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Century-long apparent decrease in iWUE with no evidence of progressive nutrient limitation in African tropical forests

Running title: A century of change in the Congo Basin forest

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Abstract

Forests exhibit leaf and ecosystem level responses to environmental changes. Specifically, rising carbon dioxide (CO_2) levels over the past century are expected to have increased the intrinsic water-use efficiency (iWUE) of tropical trees while the ecosystem is gradually pushed into progressive nutrient limitation. Due to the long-term character of these changes, however, observational datasets to validate both paradigms are limited in space and time. In this study, we used a unique herbarium record to go back nearly a century and show that despite the rise in CO_2 concentrations, iWUE has decreased in central African tropical trees in the Congo basin. Although we find evidence that points to leaf-level adaptation to increasing CO_2 – i.e. increasing photosynthesis-related nutrients and decreasing maximum stomatal conductance, a decrease in leaf $\delta^{13}C$ clearly indicates a decreasing iWUE over time. Additionally, the stoichiometric carbon to nitrogen and nitrogen to phosphorus ratios in the leaves show no sign of progressive nutrient limitation as they have remained constant since 1938, which suggests that nutrients have not increasingly limited productivity in this biome. Altogether, the data suggest that other environmental factors, such as increasing temperature, might have negatively affected net photosynthesis and consequently downregulated the iWUE. Results from this study reveal that the second largest tropical forest on Earth has responded differently to recent environmental changes than expected, highlighting the need for further on-ground monitoring in the Congo Basin.

Keywords: Congo basin, CO_2 fertilization, herbarium, photosynthesis, stomata, aggravated nutrient limitation, tropical forest, water use efficiency

Introduction

The Earth system has been subjected to unprecedented changes over the past century, including increasing atmospheric carbon dioxide (CO_2) levels, shifting rainfall regimes, and changes in global biogeochemical cycles (Steffen et al. 2015). Uncertainties in the future response of forest ecosystems to these environmental changes are perhaps most prominent in the tropics, where monitoring is underdeveloped compared to temperate regions (Schimel et al. 2015). Tropical forests comprise 55% of the current carbon (C) stock of the world's forests and exhibit high gross (GPP) and net (NPP) primary productivity (Beer et al. 2010, Pan et al. 2011). As such, tropical forests play a pivotal role in the global C cycle. The effect of human-induced changes on this biome is thus a central question in global change research (Bonan 2008a, Gibson et al. 2011). Large-scale permanent monitoring plots and tree-ring research in tropical forests have shown varying trends in tree growth over the last decades, from increased (Phillips 1998, Baker et al. 2004, Lewis et al. 2009) to stable or decreased growth (Feeley et al. 2007, Clark et al. 2010, Groenendijk et al. 2015, Van Der Sleen et al. 2015). One of the proposed drivers for a growth acceleration is the global increase in atmospheric CO_2 concentration, i.e. CO_2 fertilization, which supposedly increases the intrinsic water-use efficiency (iWUE) – i.e. the ratio of C gain to water loss- or photosynthetic rates of terrestrial plants (Ballantyne et al. 2012, Keenan et al. 2013, Lavergne et al. 2019). Indeed, increases of iWUE have been widely noted across the tropics (Hietz et al. 2005, Brienen et al. 2010, Nock et al. 2011, Van Der Sleen et al. 2015), but few studies have disentangled whether this iWUE adaptation is controlled by shifts in photosynthesis (A) or stomatal conductance (g_s) (Bonal et al. 2011).

Consequently, the question arises whether nitrogen (N) and phosphorus (P) supply can meet the increased biomass accrual from CO_2 fertilization of forests globally. Modeling efforts have initially predicted a dampening of the land C storage sensitivity to CO_2 caused by N limitation (Bonan 2008b, Sokolov et al. 2008). More recent simulations predict a reduction of 25% of the projected NPP by 2100 if both N and P limitations are taken into account (Wieder et al. 2015). To date, the empirical evidence of this progressive nutrient limitation remains inconclusive. A limited number of studies showed that long-term N effects are regionally distinct, including decreasing N availability in North American grasslands and forests (McLauchlan et al. 2010, 2017) and European forests (Jonard et al. 2015), but increasing N availability in Panama and Thailand (Hietz et al. 2011). Moreover, recent evidence suggests a general decrease in N supply relative to the N demand in natural ecosystems worldwide (Craine et al. 2018). At present, the long-term effects of changes in N availability within these biomes remain poorly understood. Additionally, despite the fact that P is considered the major limiting nutrient for tropical forest ecosystems, only a few studies have looked directly into progressive P limitation (Vitousek et al. 2010). Theory predicts that an initial P limitation might be exacerbated under CO_2 fertilization and/or

elevated reactive atmospheric N deposition because litter stoichiometry will become increasingly depleted in P, which results in lower net P mineralization rates and finally further P limitation to plant growth (Peñuelas et al. 2013, Fernandez-Martinez et al. 2014, Wieder et al. 2015, Fleischer et al. 2019). Evidence from primary succession and nutrient addition experiments shows that progressive P limitation results in a shift in leaf P content and the N:P ratio in the canopy (Izquierdo et al. 2013, Li et al. 2016), while progressive P limitation over time has only been observed in a few sites (Huang et al. 2016).

Most knowledge that we have gained on ecosystem responses to environmental change comes from short-term experiments or modeling studies. While these studies increase our process-based understanding of separate global change drivers, long-term empirical data are required to verify many of the paradigms that have been put forth. Indeed, empirical datasets are constrained by experiment duration, funding timelines, and the historic absence of researchers in many tropical sites. However, historical herbarium records can overcome some of these experimental limitations and enable us to go back in time to validate the overall response of tree species (Meineke et al. 2018). The UNESCO Yangambi Man and Biosphere Reserve, in the heart of the Congo basin, holds a research center founded in the 1930s by the colonial Belgians and passed on to a Congolese research institute in 1962. The continuous presence of researchers since 1930 has led to one of the most extensive and oldest herbarium collections in central Africa. From that collection, we selected 23 tree species common to central African tropical forests that cover a range of ecological life-history traits. We used herbarium specimens from three different time periods to accomplish three goals: 1) quantify responses in iWUE from 1938 until present, 2) determine whether this response is caused by changes in g_s or photosynthesis, or a combination of both, and 3) provide evidence of increasing nutrient limitation, i.e. to assess whether the relative N and P demand kept pace with the induced changes on an ecosystem level. These goals were accomplished through the measurement of foliar nutrient content, isotopic signatures, and stomatal traits. Furthermore, we used an additional extensive sample set, along with a modelling effort, to rule out potential sampling biases in the effects that we quantified on the herbarium specimens.

