Leaf trait plasticity in six forest tree species of central Amazonia

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Abstract

Tropical rainforest trees adjust leaf traits during ontogeny to cope with changes in the physical environment and maximize their carbon uptake. The aim of this study was to determine the plasticity index (P_I) of leaf traits in understory and canopy leaves of six Amazonian tree species. In four of the six species the P_I of leaf traits varied within species, and in four of the ten leaf traits assessed, the P_I differed between species. The greatest P_I values were found for stomatal density (D_s) and CO_2-saturated photosynthesis, and the lowest ones were found for stomatal size, and leaf thickness. Despite the differences in P_I values within species, the mean P_I was similar in all the six species. As the saplings grow toward the canopy, the strategy to increase carbon uptake involves increasing D_s and leaf nitrogen and reducing stomatal size.

Additional key words: gas exchange; *Minquartia guianensis*; *Pouteria macrophylla*; *Protium apiculatum*; *Rinorea paniculata*.

Introduction

Plants as sessile organisms must be able to endure changes in ambient conditions during its lifetime. Over generations, individuals of a population can adapt (evolve by natural selection) to long-term changes in the physical environment *via* natural selection, and eventually new species can arise. In tropical rainforest, a tree often experiences drastic changes in illumination during its lifetime as it grows toward the canopy, from severe shading in the forest understory to direct solar radiation at the top of the forest canopy. Individual plants cannot deal with changes that occur during their lifetime by genetic adaptation, as a population does over generations. Thus, over the lifetime of a tree, any morphological or physiological change can occur only due to phenotypic plasticity, which is an induced environmental response (epigenetic change), specific for a given character and environment (Bradshaw 1965, Stearns 2014). Charac-teristics, which are essential for survival (*e.g.*, a seed size), often show greater stability (canalized traits) than others (plastic traits). Plasticity is often found in traits that do not affect the reproductive success of the individual (Bell and Lechowicz 1994). Plastic adjustment to a specific environment involves production and maintenance costs; hence, it is unlikely to find a universal adjustment to all environments (Stearns 2014).

Several leaf traits, such as leaf thickness, stomatal density (D_s) and size (S_s), and leaf photosynthesis can adjust to the physical environment, with substantial differences between sun and shade leaves (Givnish 1988, Valladares and Niinemets 2008). Understanding D_s responses to environmental factors is of major importance because a stomatal number on the leaf surface may affect carbon assimilation and water-use efficiency, as it has been found that stomatal conductance (g_s) can increase with increasing D_s (Galmés *et al.* 2007, Franks *et al.* 2009). Distribution of stomata on the leaf surface (one or both sides) affects both CO_2 diffusion into carboxylation sites and latent heat flux (amount of energy used in transpiration), as transpiration is related to the distribution of stomata on the leaf surface (Jarvis and McNaughton 1986). Stomatal density can vary within leaves, plants, and individuals of a single species (Al Afas *et al.* 2006), and it is usually higher in sun leaves than that in shade leaves (Givnish 1988, Magalhães *et al.* 2009, Kenzo *et al.* 2012). Besides genetic factors (Berger and Altmann 2000, Sugano *et al.* 2010), D_s can also vary due to environmental

factors, such as light and air humidity (Bakker 1991, Nejad and Van Meeteren 2005), water availability (Gindel 1969, Quarrie and Jones 1977), and ambient CO_2 concentration (Woodward and Kelly 1995). In several species, it has been found that D_s tends to increase under dry conditions or water stress (Salisbury 1927, Gindel 1969). Along the vertical canopy profile, light availability is highly variable and often the most important factor limiting carbon uptake, particularly in understory vegetation, as the forest floor only receives 0.5 to 2% of full sun light, and with a reduced red to far red ratio (Smith 1982).

