradigm, carbon assimilation in young, developing leaves 'ramps up' with time as leaves reach maturity due to ontogenetic processes involving the light reactions machinery (Welsch *et al.*, 2000; Niinemets *et al.*, 2012), the Calvin-Benson cycle (Wilson *et al.*, 2001; Eichelmann *et al.*, 2004), and stomatal conductance (Willmer & Fricker, 1996; Pantin *et al.*, 2012). Mature leaves have the highest capacity for photosynthesis (Wilson *et al.*, 2001), which remains at a high level for some amount of time (depending upon total leaf lifespan and other factors) before declining as leaves approach senescence. In this study, support for the hypothesis that leaf PC peaks in recently mature leaves before declining in old leaves was found in the A/C_i parameters (V_{cmax}, J_{max}, and TPU; Fig. 4), and also in total chlorophyll content (Fig. 5c). Percent nitrogen was higher in mature leaves than old leaves (Fig. 5d), perhaps because trees were beginning to resorb nitrogen for use in new leaf buds and developing leaves (Hikosaka, 2004). Old leaves in this study also showed physiological changes that may be specific to tropical forests. In general, the ratio of chlorophyll a:b is lower in shade leaves, enabling them to absorb more light in the wavelengths less absorbed by sun leaves above (Boardman, 1977). Thus the shift in chlorophyll a:b ratio with leaf age (Fig. 5b) could represent a strategy to harvest more light in older leaves as they become increasingly self-shaded by younger (and more apical) leaves in evergreen trees. Epiphyll colonization, common in tropical forests, also increases in old leaves (Coley *et al.* 1993; Roberts *et al.*, 1998). Epiphylls likely influence photosynthesis via light absorption, nitrogen fixation, and perhaps even hormones (Coley & Kursar, 1996; Anthony *et al.*, 2002). Although we observed less epiphyll cover on the canopy leaves we studied compared to longer-lived

understory leaves in the TNF, more studies of epiphyll effects on photosynthesis are needed in Amazônia.

Importantly, leaf PC, as indicated by metrics of efficiency related to the light reactions and the Calvin-Benson cycle (e.g. chlorophyll concentration, J_{max}, and V_{cmax}), does not solely determine apparent photosynthesis. Stomata may influence the intercellular concentration of CO_2 by opening or closing. Our data suggest that mature leaves have higher stomatal conductance than old leaves co-occurring on the same tree (Figs 5a, S2, S3), and therefore higher PC should indeed manifest as higher apparent photosynthesis. We speculate that during drought, stomatal closure might limit photosynthesis and outweigh the leaf age effect.

Leaf demography reveals dry season leaf turnover

This study found evidence of leaf turnover, the exchange of old leaves for new leaves, during the dry season within evergreen crowns at K67 (Fig. 6). As old leaves senesced and abcised, the average number of old leaves on 1-m branches decreased from 94 to 32, from early in the dry season (pre-15 October) to late in the dry season (post-15 October). Meanwhile the average number of mature leaves across trees increased from 170 to 273, a 60% increase, as newly produced leaves reached maturity. This leaf turnover occurred even though the total quantity of leaves on 1-m branches did not change significantly from early in the dry season to late in the dry season (z=-1.21, P = 0.23). This finding is consistent with the leaf demography-ontogeny hypothesis that many evergreen trees exchange old leaves (with low PC) for recently mature leaves (with high PC) during the dry season while maintaining high LAI (Doughty & Goulden, 2008; Wu *et al.*, 2016). Our findings for this evergreen Amazon forest are in stark contrast with broadleaf deciduous forests where LAI tracks changes in canopy PC (Barr *et al.*, 2004). Instead, the PC of this forest tracks leaf age demographics rather than total leaf area, a finding that agrees with ecosystem-scale analyses at this site (Wu *et al.*, 2016).

The finding of leaf turnover within evergreen crowns does not indicate that *all* species or individuals behave similarly. Due to the intensive effort required to survey leaf demography in tall evergreen trees, our study was limited to a small percentage of trees in one diverse tropical forest, and thus needs to be interpreted as a subsample of phenological behaviors present in evergreen tropical forests. Although all of the trees we studied were evergreen (new leaves were

produced before old leaves dropped), multiple phenological patterns co-occur in many tropical forests (Rivera *et al.*, 2002; Chavana-Bryant *et al.*, 2017; Lopes *et al.*, 2016). Furthermore, the *Manilkara elata* tree we examined showed signs of more constant leaf production than other trees examined in this study, suggesting that some tree species have more constant leaf age distribution than others (Fig. 6c,d). Another likely source of variation in leaf phenological behavior is the range of leaf life-spans found in tropical forests (Reich *et al.*, 2004). The present study focused on canopy trees, but understory plants account for *c*. 50% of LAI in this forest (Stark *et al.*, 2012), and generally have longer leaf life-spans (Reich *et al.*, 2004), so leaf turnover in understory plants may affect a small fraction of understory leaves.