Methods

Historic sample set. We used herbarium specimens of 23 tropical tree species that are common to central Africa, belonging to 14 flowering plant families (Table S1) and originating from the same reserve in the Democratic Republic of Congo. Leaves were collected from the African herbarium collection of Meise Botanic Garden, Belgium. The herbarium specimens were all collected in the Yangambi Man and Biosphere Reserve (N 00°47’; E24°30’), situated on the Northern bank Congo River 100 km west of Kisangani. The region has an Af-type tropical rainforest climate, with an annual rainfall of 1750 mm, a

bimodal rainfall distribution exhibiting a longer and shorter dry season, and a stable temperature of 24.5°C throughout the year. The site is dominated by ferralsols (Van Ranst et al. 2010). Material was sampled from specimens collected at three different time points: (1) 1935-1938 (hereafter 1938), (2) 1951-1953 (hereafter 1953), (3) 2012-2013 (hereafter 2013). For most species, we sub-sampled three specimens per time period, resulting in nine specimens per species (Table S1). We specifically targeted samples from the same reserve for the entire sample set, to eliminate inter-site variability or local climate effects. Additionally, the three dates were specifically selected to maximize the time range: sample collection started in the Democratic Republic of Congo around 1935 and stopped momentarily after the independence in 1960. To our knowledge, the historic samples were taken from sunlit, flowering or fruiting branches from adult trees (pers. comm., Piet Stoffelen). The samples in 2013 were collected with tree climbers, which implies that this sample set comprises both sun and shade leaves, because of the practical difficulties of access in the upper canopy. In all cases, only fully expanded, adult leaves were sampled.

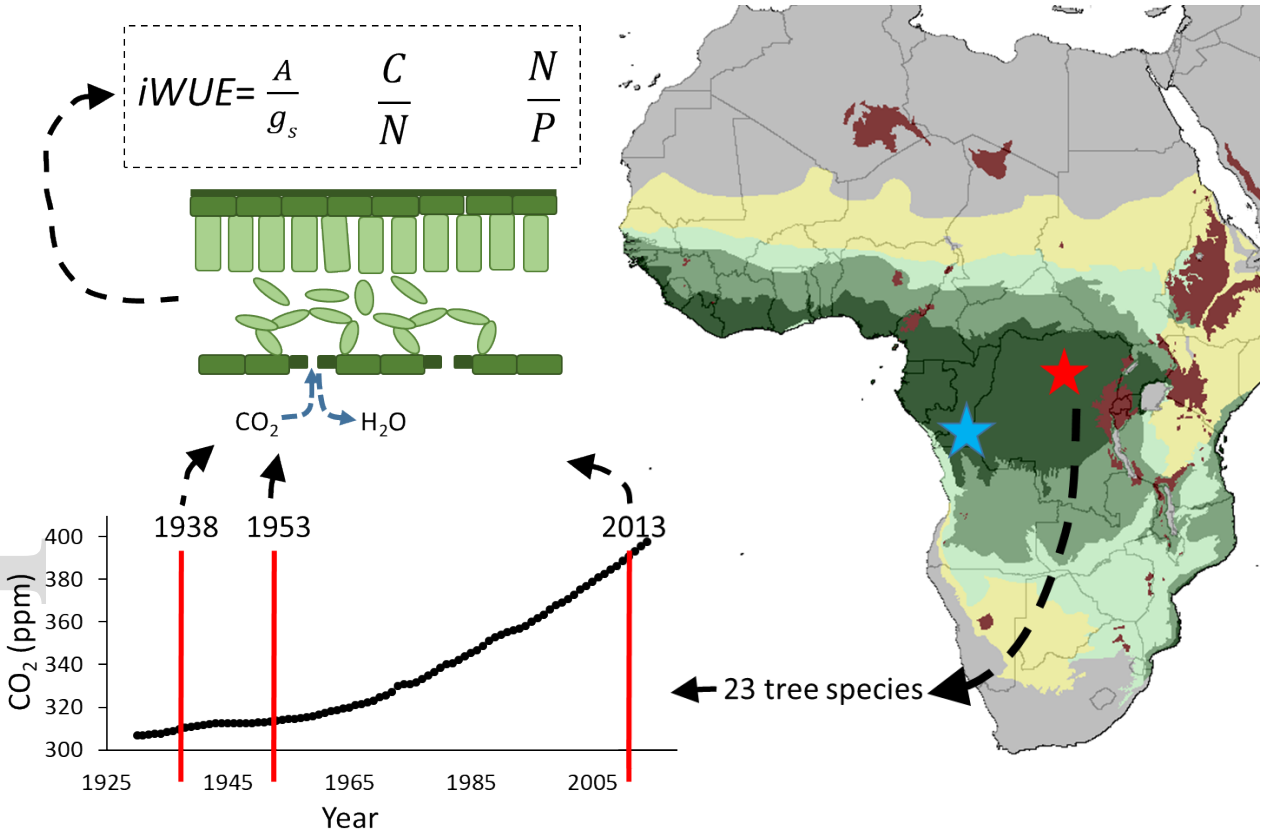

Figure 1. The location of Yangambi (red star), where triplicate samples were taken from 23 different tree species around the years 1938, 1953 and in 2013. The samples were all analyzed to detect changes over time via proxy variables for photosynthesis (A), stomatal conductance (g_s), intrinsic water-use efficiency (iWUE) and leaf stoichiometric carbon to nitrogen (C:N) and nitrogen to phosphorus (N:P) ratios. The coloration on the map shows the ecosystem type delineation, with tropical wet forest in dark green. The

blue star indicates the location of the Luki reserve, where some of the samples to quantify variability in stomatal traits were taken.