Low irradiance often leads to a decrease in D_s, fresh leaf thickness (T_{leaf}), light-saturated net photosynthetic rate (P_{Nmax}), and specific leaf mass (SLM) – the mass to leaf area ratio (Björkman 1981, Kenzo *et al.* 2015, Niinemets

2016). In trees, D_s often ranges from 30 to 1,190 mm^{-2} and a stomatal size (length of guard cells, S_s) from 10 to 50 μm, and with a negative correlation between D_s and S_s (Hetherington and Woodward 2003, Camargo and Marenco 2011). Although an increases in D_s can lead to an increase in g_s (Galmés *et al.* 2007), more often a change in D_s does not lead to variation in g_s (Hetherington and Woodward 2003), and because P_{Nmax} is highly dependent on g_s, the effect of D_s on P_{Nmax} still remains to be elucidated (Schlüter *et al.* 2003). The aim of this work was to determine the plasticity of leaf traits often involved in plant acclimation during the ontogeny of the tree, as it grows from the understory toward the canopy and undergoes the effects of variation in irradiance, humidity, and other parameters of the physical environment.

Materials and methods

Study area and plant material: The study was conducted at the Tropical Forest Experimental Station (Reserve ZF2, 02° 36' 21" S, 60° 08' 11" W) of the National Institute for Research in the Amazon (INPA) between June and October of 2012. The study area is a pristine *terra firme* rainforest. Mean annual rainfall is about 2,400 mm, with a mild dry season (≤ 100 mm per month) between June and September, while October is a transition month. The soil is an oxisol with clay texture, low fertility, and a pH of 4.5. Mean maximum irradiance above the forest canopy is about 1,200 μmol(photon) m^{-2} s^{-1} and 20 μmol(photon) m^{-2} s^{-1} in the forest understory (0.6 mol m^{-2} per d). Other physical characteristics of the study area were previously described (Marenco *et al.* 2014). For this study, six tree species were selected (*Protium apiculatum*, *P. decandrum*, *Minquartia guianensis*, *Pouteria macrophylla*, and *Rinorea paniculata)* taking into account the availability of saplings and adult trees in the study area (Table 1). Although the biodiversity of trees is very high in the region, most tree species occur at low density (≤ 1 tree ha^{-1}, Rankin-de-Mérona *et al.* 1992). Besides, the number of replications per species was limited by natural constraints endured by selected saplings (*e.g.*, stem damage, herbivory

and defoliation by animals). From each species, we collected data from four adult trees and four juveniles (saplings) growing at the understory.

Leaf traits, plasticity index and gas-exchange data: Stomatal density (D_s), stomatal size (S_s), and fresh leaf thickness (T_{leaf}) were measured in five leaves per plant and four plants per species. Data were collected from both saplings and adult trees (herein after referred to as canopy trees). Clear nail polish impressions (four per leaf) from both the upper and lower leaf surface were taken to determine D_s and S_s under a light microscope (*Leica DM500, Leica Microsystems*, Wetzlar, Germany), at a magnification of 400 × (for D_s) and 1,000 × for measuring S_s in a sample of 20 stomata per leaf (Camargo and Marenco 2011). Fresh leaf thickness was measured in a free-hand cross-section of the leaf blade at 1,000 ×. An optical reticle (accuracy of 1 μm) mounted on the eyepiece was used for determining D_s, S_s, and T_{leaf}. The microscope eyepiece reticle was calibrated against a micrometer slide (*Nachet*, Paris, France). We also determined specific leaf mas (SLM, leaf mass to leaf area ratio). Leaf area was measured with an area integrator (*Li-3050, Li-Cor,*

Table 1. Sapling and adult tree height of the species used in the study. Each value denotes the mean of four plants (± SD).

Species	Family	Adult tree height [m]	Sapling height [m]	Use
Protium apiculatum Swart	Burseraceae	21.2 ± 4.0	1.98 ± 0.2	Phytochemical and pharmacological potential
Protium decandrum (Aubl.) Marchand	Burseraceae	19.4 ± 2.2	1.58 ± 0.6	Phytochemical and pharmacological potential
Eschweilera bracteosa (Poepp. ex O.Berg) Miers	Lecythidaceae	20.3 ± 3.3	1.7 ± 0.8	Timber industry
Minquartia guianensis Aubl.	Olacaceae	25.6 ± 6.2	1.21 ± 0.1	Timber industry and pharmacological properties
Pouteria macrophylla (Lam.) Eyma	Sapotaceae	28.7 ± 5.0	1.76 ± 0.3	The tree produces edible fruits
Rinorea paniculata (Mart.) Kuntze	Violaceae	19.3 ± 3.1	1.53 ± 0.1	Timber industry

Lincoln, USA). Leaf nitrogen (N_{leaf}) was determined by the classic Kjeldahl method. Leaf dry matter was determined after oven-drying at 72°C to constant mass (about 72 h). SLM and N_{leaf} were determined in five leaves per plant and four plants per species, collected from the canopy and understory.