In addition to species diversity within a site, there is environmental variation across sites that could impact leaf phenology. The Amazon basin includes a gradient in precipitation seasonality from the northwestern Amazon, which is consistently wet, to the south and the east, which experience dry seasons up to 5 months long (Sombroek, 2001; Restrepo-Coupe *et al.*, 2013). Satellite-based remote sensing studies suggest that there is a threshold amount of precipitation below which productivity in tropical forests is driven by moisture seasonality (Guan *et al.*, 2015). Average precipitation at the K67 site slightly exceeds that 2000 mm yr^{--1} precipitation threshold, and so productivity may not be limited by water availability during non-drought years. Field studies of leaf demography and photosynthesis should be conducted at more tropical forest sites with various rainfall regimes to further describe the relationship between wet/dry seasonality, leaf phenology, and productivity across tropical forests.

Implications of evergreen leaf phenology for LSMs

Our findings suggest that recent incorporations of leaf demography and leaf age dependence of physiology into LSMs are progress towards accurate representation of evergreen tropical forests. Studies with ORCHIDEE, a global process-based vegetation model (Krinner *et al.*, 2005), showed that when V_{cmax} was parameterized as a function of leaf age (four age classes), and LAI was more or less constant, the model produced seasonality in litterfall, leaf age, and V_{cmax} (De Weirdt *et al.*, 2012). In Ecosystem Demography module version 2 (ED2; Medvigy *et al.*, 2009) a phenology module that linearly related leaf turnover to incoming shortwave radiation, then related leaf lifespan to PC using the empirically based relationship from the leaf economic

spectrum (Wright *et al.*, 2004; Kim *et al.*, 2012) created seasonality in LAI and litterfall and generally improved the ability of ED2 to simulate the carbon fluxes of the K67 site.

Here we attribute the observed seasonal GPP patterns to phenological mechanisms, and the next step is to resolve and model the eco-evolutionary constraints that give rise to these patterns. One such constraint could be carbon acquisition. A recent model that optimizes carbon acquisition as a function of leaf lifespan, when used to analyze 49 species across tropical and temperate forests (including data from this study), successfully predicted leaf lifespan and the rate of decline in PC with age in many tropical species, offering an approach useful for LSMs (Xu *et al.*, 2017). The current study reinforces the argument that providing more mechanistic detail relating tropical vegetation responses to climate variability, including seasonal variation, could help improve earth system model projections of Amazon forests under climate change scenarios (Malhi *et al.*, 2009; Good *et al.*, 2011; Huntingford *et al.*, 2013; Restrepo-Coupe *et al.*, 2017, Wu *et al.* 2017c), a long-standing modeling challenge (White *et al.*, 1999; Cox *et al.*, 2000).

Dry season leaf turnover as adaptation to seasonal light variation

The leaf demography results suggest that leaf production of many canopy tropical trees at the K67 site is synchronized with the dry season. This timing of leaf turnover may represent a strategy for reducing leaf herbivory (Lowman, 1985; Aide, 1988; Coley & Barone, 1996), and also for enhancing carbon gain. Tropical evergreen trees with sufficient water supply that experience wet-dry seasonality may align their period of maximum PC (when they have many recently-mature leaves) with the dry season period of high light availability. Experimental manipulations of light quantity show that high light induces leaf production in tropical trees (Graham *et al.*, 2003), and across tropical latitudes observations of leaf production are linked to seasonal increases in daily light quantity (Borchert *et al.*, 2014), showing that high light can be a cue or proximate control over leaf production. The degree to which wet–dry seasonality has ultimately shaped temporal strategies of acquisition and allocation in tropical evergreen trees through natural selection still requires more investigation, but the current study suggests that dry season leaf turnover contributes to optimal light-use across the year.

Conclusions

This field-based study shows that the combination of age-dependent leaf physiology and canopy leaf demography increase branch-level estimates of V_{cmax} as the dry season progresses, consistent with independent estimates of ecosystem-level PC (Fig. 7c). These findings provide the first evidence from coupled leaf and branch-level measurements supporting the leaf demography-ontogeny hypothesis for dry season GPP increases in central Amazonian forests (Wu *et al.*, 2016), and emphasize the active role of leaf phenology in controlling carbon and water exchange in central Amazon broadleaf evergreen forests. Thus, phenological rhythms of tropical evergreen forests, although subtle compared with those of temperate deciduous forests, may modulate similar vegetation feedbacks to the climate system (Richardson *et al.*, 2013) including not only seasonality of carbon uptake, but transpiration (Wright *et al.*, 2017) and canopy reflectance (Chavana-Bryant *et al.*, 2017; Wu *et al.*, 2017a).

Acknowledgements

This research was supported by US NSF (OISE-0730305), US DOE (DE-SC0008383), the Philecology Foundation through UA Biosphere 2, and a Marshall Foundation of Arizona dissertation fellowship to L.P.A.. J.W. was partly supported by DOE (BER) NGEE-Tropics at Brookhaven National Laboratory. We thank Mick Eltringham for invaluable help with tree canopy access. We thank Cecilia Chavana-Bryant and Ana Paula Florentino for methods advice. We thank Kleber Silva Santos, Aldeize Santos, Mariáh Miranda, Ana Paula Bicalho, Matthew Belheir and Michael Kwon for their fieldwork and data-processing efforts. We thank the LBA and EMBRAPA staff in Santarém for organizational support. Luciana Alves provided tree inventory information for focal tree selection. Ty Taylor contributed voucher specimens for identification at Herbario IAN.