Stoichiometry and isotopic composition. For each herbarium specimen, one leaf was sampled in the least invasive way possible by punching a hole with a hole-puncher in the center of the leaf on the right side of the central vein (upper side of the leaf pointing upwards). Leaf C, N and $\delta^{13}C$ of plant samples were analyzed using an elemental analyzer (Automated Nitrogen Carbon Analyser; ANCA-SL, SerCon, UK), interfaced with an Isotope Ratios Mass Spectrometer (IRMS; 20-22, SerCon, UK). To check if the bulk $\delta^{13}C$ signal was consistent with the cellulose $\delta^{13}C$ signal, we analyzed cellulose $\delta^{13}C$ for a subset of the samples (n=27). For this, we used an α-cellulose extraction protocol, modified for speed and small sample extraction (Brendel et al. 2000, Evans and Schrag 2004). In short, we weighed ca. 2 mg of bulk leaf material into 1.5 mL screw-cap polyethylene tubes and added 240 µl 80% acetic acid and 24 µl 69% nitric acid. The tubes were capped and placed in a 120°C oil bath for 30 minutes. After cooling of the samples to room temperature, 800 µl 100% ethanol was added to the tubes, and the tubes were centrifuged for 5 minutes at 15500 g. Next, three sequential rinse steps were performed by adding 1) 600 µl deionized water, 2) 300 µl 100% ethanol and 3) 500 µl acetone with the centrifugation step between each rinse to remove the supernatant. Finally, the tubes were dried in an oven for 30 minutes at 50°C. The $\delta^{13}C$ signal of the extracted cellulose was analyzed as described for the bulk leaf material above. We sampled the same leaves a second time with a hole puncher and analyzed the bulk material for the for the leaf $\delta^{18}O$ values using a high temperature Thermal Conversion Elemental Analyzer (TC-EA), interfaced with an IRMS (IRMS; 20-20, SerCon, UK). In addition to C and O isotope analysis, between 0.2 and 0.5 g of leaf sample was dry-ashed at 550°C for 5.5 hours; the ash was dissolved in 2M HCl solution and subsequently filtered through a P-free filter. The aliquots were then analyzed for P and Mg by inductively-coupled plasma atomic emission spectroscopy (ICP AES, IRIS interpid II XSP, Thermo scientific, USA; Ryan *et al.* 2001). Stoichiometric C:N, N:P and C:P ratios that were calculated are all mass ratios.

Stomatal traits. Leaf impressions were made from the abaxial side of five leaves per specimen halfway between the main vein and margin of the leaf, equidistant from the tip and base of the leaf blade. Transparent varnish was used to make the impressions, which were mounted with double-sided tape on a microscope slide after drying. Three photomicrographs of 1600x1200 pixels were taken per leaf print (dimensions = 344x258 µm; area view field = 0.09 mm^2) using a digital stacking microscope (VH-5000 Ver 1.5.1.1, KEYENCE CORPORATION, Osaka, Japan) with full coaxial lightning and default factory settings for shutter speed at ×1000 lens magnification (VH-Z250R). In order to determine the stomatal density of our dataset, we first trained a stomata detector model. Briefly, we started from the deep learning approach discussed in Meeus et al. (under review), which comprises a patch-based approach and starts from the

pre-trained convolutional layers of the VGG19 architecture (Simonyan and Zisserman 2014) by using the imagenet dataset (Deng et al. 2009). The output of the convolutional layers is then fed into a classifier network consisting of two dense layers, with 4096 and 2048 neurons, respectively and one output neuron. The weights of the classifier network were trained using the Adam learning rule (Kingma and Ba 2015) with batch size 128. The training set consisted of 8,500 positive and 48,500 negative patches, which were sampled from the 18 species for which leaf prints and high-quality microphotographs were available. In order to avoid over-fitting, the weights of the dense layers were trained using dropout. Furthermore, data augmentation was used to enrich the training set by flipping and rotating the patches as well by varying the contrast, brightness, and sharpness. The model described in Meeus et al. (under review) was adjusted to increase accuracy of stomatal detection by optimizing the threshold for each of the species separately on a validation set consisting of three microphotographs per species. Threshold and information retrieval standard measures such as precision, recall and F-score to evaluate the model's performance are shown in Table S2. Stomatal counts were converted to stomata per square millimeter. Guard cell length was manually measured in one stoma per picture on a subset of on average 10 pictures per herbarium specimen using Fiji (Schindelin et al. 2012).

Variance sample set. To gain insight in how variance is structured within crown, individual, and species, we performed a decomposition of intra-specific variance for a subset of the studied species. For the leaf chemical and isotope composition, we looked at intra-species variability for two species: *Gilbertiodendron dewevrei* (four trees) and *Mammea africana* (two trees) sampled in 2012, in the same reserve as where the herbarium samples were taken. From each individual tree, triplicate leaf samples were collected at three canopy heights (low, middle, and upper) during six different sampling events. For this sample set, we used whole ground leaves, which were analyzed the same way as the time series sample set. For the stomatal traits, we used samples of four species: *Prioria balsamifera* (four individuals), *Prioria oxyphylla* (two individuals), *Polyalthia suaveolens* (three individuals), *Trichilia gilgiana* (three individuals). For each individual, 3 specimens were collected: one at the base of the crown, one in the middle, and one at the top. The latter samples were collected in the Luki reserve at a different location in the Democratic Republic of Congo, Eastern DRC, in 2016 (Figure 1).

Data analyses. For the calculation of the iWUE we derived historic $\delta^{13}C\text{-}CO_2$ ($\delta^{13}C_a$) values from the equation in Bonal et al. (2011), which are in turn based on earlier work by Keeling et al. (1989) and Friedli et al. (1986), correcting for the Suess effect. For historic atmospheric CO_2 concentrations (C_a), we fitted a second-order polynomial regression to monitoring data from Mauna Loa and extrapolated back to 1935 and 1953 (dataset available at ftp://aftp.cmdl.noaa.gov/). We used the classic model of C isotope

discrimination during photosynthesis to derive leaf $\Delta^{13}C$ from these data and the cellulose $\delta^{13}C$, which were obtained via the bulk leaf $\delta^{13}C$ measurements (Figure S2, Farquhar et al. 1982):

$$\Delta^{13}C_{cell} = \frac{\delta^{13}C_a - \delta^{13}C_{cell}}{1 + \delta^{13}C_{cell}} \quad (1)$$