The plasticity index (P_I) of a given leaf trait parameter was calculated as the maximum minus the minimum value divided by the maximum value, a 0–1 scale (Valladares *et al.* 2002). Gas-exchange data were measured in two fully expanded leaves per plant and four plants per species, from each forest stratum (saplings and canopy trees). We determined light-saturated net photosynthetic rate (P_{Nmax}) and light- and CO_2-saturated net photosynthetic rate (herein after referred to as potential photosynthesis, P_{Npot}), and stomatal conductance (g_s) with an infrared gas analyzer (*Li-6400, Li-Cor,* Lincoln, USA). The g_s was measured at light-saturated conditions and CO_2 concentration of 380 ppm. P_{Nmax} was measured at light saturation [1,000 μmol(photon) m^{-2} s^{-1}], ambient temperature (27 ± 0.5°C), and a CO_2 concentration of 380 ppm, whereas P_{Npot} was measured at a CO_2 of 2,000 ppm. Gas-exchange data were collected after a stabilization period of about 10–15 min, using a 6-cm^2 leaf chamber. The leaf was illuminated with a red–blue light source (*Li-6400-02B, Li-Cor*) and CO_2 kept constant using an onboard CO_2 injector system (*Li-6400-01, Li-Cor*). The system was set to control the airflow rate (kept constant at 400 μmol s^{-1}). Photosynthetic measurements were made between 08:00 and 15:00 h, when g_s values were higher.

Water-use efficiency was calculated as the photosynthesis to transpiration ratio (WUE = P_{Nmax}/E), and

nitrogen-use efficiency (NUE) as the relationship between P_{Npot} (to minimize the effect of g_s) and leaf nitrogen (N_{leaf}) on an area basis (NUE = P_{Npot}/N_{leaf}).

Gas-exchange data from saplings were collected from intact leaves. In a previous experiment, using an observation tower to reach the canopy of a 26 m tall tree and two IRGAs synchronically connected to branches (2 cm in diameter, 1 m in length) of the same tree. One of this branches kept intact and the other, after a stabilization period, was detached from the tree; it was found that g_s remained stable for at least 45 min after the branch was cut (Bento 2012). Thus, in order to collect data from canopy trees, we climbed up the tree boles and cut branches (3–5 cm in diameter, 1–2 m in length) from the upper canopy and then data were collected in the next 30 min at the forest floor.

Environmental data and statistical analyses: Irradiance, air temperature, air humidity, and precipitation were measured above the forest canopy at the nearest open place, the top of a 40-m tall observation tower. Irradiance, air temperature, and air relative humidity data were recorded at the observation tower with specific sensors connected to a datalogger (*Li-1400, Li-Cor*, Lincoln, USA) as previously described (Marenco *et al.* 2014). All data were subjected to analysis of variance (*ANOVA*) in order to examine the difference between species and forest strata, and the *Tukey*'s test ($p = 0.05$) was used for mean separation. *Pearson*'s correlation was used to assess the relationship between parameters. For statistical analysis we used *SigmaPlot 11.0* (*Systat Software, Inc.*).

Results

During the study period (June–October), mean monthly rainfall was 163.2 mm, and above the forest canopy mean PAR was 26.97 mol(photon) m^{-2} per day; during the whole day period, mean air temperature and mean relative humidity were 25.03°C and 83.6%, whereas mean temperature and RH were during day time 26.7°C and 77.9%, respectively.