Author contributions

L.P.A., S.R.S., T.E.H. and J.W. planned and designed the research. J.W., L.P.A. and N.P. surveyed leaf demography, and J.W. curated these surveys. L.P.A., M.N.S. and N.P. performed gas exchange. J.W., N.P., E.S.T., and L.P.A. extracted chlorophyll. P.B.d.C. contributed leaf chemistry. N.R-C., R.C.O.J., S.R.S. and R.d.S. obtained EC measurements. V.Y.I. and R.S.O.

installed stem psychrometers. L.P.A. performed analyses on gas exchange and leaf demography. J.W. calculated PC. L.P.A., made figures with contributions from S.G., and M.N.S. S.R.S., T.E.H., G.A.M., D.V.P., S.C.S., E.S.T. and L.P.A. interpreted the analyses. L.P.A. wrote the first draft and all authors contributed to editing the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Examples of leaves from each age category for trees used in leaf demography surveys.

Fig. S2 Stomatal conductance by leaf age and time of day trees early in the dry season.

Fig. S3 Stomatal conductance by leaf age and time of day trees late in the dry season.

Table S1 Focal tree information

Table S2 Early vs late dry season A_{net} information by tree

Table S3 Young and mature leaf V_{cmax} information by tree

Table S4 Mature and old leaf V_{cmax} information by tree

Table S5 Young and mature leaf J_{max} information by tree

Table S6 Mature and old leaf J_{max} information by tree

Table S7 Young and mature leaf TPU information by tree

Table S8 Mature and old leaf TPU information by tree

Table S9 Mature and old leaf stomatal conductance information by tree

Table S10 Young and mature leaf Chl*a* : *b* ratio information by tree

Table S11 Mature and old leaf Chl*a* : *b* ratio information by tree

Table S12 Young and mature leaf total chlorophyll information by tree

Table S13 Mature and old leaf total chlorophyll information by tree

Table S14 Young and mature leaf percent nitrogen information by tree

Table S15 Mature and old leaf percent nitrogen information by tree

Table S16 Young and mature leaf carbon to nitrogen ratio information by tree

Table S17 Mature and old leaf carbon to nitrogen ratio information by tree

Table S18 Early vs late dry season number of young leaves on *c*. 1 m branches

Table S19 Early vs late dry season number of mature leaves on *c*. 1 m branches

Table S20 Early vs late dry season number of old leaves on *c*. 1 m branches

Table S21 Linear regressions of branch-level V_{cmax} vs day of year

Methods S1 Stem water potential.

Methods S2 Chlorophyll concentration calculations.

Methods S3 Meta-analysis equations.

Methods S4 Branch-level V_{cmax} calculations.

Fig. 1 Annual cycle of eddy-covariance derived gross primary productivity (GPP) and canopy-scale photosynthetic capacity (PC), averaged over years (2002–2005 and 2009–2011) at K67 in the Tapajós National Forest, Brazil. The shaded gray region indicates the dry season. GPP is

derived from net ecosystem exchange (NEE) by assuming nighttime NEE is representative of daytime ecosystem respiration. PC, the canopy scale rate of carbon fixation per unit of light under reference environmental conditions, is derived from averaging the measured GPP : photosynthetically active radiation (PAR) ratio when PAR, vapor pressure deficient, air temperature, and cloudiness all fall within fixed narrow ranges (replotted from Wu *et al*., 2016). Error bars are ± 1 SE.

Fig. 2 Hypothesized causes for increases in gross primary productivity (GPP) with dry season progression in Amazonian tropical evergreen forests divided into two general categories: changes due to seasonal shifts in environmental drivers (far left panel), and/or changes due to vegetation structure and function (remaining panels). The latter are additionally divided between changes due to leaf quantity (e.g. leaf area index) and/or due to leaf-level physiological function. Leaf-level function may change as a result of ontogeny, plant water status, and/or biotic interactions.

Fig. 3 Time series during the dry season (gray) showing progression of (a) weekly mean pre-dawn (05:30 h) stem water potential for three trees (an *Erisma uncinatum*, a *Tachigali cf. chrysophylla*, and a *Chamaecrista xinguensis*) using available data from 2012 to 2014), (b) weekly mean net assimilation rate (A_{net}) of only mature leaves using within-tree means, and (c) weekly mean stomatal conductance (g_s) of only mature leaves using within-tree means (Supporting Information Table S9; Figs S2, S3). Within-tree means in (b, c) are for seven trees (an *Erisma uncinatum*, a *Tachigali* cf *chrysophylla*, a *Chamaecrista xinguensis*, a *Mezilaurus itauba*, a *Coussarea paniculata*, and two *Manilkara elata* trees). Reference cell [CO_2] was 350 µmol mol^{-1} for A_{net} and g_s from LI-COR 6400. For g_s, circles show conductance from a LI-COR 6400, and triangles show conductance from a porometer. Net assimilation in (b) shows a decrease from early (before October 15) to late (after October 15) in the dry season when mature leaves—the age conventionally chosen for ecophysiology studies—are examined (Table S2), a contrast with canopy scale gross primary productivity (GPP) (Fig. 1). Error bars show data range in (c), and ± 1 SD of tree means in (b, c).