Likewise, if we do not take into account respiration-related fractionation, we know that carbon isotope discrimination can be described by:

$$\Delta^{13}C_{cell} = a + (b - a)\frac{C_i}{C_a} - \frac{f\Gamma^*}{C_a} \quad (2)$$

where the first term is the fractionation during CO_2 diffusion through the stomata (a=4.4‰; O'Leary, 1981), the second term the fractionation associated with reactions by Rubisco and PEP carboxylase (b=27‰; Farquhar and Richards 1984), and the third term fractionation through photorespiration (*f*=12‰ with Γ* the CO_2 compensation point in the absence of day respiration≈40 ppmv; Farquhar et al. 1982, Keeling et al. 2017, Schubert and Jahren 2018, Lavergne et al. 2020). Hence, the CO_2 concentration in the stomatal cavity (C_i) can therefore be calculated as follows:

$$C_i = \frac{C_a(\Delta^{13}C_{cell} - a) + f\Gamma^*}{b - a} \quad (3)$$

Furthermore, we know that iWUE is related to the ratio of photosynthesis (A) to stomatal conductance (g_s), and given by:

$$WUE = \frac{A}{g_s} = \frac{C_a}{1.6}(1 - \frac{C_i}{C_a}) \quad (4)$$

For the trends of all chemical or stomatal traits over time, we fitted linear mixed effects models with species as a random effect and the sampling period as a categorical fixed effect. Models were then fitted using maximum likelihood methods in the '*lme4*' package in R (Bates et al. 2007). P-values for fixed effects were determined based on the denominator degrees of freedom calculated with the Satterthwaite approximation, in the '*lmerTest*' package (Kuznetsova et al. 2014). Given the distinctness of legumes in plant physiology (Adams et al. 2016), we repeated the analysis while adding a two-level factorial fixed effect to separate *Fabaceae* and non-*Fabaceae* trees as potential N fixers, allowing for the interaction between sampling data and this new grouping variable.

For the decomposition of intraspecific variance in leaf chemistry and stomatal traits into inter-individual and intra-crown variance, we first fitted a random effects model with nested random effects, i.e. crown level nested in individual, and with species as a fixed effect. We subsequently extracted the variance that was estimated to be associated with the different nesting levels and considered it to be the 'structural

variation' with the respective level. Second, we re-fitted a mixed effect model for all response variables, but now with the crown position (upper canopy, middle, and lower canopy) as an additional fixed effect with species, instead of a random effect nested in individual. This was done to explicitly estimate the effects of sampling height in the canopy, on the different response parameters. For this decomposition of variance, we used the Bayesian multilevel model package *'brms'* (Bürkner 2017), with weakly informed Gaussian prior distributions for all effects. After fitting, the estimates of variance were extracted via the posterior distributions of the random error terms, and additionally also via the posterior distributions of the factor levels for the fixed effects for the second model fits including crown position as a fixed effect. For all statistical analysis, R was used (R Development Core Team 2018).

Sensitivity of $\delta^{18}O$ to changes in g_s. Earlier work has pointed out the limited sensitivity of $\delta^{18}O$ in conditions of high relative humidity (Farquhar et al. 2007, Roden and Siegwolf 2012). To quantify the sensitivity of $\delta^{18}O$ to changes in g_s, we used a recent model from dendrochronology, developed by Barbour et al. (2004), and further improved by Lorrey et al. (2016). For ease of interactive use, we translated the model into a Microsoft Excel tool that simulates changes in leaf $\delta^{18}O$ as function of stomatal conductivity shifts, relative humidity, temperature, photosynthetic active radiation, and source water and atmospheric water $\delta^{18}O$ signature (Appendix 1, Figure S1). We parameterized this for our central African site, and assessed potential changes in leaf $\delta^{18}O$ as a response to changes in g_s, at different levels of relative humidity. For this, we assumed a constant temperature of 25°C, a wind speed of 3.1 m s^{-1}, a photosynthetic active radiation of 1000 µmol m^{-2} s^{-1}, and an effective path length of 0.1 m, which corresponds to recent on-site measurements. Additionally, we used this tool to assess how sensitive a stomatal conductance-induced change in leaf $\delta^{18}O$ was to changes in temperature, leaf width, effective path length, photosynthetic active radiation, atmospheric pressure, wind speed and source water isotope composition, at high relative humidity (90%).

Results

Trends in leaf stable isotope signatures and nutrients. The sampled species set comprises species with leaf N values ranging from 1.30 to 4.29% and wood density values from 219 to 841 kg m^{-3}, including both N fixers and non-N fixers. Overall, leaf $\delta^{13}C$ decreased from –26.6‰ in 1938 to -31.9‰ in 2013 (Figure 2a). This decrease implies an overall increase in $\Delta^{13}C_{leaf}$ from 20.2‰ in 1938 to 23.9‰ in 2013 (Figure 2b). Consequently, estimated iWUE decreased on average from 55.8 to 27.4 (Figure 2c). The leaf cellulose $\delta^{13}C$ showed a strong positive correlation with bulk leaf $\delta^{13}C$ (Figure S2; R^2=0.75; P-value < 0.001). Leaf P and Mg showed an increase from 1953 to 2013 (Figure 2e and 2f), while leaf N did not change significantly (Figure 2d). Likewise, the leaf $\delta^{18}O$ signature did not change over time (Figure 2i).

The analysis, including the interaction between non- and potential N-fixers, revealed that iWUE significantly decreased in both non N-fixers and in N-fixers, but faster in non N-fixers (FIgure S3c).

Trends in stomatal traits. Automatic detection of stomata gained accurate stomatal counts except for one species, *Irvingia grandifolia* with a F-score of only 0.58 (Table S2) which was therefore omitted from further stomatal analyses. Stomatal densities across species and time ranged from 22 mm^{-2} (*Strombosiopsis tetrandra*) to 1089 mm^{-2} (*Entandrophragma candollei*) (Figure S5). Guard cell size ranged from 6 µm to 37 µm. The stomatal density decreased from an average of 368 to 245 stomata mm^{-2}, while the average guard cell length did not change significantly over time. Additionally, the guard cell length (GCL) increased in N fixers contrary to non-N fixers (Figure S3h). The leaf stoichiometric responses were not different in potential N fixers versus non-fixers (Figure S4).