Morpho-anatomical leaf traits: Irrespective of forest stratum (canopy or understory), stomata were present only on the abaxial leaf surface (hypostomatous leaves), and in comparison with understory leaves, mean D_s increased by 101.7% in canopy leaves, and over strata it ranged from 142.6 mm^{-2} in *P. macrophylla* to 775.5 mm^{-2} in *P. decandrum* (Table 2). SLM showed a larger interspecific variation in canopy leaves (in *R. paniculata* 83.2 *vs.* 150.1 g m^{-2} in *P. macrophylla*) than in those from the understory, 60.4 g m^{-2} in *R. paniculata* against 82.7 g m^{-2} in *P. apiculatum* (Table 2). Despite the significant difference between species, stomatal size and leaf thickness were the leaf traits that varied the least. Mean S_s varied

between 11.87 and 13.40 μm, and T_{leaf} from to 0.212 to 0.159 mm, in canopy and understory leaves, respectively (Table 2).

Physiological leaf traits: Leaf N content was greater in canopy leaves than in understory leaves, with significant difference between species (Table 3), and it ranged across strata between 0.074 mol m^{-2} in *M. guianensis* to 0.224 mol m^{-2} in *P. macrophylla*. P_{Nmax} and P_{Npot} were also higher in leaves of the canopy than in understory leaves (Table 3). However, within strata, the interspecific variation in P_{Nmax} values was rather low, from 3.07–5.54 μmol m^{-2} s^{-1} (understory) to 4.09–7.17 μmol m^{-2} s^{-1} at the canopy. P_{Npot} differences between species and within strata were significant and larger in the canopy (13.02–24.91 μmol m^{-2} s^{-1}) than in the understory (7.38–10.06 μmol m^{-2} s^{-1}). The g_s values differed between species and strata (Table 3), but contrary to expectation, mean g_s was higher in the understory (0.082–0.203 mol m^{-2} s^{-1}) than that in the canopy (0.075–0.122 mol m^{-2} s^{-1}), and across species and strata, WUE tended to remain rather constant (Table 4).

Table 2. Stomatal density (D_s), stomatal size (S_s), fresh leaf thickness (T_{leaf}), and specific leaf mass (SLM) in leaves of six Amazonian tree species. Within columns, means followed by the same small letter do not differ significantly (*Tukey's* test at p=0.05), and between strata in the same species, means followed by *the same capital letter* are not significant (*Tukey's* test at p=0.05). Each value denotes the mean (± SD) of four plants (n = 4). *Bold numerals* indicate significance (p≤0.05).

Species	D_s [mm^{-2}] Canopy	Understory	S_s [µm] Canopy	Understory	T_{leaf} [mm] Canopy	Understory	SLM [g m^{-2}] Canopy	Understory
P. apiculatum	680.9 ± 299^{abA}	279.7 ± 31^{bB}	9.05 ± 1.7^{cdB}	11.64 ± 0.6^{cA}	0.200 ± 0.011^{bcA}	0.176 ± 0.016^{abA}	102.0 ± 10.6^{bcA}	82.7 ± 14.4^{aA}
P. decandrum	775.5 ± 199^{aA}	330.2 ± 80^{bB}	10.56 ± 1.0^{bcdA}	9.94 ± 1.3^{cA}	0.222 ± 0.038^{abcA}	0.155 ± 0.008^{abB}	130.4 ± 40.7^{abA}	72.5 ± 6.3^{aB}
E. bracteosa	364.3 ± 40^{cA}	274.7 ± 35^{bB}	12.01 ± 0.8^{bcA}	11.42 ± 0.4^{cA}	0.227 ± 0.028^{abA}	0.179 ± 0.002^{aB}	118.7 ± 9.4^{abcA}	80.3 ± 12.9^{aB}
M. guianensis	339.2 ± 104^{cA}	221.5 ± 51^{bB}	8.19 ± 0.8^{dB}	11.06 ± 1.8^{cA}	0.184 ± 0.005^{cA}	0.143 ± 0.011^{abB}	97.4 ± 16.6^{bcA}	61.3 ± 10.0^{bB}
P. macrophylla	440.6 ± 76^{bcA}	142.6 ± 26^{bB}	13.56 ± 2.5^{bA}	15.39 ± 1.1^{bA}	0.250 ± 0.016^{aA}	0.139 ± 0.020^{bB}	150.1 ± 17.2^{aA}	73.8 ± 13.9^{aB}
R. paniculata	219.2 ± 46^{cA}	149.5 ± 29^{bB}	17.84 ± 2.5^{aB}	20.93 ± 2.9^{aA}	0.187 ± 0.021^{cA}	0.161 ± 0.016^{abA}	83.2 ± 19.2^{cA}	60.4 ± 10.1^{bA}
Mean	469.95 ± 244^{A}	233.03 ± 82^{B}	11.87 ± 3.6^{B}	13.40 ± 4.1^{A}	0.212 ± 0.031^{A}	0.159 ± 0.020^{B}	113.63 ± 29.7^{A}	71.83 ± 13.5^{B}
P value (strata)	**0.000**		**0.003**		**0.000**		**0.000**	
P value (species)	**0.000**		**0.000**		**0.002**		**0.000**	