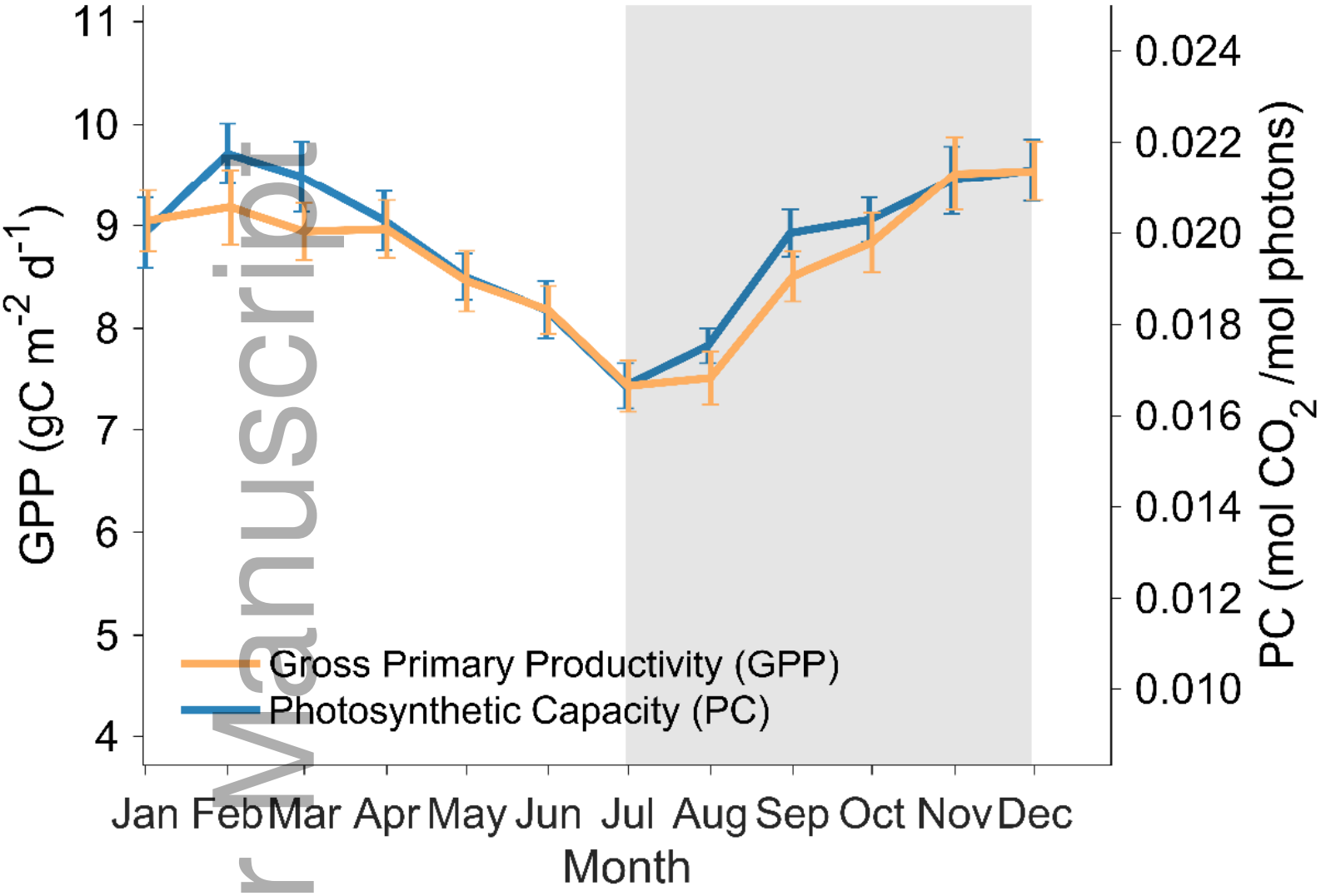
Fig. 4 Age dependence of photosynthetic parameters: (a) maximum rate of carboxylation (V_{cmax}), (b) maximum rate of electron transport (J_{max}), and (c) triose phosphate use (TPU) derived from A/C_i curves measured for leaves from six trees (sun leaves and shade leaves combined) at the K67 site. Leaf age categories are: young (yellow), mature (green) and old (brown) leaves (where age is defined as described in the text). Boxplots represent the distribution of individual tree means, with the bold horizontal line indicating the median, the top and bottom of each box indicating first and third quartiles, the whiskers extending to the highest and lowest values that are within 1.5 × the interquartile range (IQR) of the upper and lower quartiles (respectively), and the circular dots indicating outliers. Individual tree means come from an *Erisma uncinatum*, a *Tachigali* cf *chrysophylla*, a *Chamaecrista xinguensis*, a *Mezilaurus itauba*, a *Coussarea paniculata*, and a *Manilkara elata* tree (see Supporting Information Tables S3–S8 for means of all available data by tree).

Fig. 5 Age dependence of leaf physiological traits: (a) stomatal conductance from porometer measurements, (b) Chl*a* : *b* ratio, (c) total chlorophyll, d) percent nitrogen, (e) C : N ratio. Leaf age categories are: young (yellow), mature (green) and old (brown) leaves (where age is defined as described in the text). Shown are leaf age by tree means from trees that had all leaf age categories sampled: in (a–c) six trees for leaf chemistry (an *Erisma uncinatum*, a *Tachigali* cf *chrysophylla*, a *Chamaecrista xinguensis*, a *Mezilaurus itauba*, a *Coussarea paniculata*, and a *Manilkara elata* tree); in (d) two trees for g_s (an *Erisma uncinatum* and a *M. elata* tree); and in (e, f) two trees for chlorophyll (an *Erisma uncinatum* and a *C. xinguensis* tree). Boxplots represent the distribution of individual tree means (see Supporting Information Tables S9–S17 for all available data by tree), with the bold horizontal line indicating the median, the top and bottom of each box indicating first and third quartiles, the whiskers extending to the highest and lowest values that are within 1.5 × the interquartile range (IQR) of the upper and lower quartiles (respectively), and the circular dots indicating outliers.