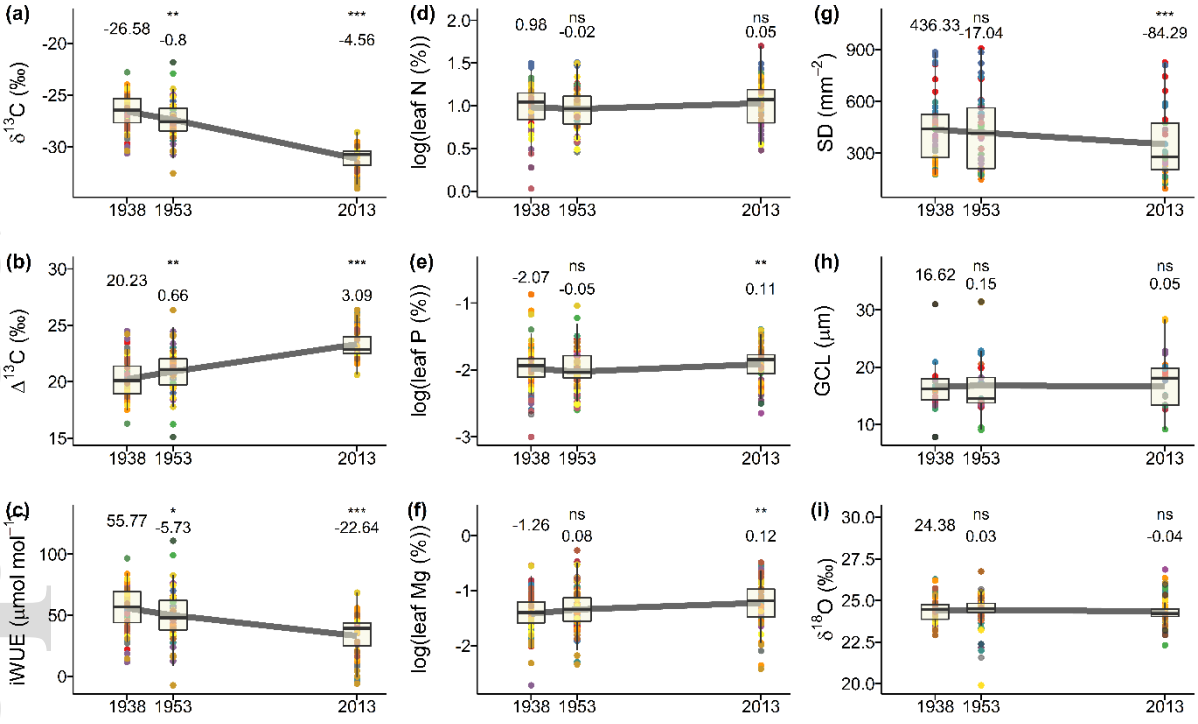

Figure 2. Trends of leaf carbon stable isotopic composition (a and b) and intrinsic water-use efficiency (iWUE, c); leaf nitrogen (N, d), leaf phosphorus (P, e) and leaf magnesium (Mg, f) which are nutrient proxies related to photosynthesis; and stomatal density (SD, g) and guard cell length (GCL, h) and the stable oxygen isotopic signature (i) over the last century in central African tropical forest. In all plots the left value is the baseline value for 1938, followed by the significance of change with the effect estimates for 1953, and the significance and effect size for 2013 with respect to 1953, with three levels of significance: $P < 0.001$ (***), $P < 0.05$ (**) and $P < 0.1$ (*). Different colors indicate the 23 different species that were included in the analyses, with the grey line the overall fixed effect of the fitted models.

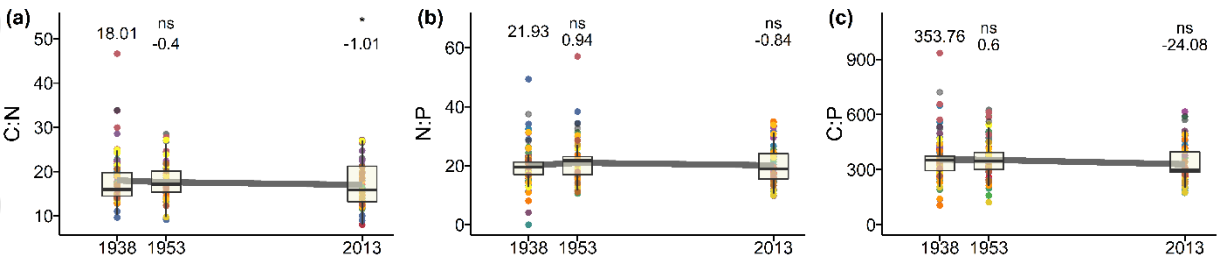

Figure 3. Shifts in foliar C:N (a), N:P (b) and C:P (c) mass ratio stoichiometry since 1938 for central African trees. In all plots the left value is the baseline value for 1938, followed by the significance of change with the effect estimates for 1953, and the significance and effect size for 2013 with respect to 1953, with three levels of significance: $P < 0.001$ (***), $P < 0.05$ (**) and $P < 0.1$ (*). Different colors indicate the 23 different species that were included in the analyses, with the grey line the overall fixed effect of the fitted models.

Variance of isotopes, leaf chemistry and stomatal traits within the canopy. The structural variance associated with crown sampling height for leaf N, P, and N:P was higher within an individual than between individuals of the same species, but lower for all other measured variables (Figure 4). For leaf N and P, 16% and 35% of the intraspecific variation was associated with the sampling position in the crown, respectively, resulting in 29% for the leaf N:P stoichiometry. The isotope signatures were much less sensitive to crown sampling level for leaf $\delta^{13}C$ and leaf $\delta^{18}O$ with 17% and 16% of the variation, respectively. Stomatal density (SD) and leaf magnesium exhibited especially high inter-individual variability (45% and 32%, respectively) and intra-crown variance (25% and 13%, respectively). The additional model fits including crown-height as a fixed effect revealed that sun leaves exhibit higher leaf $\delta^{13}C$, leaf N, P, and Mg, and stomatal density values than shade leaves, while the inverse was noted for leaf C:N, N:P, and $\delta^{18}O$ signatures. The largest relative effects were for leaf P (16% higher in upper canopy vs. middle canopy), and leaf N:P (-17%), with all other variables exhibiting effects < 10% (Figure 4).