Table 3. Leaf nitrogen (N_{leaf}), light-saturated net photosynthetic rate (P_{Nmax}), light and CO_2-saturated net photosynthetic rate (P_{Npot}), and stomatal conductance (g_s) in leaves of six Amazonian tree species. Within columns, means followed by *the same small letter* do not differ significantly (*Tukey's* test at p=0.05), and between strata in the same species, means followed by *the same capital letter* are not significant (*Tukey's* test at p≤0.05). Each value denotes the mean (± SD) of four plants (n = 4). *Bold numerals* indicate significance (p≤0.05).

Species	N_{leaf} [mol m^{-2}] Canopy	Understory	P_{Nmax} [µmol(CO$_2$) m^{-2} s^{-1}] Canopy	Understory	P_{Npot} [µmol(CO$_2$) m^{-2} s^{-1}] Canopy	Understory	g_s [mol(H$_2$O) m^{-2} s^{-1}] Canopy	Understory
P. apiculatum	0.124 ± 0.035^{bA}	0.097 ± 0.015^{aaA}	4.09 ± 1.41^{bA}	4.21 ± 0.99^{aA}	16.89 ± 3.12^{bcdA}	9.32 ± 2.92^{aB}	0.075 ± 0.026^{aA}	0.115 ± 0.034^{bA}
P. decandrum	0.145 ± 0.046^{bA}	0.075 ± 0.007^{aB}	4.99 ± 0.60^{aA}	3.07 ± 1.43^{aB}	13.02 ± 0.99^{dA}	7.38 ± 1.29^{aB}	0.100 ± 0.018^{aA}	0.090 ± 0.016^{bA}
E. bracteosa	0.182 ± 0.040^{abA}	0.102 ± 0.016^{aB}	6.04 ± 1.44^{aA}	3.97 ± 1.18^{aB}	19.80 ± 3.33^{abA}	9.37 ± 1.88^{aB}	0.080 ± 0.023^{aA}	0.082 ± 0.031^{bA}
M. guianensis	0.123 ± 0.028^{bA}	0.074 ± 0.012^{aB}	7.17 ± 1.53^{aA}	3.14 ± 0.68^{aB}	24.91 ± 2.38^{aA}	7.61 ± 2.97^{aB}	0.084 ± 0.022^{aA}	0.093 ± 0.033^{bA}
P. macrophylla	0.224 ± 0.057^{aA}	0.092 ± 0.021^{aB}	5.44 ± 1.68^{aA}	5.54 ± 1.21^{aA}	18.91 ± 2.24^{bcA}	10.06 ± 2.67^{aB}	0.122 ± 0.044^{aB}	0.203 ± 0.059^{aA}
R. paniculata	0.138 ± 0.041^{bA}	0.096 ± 0.020^{aA}	4.65 ± 1.27^{aA}	3.58 ± 0.97^{aA}	14.44 ± 1.37^{cdA}	9.29 ± 2.70^{aB}	0.089 ± 0.047^{aA}	0.133 ± 0.015^{abA}
Mean	0.156 ± 0.052^{A}	0.089 ± 0.018^{B}	5.40 ± 1.58^{A}	3.92 ± 1.29^{B}	18.00 ± 4.50^{A}	8.84 ± 2.42^{B}	0.092 ± 0.033^{B}	0.119 ± 0.052^{A}
P value (strata)	**0.000**		**0.000**		**0.000**		**0.007**	
P value (species)	**0.006**		0.092		**0.000**		**0.000**	