Fig. 6 Leaf demography time series focusing on the dry season (gray) in the Tapajós National Forest (TNF) for five trees surveyed in 2012, 2013, and 2014. Sun branches are shown for five trees, and shade branches are shown for four of the trees (the *Mezilaurus itauba* had few shade

branches due to the shape of its crown). The proportions of young (yellow circles), mature (green circles) and old (brown circles) leaves are shown for 1 m branches from *Erisma uncinatum* sun (a) and shade (b), *Manilkara elata* sun (c) and shade (d), *Chamaecrista xinguensis* sun (e) and shade (f), *Mezilaurus itauba* sun (g) and shade (h), and *Tachigali* cf *chrysophylla* shade (i). Colored lines are polynomials fit to the demography proportion data for the purpose of visualization. Lower right hand panel (j) shows a time series of leaf area index for this forest from Brando *et al*. (2010) with error bars showing ± 1 SE.

Fig. 7 Branch-level V_{cmax} (estimated by weighting V_{cmax} by leaf age proportion) for (a) branches with sun leaves, (b) branches with shade leaves, and (c) compared to canopy photosynthetic capacity (PC) derived from eddy covariance (as in Fig. 1). In (a, b), dotted lines show linear regressions for individual trees, and solid lines show mean of all other lines, with ± SE and statistical significance of slope of composite black lines indicated. The full species names abbreviated in (a) are *Erisma uncinatum, Manilkara elata, Chamaecrista xinguensis, Tachigali* cf *chrysophylla,* and *Mezilaurus itauba*. In (c) the eddy covariance-derived canopy PC (orange line), is compared to mean branch-level V_{cmax} for sun (dashed black line) and shade (solid black line), with the vertical axes for PC (right) and branch-level V_{cmax} (left) each scaled to range from 50% to 150% of their respective minimum values, and the dry season is indicated by the gray box. Month ticks are at the first of each month.



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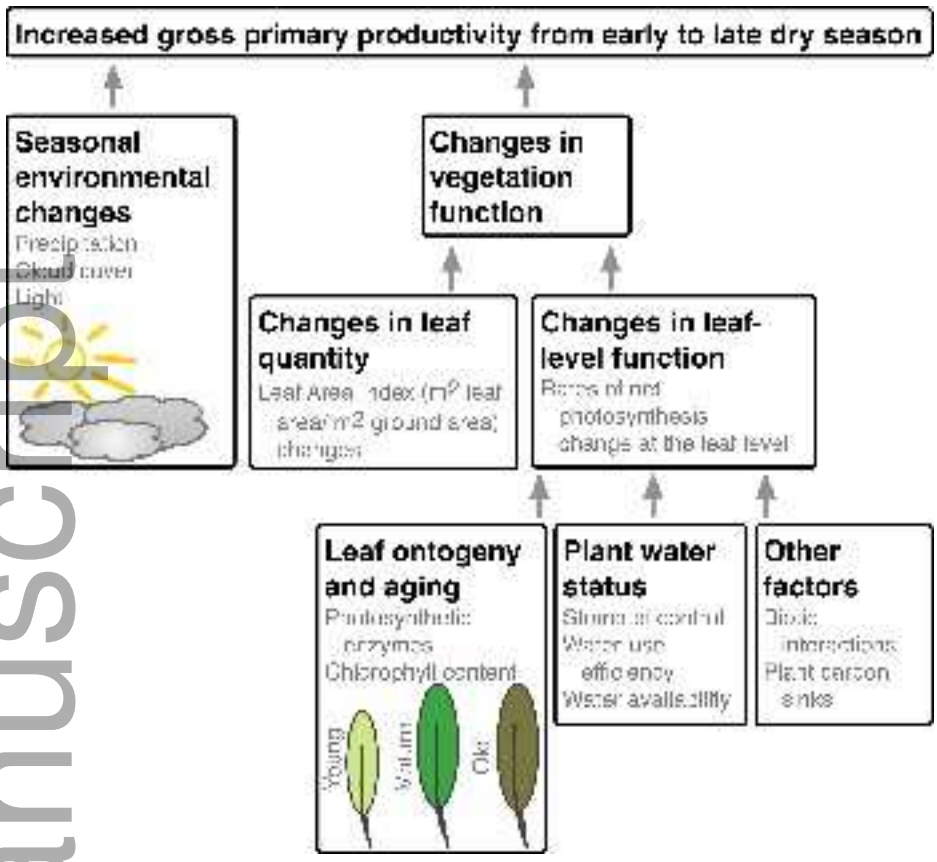