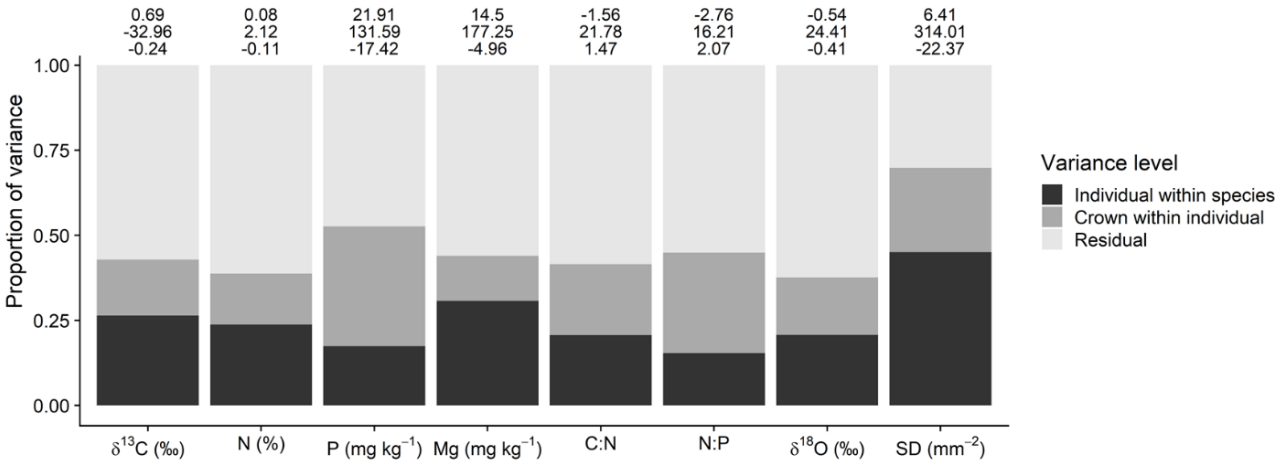

Figure 4. Intra-specific variability, decomposed into inter-individual and intra-crown variability of the measured stable isotope signatures, leaf nutrients and the stomatal density (SD) of the leaves based on two present-day sample sets from Yangambi and Luki, respectively, in the Democratic Republic of Congo. The bars indicate the relative variance associated with each of the levels; numbers above the plot give the specific effect of sampling in the upper or lower canopy (respectively highest and lowest line) versus the canopy values in the center of the canopy (middle line).

Sensitivity of $\delta^{18}O$ to changes in stomatal conductance. The simulated leaf $\delta^{18}O$ value as a function of g_s clearly shows a decreasing $\delta^{18}O$ response with increasing relative humidity, all other parameters kept constant. If we assume a doubling of the stomatal conductivity over time, the resulting shift in leaf $\delta^{18}O$ value would be 0.6‰, if relative humidity is near 100% (Appendix 1 for additional scenarios, Figure S1). Additionally, we used the tool to assess the sensitivity of stomatal conductance-induced changes in leaf $\delta^{18}O$ to any of the other parameters that are needed for the calculations. Our sensitivity analysis (at a relative humidity of 90%, and assuming that g_s doubles), shows that increasing any of the parameter values with 50% does not change the leaf $\Delta^{18}O$ (i.e. the signal we would have to capture with an IRMS after a doubling of the g_s over time) with more than 0.3‰ (Appendix A1).

Discussion

Morphological and chemical leaf adaptation to increasing CO_2, but decreasing iWUE. Our study site in central Africa exhibits a clear decreasing iWUE across the sampled species, and is thereby apparently paradoxical in its response to environmental change. Indeed, in the wake of CO_2 fertilization, increasing iWUE has been widely reported from boreal and temperate forests (e.g. Keenan et al. 2013, Wang et al. 2018). There are only few studies for tropical forests (Cernusak et al. 2013) and most studies show either

increasing iWUE (Hietz et al. 2005, Silva et al. 2009, Brienen et al. 2010, Nock et al. 2011, van der Sleen et al. 2014) or no significant change (Bonal et al. 2011). The question arises whether our observed decrease in iWUE is driven by decreasing *A* or increasing g_s. To disentangle this, we looked at stomatal density and guard cell length, which directly signal maximal g_s. The combination of a stable guard cell length, with a decreasing stomatal density, suggests an optimization for reduced water loss on the leaf level. Indeed, it seems that leaves in central African forests are down-regulating stomatal densities as a response to increased CO_2 (Xu et al. 2016) or other factors such as increased vapor pressure deficit (Figure S7, Jiao et al. 2019) or decreased soil water availability (Bertolino et al. 2019). In addition to guard cell length and stomatal density, foliar or wood $\delta^{18}O$ signature has been widely used as an indicator for g_s and shifts therein. This $\delta^{13}C$ and $\delta^{18}O$ dual isotope approach was established to relate changes in iWUE to changes in either g_s or photosynthesis (Scheidegger et al. 2000). As there is no detectable trend in leaf $\delta^{18}O$ in our study, this would suggest that the actual g_s did not vary over time, and that the iWUE response is mainly driven by reduced photosynthesis. However, recent work has cautioned against the use of $\delta^{18}O$ as a proxy for g_s in areas were relative humidity is high (Farquhar et al. 2007, Roden and Siegwolf 2012). Indeed, after simulating the $\delta^{18}O$ with our tool (Appendix 1) and with the parameterization we used for our site in central Africa, $\delta^{18}O$ appears to be insensitive to g_s (Figure S2). As a result, the interpretation of these and other $\delta^{18}O$ data as a proxy for g_s from the tropics should be done with great care.

For photosynthesis, there is no direct proxy that integrates the photosynthetic activity of the leaves. Instead, we looked at foliar nutrients that have been widely related to photosynthetic capacity (Evans 1989, Kattge et al. 2009, Walker et al. 2014, Tränkner et al. 2018). As such, the foliar N, P, and Mg show a slight increase over time. Along with the decreasing stomatal density, this increase in leaf nutrients seems to suggest a rather strong adaption of central African trees to environmental change: less potential water loss via a ca. 30% decrease in stomatal density and a apparent higher potential photosynthetic capacity via a 5-20% increase in leaf nutrients.