Table 4. Water-use efficiency (WUE) and nitrogen-use efficiency (NUE) in leaves of six Amazonian tree species. Within columns, means followed by *the same small letter* do not differ significantly (*Tukey*'s test at $p=0.05$) and between strata in the same species, means followed by *the same capital letter* are not significant (*Tukey*'s test at $p=0.05$). Each value denotes the mean ($\pm$ SD) of four plants ($n = 4$). *Bold numerals* indicate significance ($p \leq 0.05$). In the last column it also shows the mean of P_I across leaf traits.

Species	WUE [mmol(CO_2) mol^{-1}(H_2O)]		NUE [µmol(CO_2) mol^{-1}(N) s^{-1}]		Mean P_I across leaf traits
	Canopy	Understory	Canopy	Understory	
P. apiculatum	2.002 ± 0.301^{aB}	4.078 ± 2.839^{aA}	140.7 ± 29.21^{abA}	100.2 ± 41.10^{aA}	0.308 ± 0.0386^{a}
P. decandrum	2.577 ± 0.226^{aA}	1.691 ± 0.740^{aA}	96.5 ± 29.03^{bA}	99.9 ± 25.66^{aA}	0.324 ± 0.0896^{a}
E. bracteosa	2.529 ± 0.178^{aA}	4.269 ± 0.934^{aA}	109.7 ± 8.51^{bA}	91.8 ± 13.01^{aA}	0.309 ± 0.0425^{a}
M. guianensis	3.838 ± 0.532^{aA}	2.486 ± 0.702^{aA}	206.9 ± 36.09^{aA}	102.9 ± 39.10^{aB}	0.384 ± 0.0875^{a}
P. macrophylla	3.622 ± 0.780^{aA}	3.350 ± 1.700^{aA}	91.6 ± 36.85^{bA}	114.3 ± 42.70^{aA}	0.429 ± 0.0871^{a}
R. paniculata	4.011 ± 2.075^{aA}	1.967 ± 0.765^{aB}	110.6 ± 28.29^{bA}	104.6 ± 49.66^{aA}	0.301 ± 0.0215^{a}
Mean	3.097 ± 1.141^{A}	2.974 ± 1.669^{A}	126.0 ± 47.98^{A}	102.28 ± 33.68^{B}	0.343 ± 0.077
P value (strata or P_I)	0.736		**0.020**		0.078
P value (species)	0.333		**0.016**		

Table 5. *Pearson*'s correlation coefficients (upper right section) and *p* values (lower left section) between the examined variables, light-saturated net photosynthetic rate (P_{Nmax}), light and CO_2-saturated net photosynthetic rate (P_{Npot}), stomatal conductance (g_s), water-use efficiency (WUE), stomatal density (D_s), stomatal size (S_s), fresh leaf thickness (T_{leaf}), specific leaf mass (SLM), leaf nitrogen (N_{leaf}), and nitrogen-use efficiency (NUE). *Bold numerals* indicate significance ($p \leq 0.05$). For correlation analysis data were pooled across species and forest strata ($n = 48$).

	P_{Nmax}	P_{Npot}	g_s	WUE	D_s	S_s	T_{leaf}	SLM	N_{leaf}	NUE
P_{Nmax}	-	**0.67**	**0.34**	**0.42**	0.10	–0.14	0.27	**0.33**	0.43	**0.34**
P_{Npot}	**0.000**	-	–0.20	0.22	0.25	–0.22	**0.55**	**0.55**	0.58	**0.58**
g_s	**0.019**	0.167	-	0.11	–0.25	**0.36**	–0.27	–0.16	–0.06	–0.09
WUE	**0.003**	0.133	0.478	-	–0.19	0.04	0.11	0.03	0.05	0.23
D_s	0.51	0.091	0.088	0.203	-	**–0.53**	**0.56**	**0.59**	**0.32**	0.00
S_s	0.34	0.130	**0.012**	0.799	**0.000**	-	–0.14	–0.21	0.00	–0.24
T_{leaf}	0.058	**0.000**	0.060	0.475	**0.000**	0.330	-	**0.89**	**0.76**	–0.11
SLM	**0.020**	**0.000**	0.284	0.824	**0.000**	0.160	**0.000**	-	**0.82**	–0.17
N_{leaf}	**0.002**	**0.000**	0.662	0.743	**0.027**	0.870	**0.000**	**0.000**	-	–0.27
NUE	**0.018**	**0.000**	0.528	0.113	0.980	0.105	0.463	0.243	0.060	-