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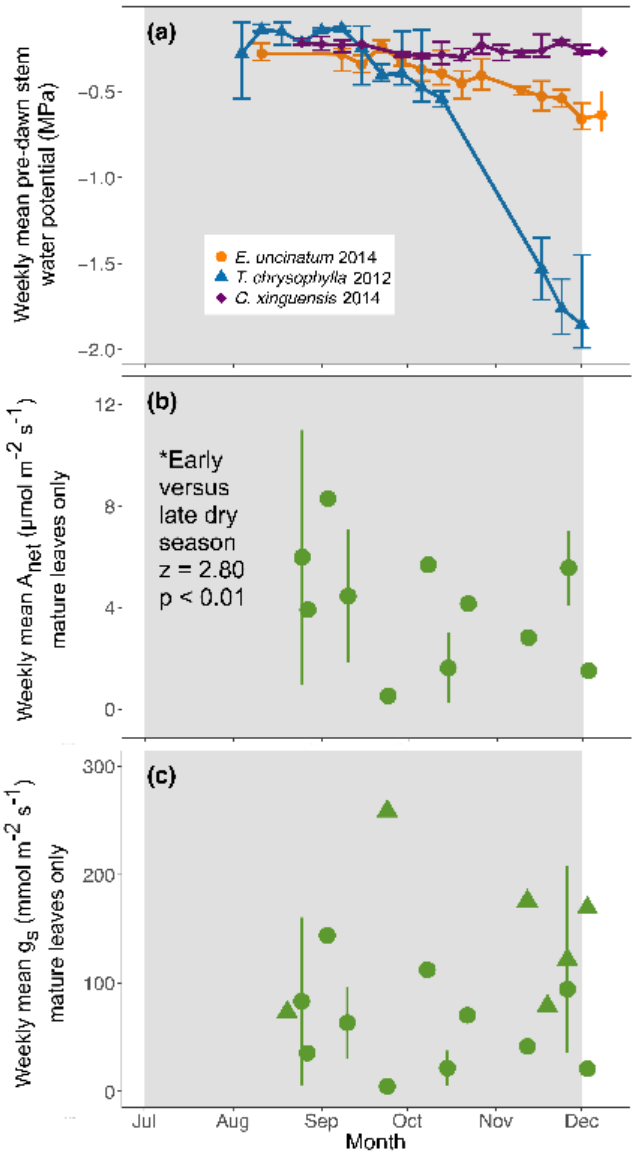

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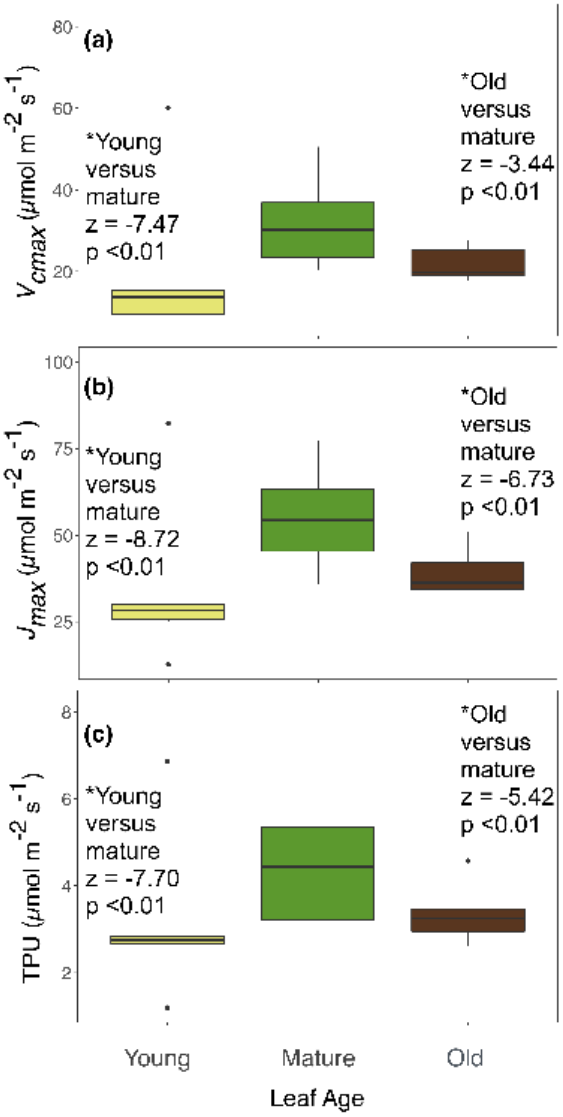