The combination of a decreasing stomatal density and apparent upregulation in photosynthetic capacity would result in an expected increasing iWUE, but yet iWUE decreases over time. This paradoxical response challenges our current paradigms of tropical rainforest responses to environmental change, and points to additional variables acting on the forest other than only CO_2 fertilization. Recent reports on the increase of boreal summer dry season length in the Congo basin (Jiang et al. 2019), in combination with a decline of the greenness of the Congo tropical forest in the last decade (Zhou et al. 2014), highlight a biome-specific change that might be linked to this unique response to a regional change. Indeed, long-term drying since the 1950s in central Africa (Dai 2013), along with longer dry seasons, higher temperatures, and increases in photosynthetic active radiation (Zhou et al. 2014) separates the

Congo basin forest from the Amazon forest, which is subjected to episodic short-term droughts (Phillips et al. 2009, Saatchi et al. 2013). Such long-term drying trend and increasing temperatures would surely impact the forest evapotranspiration via an increase in the vapor pressure deficit (VPD). Drought experiments have shown that plants maximize transpiration in that case, provided the available soil water levels are high enough. Although it is assumed that the sensitivity of photosynthesis to VPD is likely weaker than the sensitivity of g_s to VPD, it is still substantial. As a result, the overall relationship between iWUE and VPD is likely hyperbolic and iWUE can decline as VPD continues to rise (Zhang et al. 2019, Grossiord et al. 2020). More importantly, if this drying trend is accompanied by an air temperature increase, than this ensemble might shift local conditions to the extent that the temperature optimum for photosynthesis is exceeded, resulting in a depression of net photosynthesis (Lin et al. 2012, Aubry-Kientz et al. 2019, Huang et al. 2019). This holds especially for tropical forests, which already operate near a high temperature optimum, above which canopy photosynthesis may decrease with moderate air temperature warming (Huang et al. 2019). Indeed, it has been shown that the plasticity for thermal acclimation at the leaf level in tropical trees is limited, with potentially strong negative effects on leaf photosynthesis (Cheesman and Winter 2013, Dusenge and Way 2017, Slot and Winter 2017). We confirmed these climatic trends using the CRUNCEP data (Viovy 2018) for the grid cell of our study site, and looked at trends in temperature, VPD, relative humidity and precipitation, and additionally maximum temperature using the Berkeley data (Rohde et al. 2013). Although we have to keep in mind that these data are based on interpolation and re-analyses, our site seems to have experienced an increasing temperature, with maximum temperatures exceeding the 30°C since 1970 (Figure S7), which approximately corresponds to optimal temperature for photosynthesis in the tropics (Huang et al. 2019). Additionally, precipitation has slightly decreased and VPD increased over that same period. All together, these environmental changes could be responsible for a simultaneous increase in water deficit and water demand in this biome, combined with a reduction of net photosynthesis. The maximum temperature increase might push central African tropical forests over the physiological optimum temperature for photosynthesis, but with a high VPD and hence high transpiration. If this is at the basis of the decreasing iWUE trend, then this is likely of importance for other tropical forest biomes as well, as temperatures are projected to increase across the tropics. However, more experimental work is needed to test this observation in detail. Additionally, some authors have cautioned against the use of a linear, simplified relationship between $\delta^{13}C$ and iWUE, because iWUE might be influenced by other factors such as mesophyll conductance (Seibt et al. 2008). Indeed, a systematic change over time in mesophyll conductance could underlie any $\delta^{13}C$ trend and more research is needed to assess such effects. We acknowledge the importance of such biases but also note the clear practical limitation to assess mesophyll conductance in tree-ring or herbarium studies. Altogether, we can only conclude from our data that net photosynthesis in our study site cannot be upregulated proportionally to changes in water-

use, which has resulted in higher concentrations of CO_2 in the stomatal cavity and subsequently in a decreasing iWUE.

Ruling out sampling bias. Our variance decomposition of the present-day sample set from Yangambi and Luki (Figure 4) showed that up to 35% of the variation in leaf N, leaf P, and leaf Mg can be attributed to the canopy level of sampling. Therefore, we have to consider that a sampling effect is potentially contributing to the observed leaf nutrient trends. Indeed, the variance decomposition indicated that higher, sunlit leaves, have structurally higher nutrient contents (Figure 4). However, the older herbarium specimens supposedly comprised sunlit leaves, while the 2013-collected samples were sampled with climbers, and were thus a mixture of sun and shade leaves. This means that the increasing nutrient content is potentially underestimated and the real increase in nutrient content might be larger than the trends detected in this study (Figure 2). For stomata, on the other hand, there seems to be a decrease in density from samples in the lower canopy (Figure 4) but not on the order of magnitude of the temporal decrease that was noted in the herbarium specimens (Figure 2.; -22 mm^{-2} versus -84 mm^{-2}, respectively). Additionally, previous work has shown high variability in both whole-plant iWUE and the photosynthetic/stomatal responsiveness to increasing CO_2 across tropical tree species (Cernusak et al. 2007, Hasper et al. 2017). Nevertheless, the general trend across 23 common tree species that cover a wide range in the trait space is a decreasing iWUE.

One additional challenge in using $\delta^{13}C$ as a proxy for environmental information stored in plants is the influence of tree height on $\delta^{13}C$ (Brienen et al. 2017). Although there is very little structural variance in $\delta^{13}C$ associated with canopy level (Figure 4), we have used the variance dataset (2% relative positive effect of upper canopy sampling, 1% relative negative effect of sampling lower canopy) to test if an extreme sampling bias could have changed our trend in iWUE. If we hence assume that samples from 1938 were overestimated by 2%, and samples in 2013 underestimated with 1% relative to their mean, then we still see a clear and significant decreasing iWUE (Figure S6). This implies that a decreasing iWUE trend at our site cannot be caused by a sampling bias at the canopy level.