NUE was 23% higher in the canopy, and between species the range of values were larger in canopy leaves (91.6–206.9 µmol mol^{-1} s^{-1}) than in understory leaves (91.8–114.3 µmol mol^{-1} s^{-1}, Table 4). The correlations between P_{Nmax} and g_s, N_{leaf}, SLM, and P_{Npot} were significant (Table 5), but there was no correlation between P_{Nmax} and D_s or S_s. Likewise, there was no correlation between T_{leaf} and P_{Nmax}, but the relationship between T_{leaf} and P_{Npot} was significant; the association between P_{Npot} and N_{leaf} or SLM was also significant (Table 5). We found positive correlation between D_s and T_{leaf}, SLM or N_{leaf}; N_{leaf} and T_{leaf} or SLM, as well as between g_s and S_s, but a negative correlation between D_s and S_s; NUE was associated with P_{Nmax} and P_{Npot}, but WUE only correlated with P_{Nmax} (Table 5).

Plasticity index: In four of the six examined species, the plasticity index of leaf traits varied significantly within species, the mean ranged from 0.067 for S_s in *E. bracteosa* to 0.692 for P_{Npot} in *M. guianensis* (Fig. 1). In *P. apiculatum* and *R. paniculata*, P_I differences between leaf traits were insignificant, and in both species a mean P_I value of 0.30 was found (Fig. 1, Table 4). The plasticity index for S_s ($P_I = 0.182$, mean across species), SLM ($P_I = 0.337$), P_{Nmax} ($P_I = 0.365$), P_{Npot} ($P_I = 0.477$), g_s ($P_I = 0.326$), and WUE ($P_I = 0.384$) did not differ between species (Fig. 2). On the other hand, the P_I for N_{leaf}, T_{leaf}, D_s, and NUE varied interspecifically (Fig. 2). P_I values for N_{leaf} were greater in *P. macrophylla* (mean of 0.577) and *P. decandrum* (0.448). Because NUE is a function of P_{Npot} and N_{leaf}, *M. guianensis* had the greatest plasticity (0.499) for this character and *P. decandrum* the lowest one, mean of 0.115 (Fig. 2). Three of the six species (*P. apiculatum*, *P. decandrum*, and *P. macrophylla*) had high P_I for D_s (0.525–0.674), but all of them had a low plasticity for S_s, mean of 0.067–0.250 (Fig. 2). To show a general trend, we analyzed the P_I pooled over species. We found that D_s and P_{Npot} had the highest P_I values across species (mean P_I of 0.430–0.477), whereas S_s and T_{leaf} had the lowest plasticity, 0.182–0.235 (Fig. 3). Although the P_I of some leaf traits had interspecific variation (*i.e.*, D_s, T_{leaf}, N_{leaf}, and NUE), on average the mean P_I values were similar between species (Table 4).

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Discussion

The climate conditions during the study period were similar to those previously reported for this site (Nascimento and Marenco 2013, Marenco *et al.* 2014). Stomata occurrence solely in the abaxial leaf surface suggests that hypostomaty is a canalized trait (highly stable) in tropical rainforest trees (Boeger *et al.* 2004, Camargo and Marenco 2011). Canopy leaves had higher D_s than leaves of saplings (Table 2), which is consistent with results reported by others (Lockheart *et al.* 1998, Ichie *et al.* 2016). In several tree species (*e.g.*, *Sinapis alba*, *Quercus myrsinifolia*, and *Fagus sylvatica*), an increase in light intensity leads to an increase in D_s (Furukawa 1998, Lockheart *et al.* 1998), and hence leaves at a higher position on the stem tend to have smaller stomata and higher D_s than lower leaves (Medri and Lleras 1979, Camargo and Marenco 2011, Kenzo *et al.* 2012).