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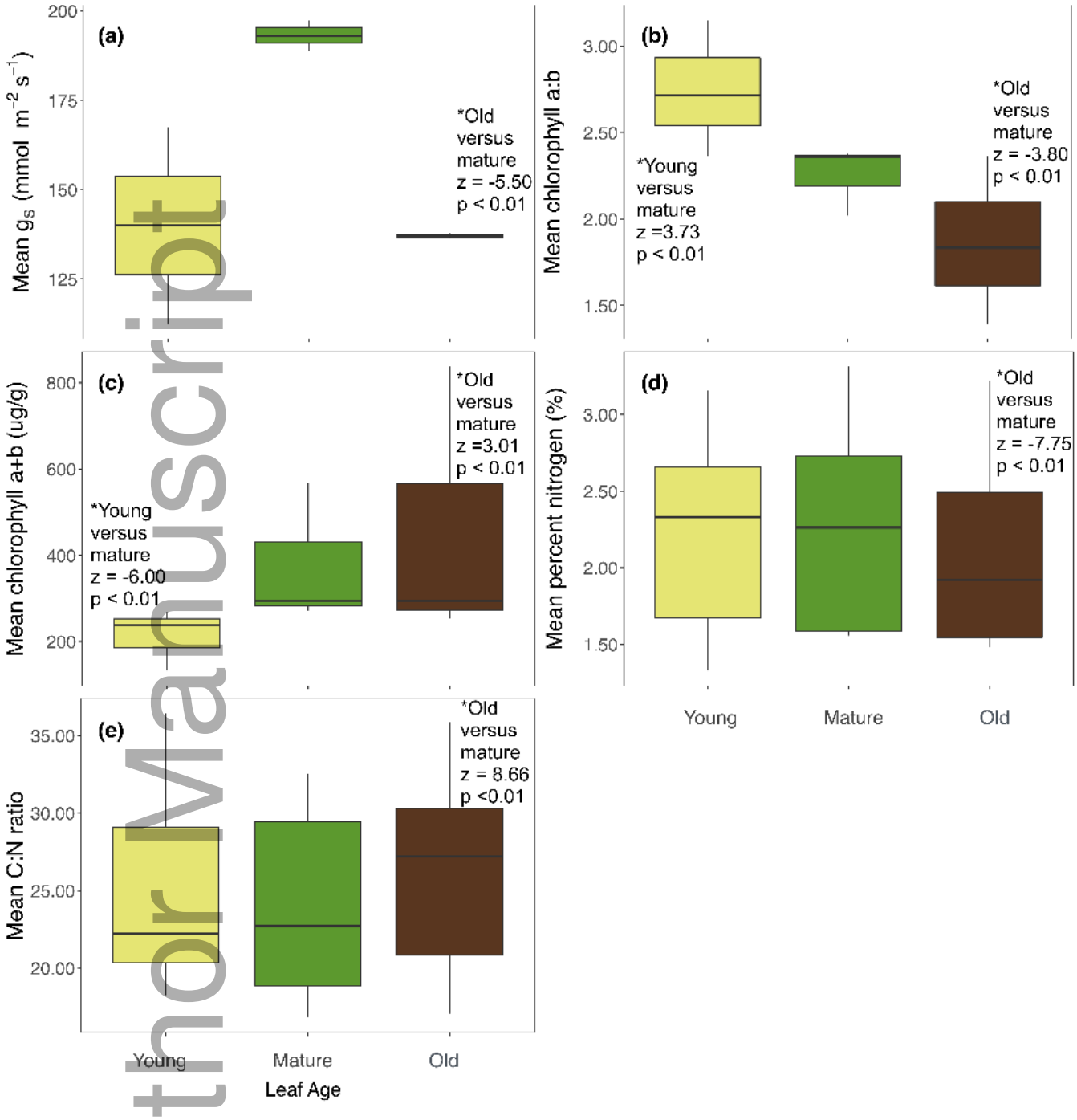

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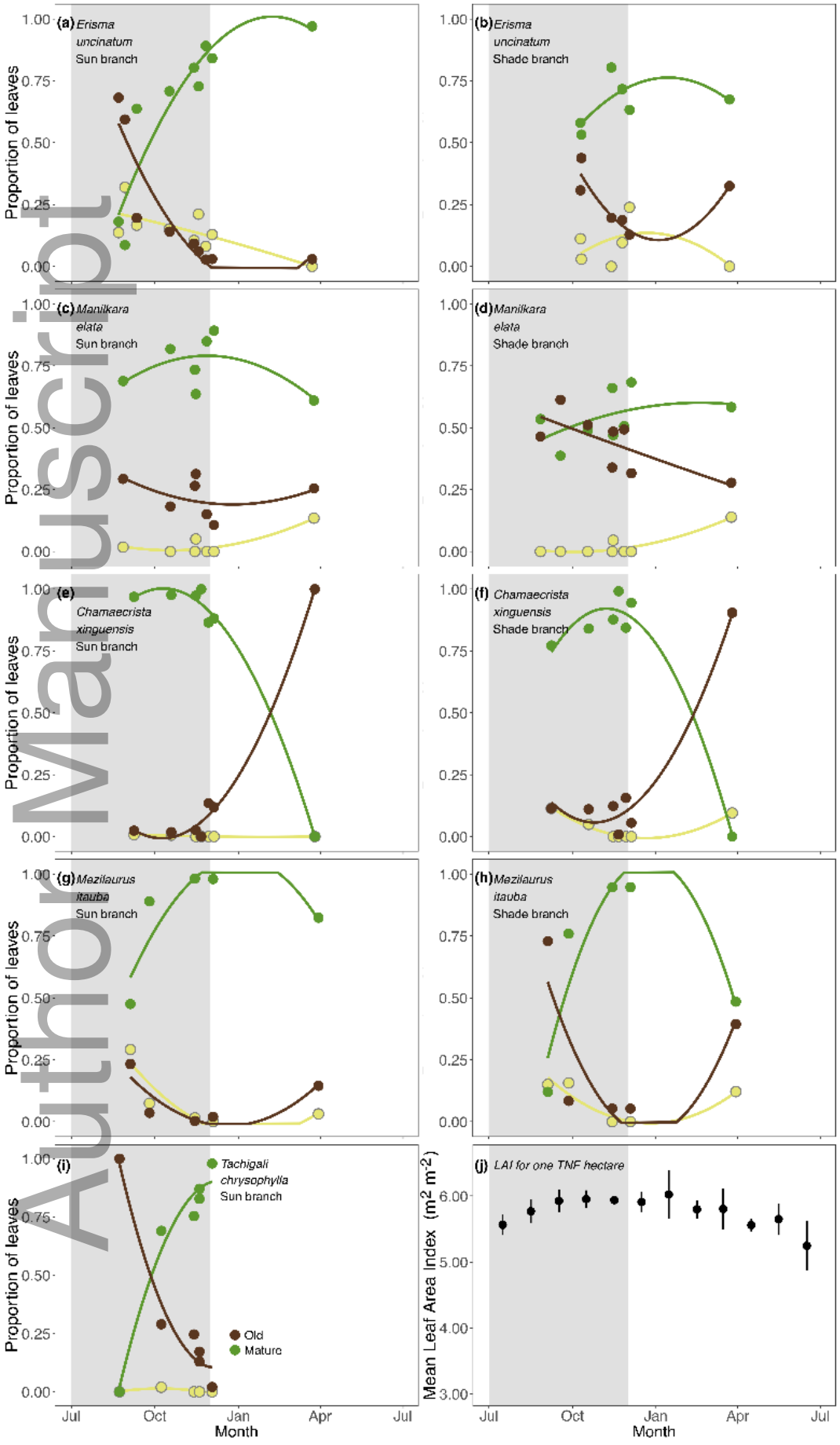