No proof of progressive nutrient limitation To our knowledge, only one study has reported a decrease in iWUE, which took place in a subtropical P-limited forest (Huang et al. 2016). The latter study links the decreasing iWUE to the combination of P limitation, aggravated by high on-site N deposition. This aggravated P limitation was apparent from both the increasing leaf N:P ratio and decreasing leaf P. Like this site in subtropical China, our central African study site is also P-limited and subjected to high N deposition (Bauters et al. 2018, 2019). However, contrary to the site in south China, no shifts in N:P ratio could be detected, while the leaf P in the leaves seems to have been increasing. Indeed, shifts in Huang

et al. in N:P ratio, along with a decreasing P content shows trends that are very similar to aggravated P limitation that was noted along a primary succession (Izquierdo et al. 2013). In contrast, the lack of a shift in C:N or N:P ratios in our data seems to suggest that there is no progressive N or P limitation in central Africa. Additionally, the overall leaf P increases over time, despite the fact that lowland tropical trees are assumed to be P limited (Vitousek et al. 2010) since they grow on strongly weathered and P-poor oxisols (Walker and Syers 1976). However, the way this species-level P limitation manifests as a community-wide response is still debated (Turner et al. 2018, Fleischer et al. 2019). In any case, we find no direct proof of N or P becoming increasingly limiting or increasingly constraining the C balance of the forest at our study site. This is contrary to what we would expect from theory and model simulations (Bonan 2008b, Wieder et al. 2015), and suggests that an increasing P limitation is either simply not reflected in the canopy stoichiometry, or is not yet occurring. In the latter case, external nutrient inputs could alleviate an increasing nutrient limitation. Biomass burning in the savanna borders on the African continent seems to give rise to an extraordinarily high N deposition on central African forests (Bauters et al. 2018), with seemingly no direct export that matches the N input (Bauters et al. 2019). Whether this also causes high organic P or airborne particle-bound P deposition on these forests, potentially alleviating an aggravated N or P limitation, is currently unknown.

Implications for the Congo Basin's C balance. The implication of the observed physiological leaf-level response, i.e. the overall interactive effect of CO_2 fertilization and other environmental change factors on the whole-ecosystem C balance, is widely debated. Model simulations and empirical results from Free Air CO_2 Enrichment (FACE) experiments suggest an increase in net primary productivity, constrained by nutrient bioavailability (Norby et al. 2005, 2010, 2017), while tree-ring research from the tropics suggests that a change in iWUE does not lead to a long-term increased biomass accrual or growth stimulation (Nock et al. 2011, van der Sleen et al. 2014). In temperate regions, an increasing iWUE coincided with decreasing growth induced by warming (Penuelas et al. 2008). Unfortunately, FACE experiments are currently lacking in the tropics, so direct evidence for the CO_2 fertilization effect on tropical productivity is still missing (Norby and Zak 2011, Cernusak et al. 2013). Additionally, long-term adaptation of plant physiology or a delayed soil nutrient constraint might also lead to an overestimation of the CO_2 fertilization effects on net primary productivity from FACE experiments (Norby and Zak 2011, Peñuelas et al. 2011, Reich and Hobbie 2013). Indeed, ecosystem-level interpretations which are based on shifts in leaf-level iWUE alone are not trivial. At the very least, however, the decreasing iWUE raises questions on the implications for the Congo basin forest's C balance at large scale. Further on-ground monitoring with repeated censuses and with more advanced ecosystem-level monitoring tools (e.g. eddy covariance towers) are needed to address this knowledge gap, given the importance of this biome for the global C cycle.

Conclusion

Tropical forests are important in our global understanding of the changing C balance, but empirical evidence of responses to environmental changes is sparse. The Congo basin's forest seems to show a unique response, exhibiting decreasing iWUE since 1938, with a downregulation of stomatal density and without a clear upregulation of photosynthetic capacity. Via an additional study on the variability of the measured variables in canopies, we can safely exclude that the iWUE trend shown in this study is not driven by sampling bias. This observed response challenges our current understanding of CO_2 fertilization on tropical terrestrial ecosystems. For now, we can only conclude that environmental factors other than increasing CO_2, e.g. increasing maximum temperature, likely overprints the expected iWUE response of central African trees. Still, more work is needed to mechanistically quantify these effects . The overall impact of this decreasing iWUE on the whole ecosystem C balance is unknown, but without doubt critical. Finally, we did not find proof of the progressive nutrient limitation hypothesis, exhibited by the lack of shifts in leaf nutrient stoichiometry since 1938 documented in this study.

Supplementary information

Figure S1. The response in leaf $\delta^{18}O$ to changes in stomatal conductance, as a function of the relative humidity, as simulated by the excel tool in Appendix 1, based on the model of Lorrey et al. (2016).

Figure S2. Bulk leaf $\delta^{13}C$ vs leaf cellulose $\delta^{13}C$.

Figure S3. The evolution of 1) leaf carbon stable isotope composition and intrinsic water-use efficiency (iWUE); 2) leaf nitrogen (N), leaf phosphorus (P) and leaf magnesium (Mg), as nutrient proxies related to photosynthesis, and 3) stomatal density (SD) and guard cell length (GCL) and the stable oxygen isotope signature over the last century in central African tropical forest, with separation of nitrogen fixers and non-fixers.

Figure S4. Shifts in C:N, N:P and C:P stoichiometry since 1938 for central African trees, with separation of nitrogen fixers and non-fixers.

Figure S5. Examples of stomata microscope images of herbarium specimens of nine tropical tree species

Figure S6. Trends in leaf carbon stable isotopic composition and intrinsic water-use efficiency with a correction for an hypothetical (and maximum) sampling bias

Figure S7. The trends in air temperature, precipitation, relative humidity, vapor pressure deficit and maximum temperature from 1935 to 2010 for our study location.

Table S1. Species and herbarium specimens that were included in the analyses for the different time periods.

Table S2. Species-specific thresholds and information retrieval standard measures (precision, recall and F-score) for a validation set of three microphotographs per species (N = 18).

Table S3. General site characteristics

Appendix

A1. An excel-tool to simulate the $\delta^{18}O$ signature in leaves as a response to changes in stomatal conductance, relative humidity, temperature, wind speed, source water $\delta^{18}O$ and water vapor $\delta^{18}O$, based on earlier models by Barbour et al. (2004) and Lorrey et al. (2016). Explanation on how to use the tool is provided in the Excel file itself. It also includes an interactive sensitivity analysis.

Data Accessibility

Data supporting the results in this paper are available via the Supplementary Information or archived in the Ghent University institutional repository, and available upon request with the corresponding author.

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