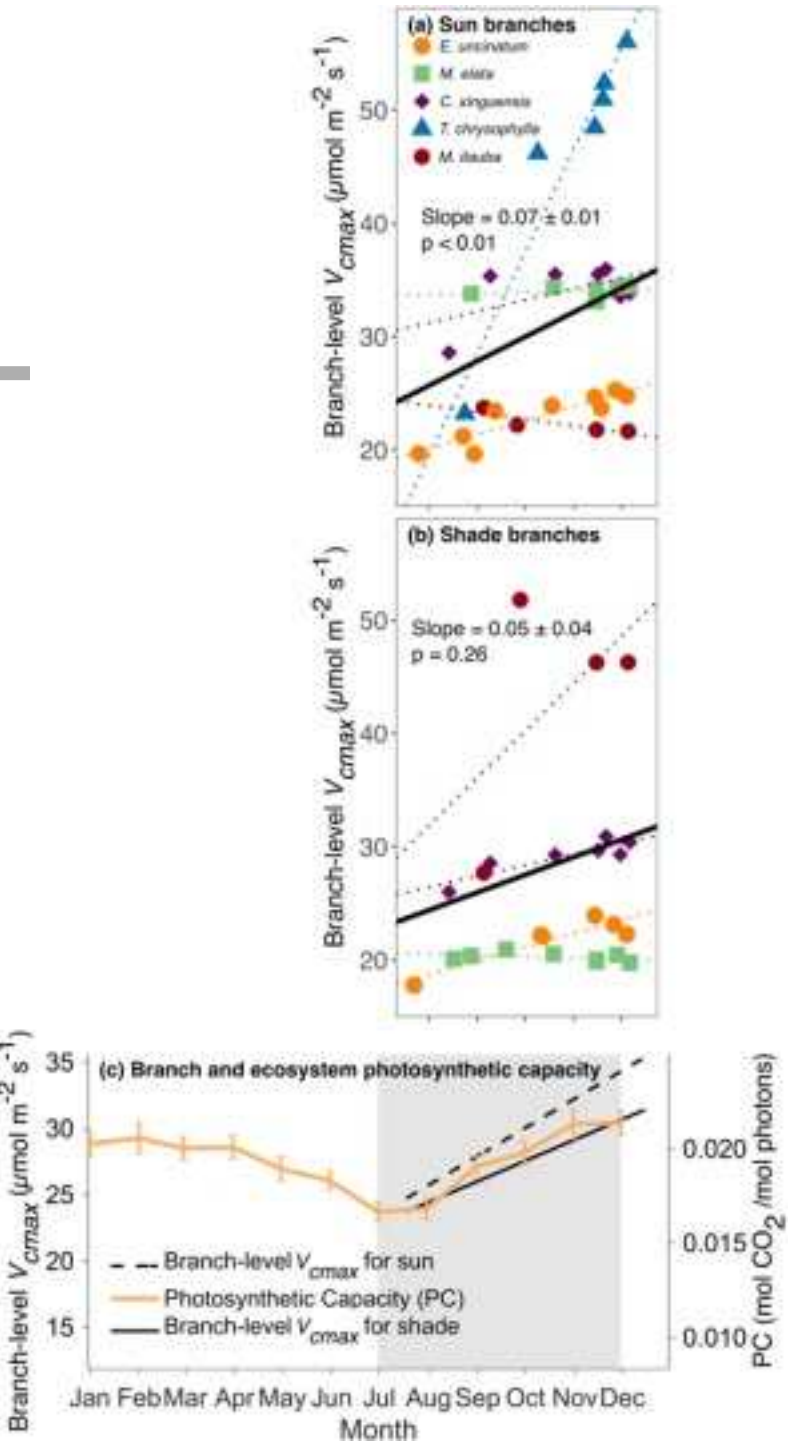

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