In comparison with sapling leaves, T_{leaf} was greater in canopy leaves, which is consistent with the positive effect of irradiance on leaf thickness (Witkowski and Lamont 1991, Terashima *et al.* 2001, Magalhães *et al.* 2009). In *Bertholletia excelsa*, for example, leaf thickness can increase from 230 μm in a 0.3 m tall sapling to 380 μm in an 18 m tall tree (Medri and Lleras 1979). Leaf thickness positively correlated with D_s ($r = 0.56$) and specific leaf mass ($r = 0.89$), which is expected because D_s and SLM increase as the tree grows towards the canopy (Givnish 1988, Kenzo *et al.* 2015, Ichie *et al.* 2016), and it reflects the capacity of juvenile trees to respond to changes in irradiance, either in response to gap opening or height increase over time. Within tree species, leaf size often decreases with increasing tree height (Medri and Lleras 1979, England *et al.* 2006, Sack *et al.* 2006), and to counterbalance the reduction in leaf size, T_{leaf}, SLM, and

N_{leaf} often increase with tree height, which explains the positive correlation between T_{leaf} and D_s or N_{leaf}. It is well-documented that sun leaves have greater D_s and SLM than shade leaves (Givnish 1988, Valladares and Niinemets 2008), which suggests that irradiance is one of the main drivers of D_s and SLM variation along the forest profile. Changes in humidity can also contribute to variation in D_s. Considerable stomatal closure can occur in canopy leaves because leaf-to-air vapor pressure difference and hydraulic resistance increases with tree height (Zimmermann 1978, Schäfer *et al.* 2000, Woodruff *et al.* 2004, Ambrose *et al.* 2010), which leads to a decline in leaf water potential (Koch *et al.* 2004, Woodruff *et al.* 2004, Kenzo *et al.* 2015). Besides, under low air-humidity conditions, the peristomatal transpiration (that may lead to stomatal closure) can be greater in high-D_s leaves. El-Sharkawy *et al.* (1985) found that g_s decreased with increasing D_s as vapor pressure deficit increased. Also, low water availability can lead to increases in D_s (Gindel 1969, Quarrie and Jones 1977). It has been reported that there is a positive correlation between g_s and D_s (Franks *et al.* 2009). However, contrary to expectation, g_s did not increase with increasing D_s, which led us to conclude that the aperture of the stomatal pore was below its maximum width, and hence g_s was lower than expected. There was a negative correlation between D_s and S_s ($r = -0.53$, $p < 0.001$), which is in agreement with the findings reported by others (Camargo and Marenco 2011, Ichie *et al.* 2016). At the canopy, high density of smaller stomata allows the leaf to keep a rapid control of stomatal movement and thereby a tight control of transpiration, as there is negative correlation between the length of the stomatal pore and sensitivity to decreasing leaf water potential (Aasamaa *et al.* 2001).

Fig. 1. Box plot plasticity index (P_I, unitless) of leaf traits within species. The boundaries of the box indicate the 25th and 75th percentile. The *solid line* within the box denotes the median. Species: *Eschweilera bracteosa* (*A*), *Minquartia guianensis* (*B*), *Protium decandrum* (*C*), *Pouteria macrophylla* (*D*), *Protium apiculatum* (*E*), and *Rinorea paniculata* (*F*). D_s – stomatal density; S_s – stomatal size; N_{leaf} – leaf nitrogen; P_{Nmax} – light-saturated net photosynthetic rate; P_{Npot} – light and CO_2-saturated net photosynthetic rate; g_s – stomatal conductance; WUE – water-use efficiency; NUE – nitrogen-use efficiency; T_{leaf} – fresh leaf thickness, SLM – specific leaf mass. P_{Nmax} and g_s were measured at light saturation 1,000 μmol(photon) m^{-2} s^{-1}.
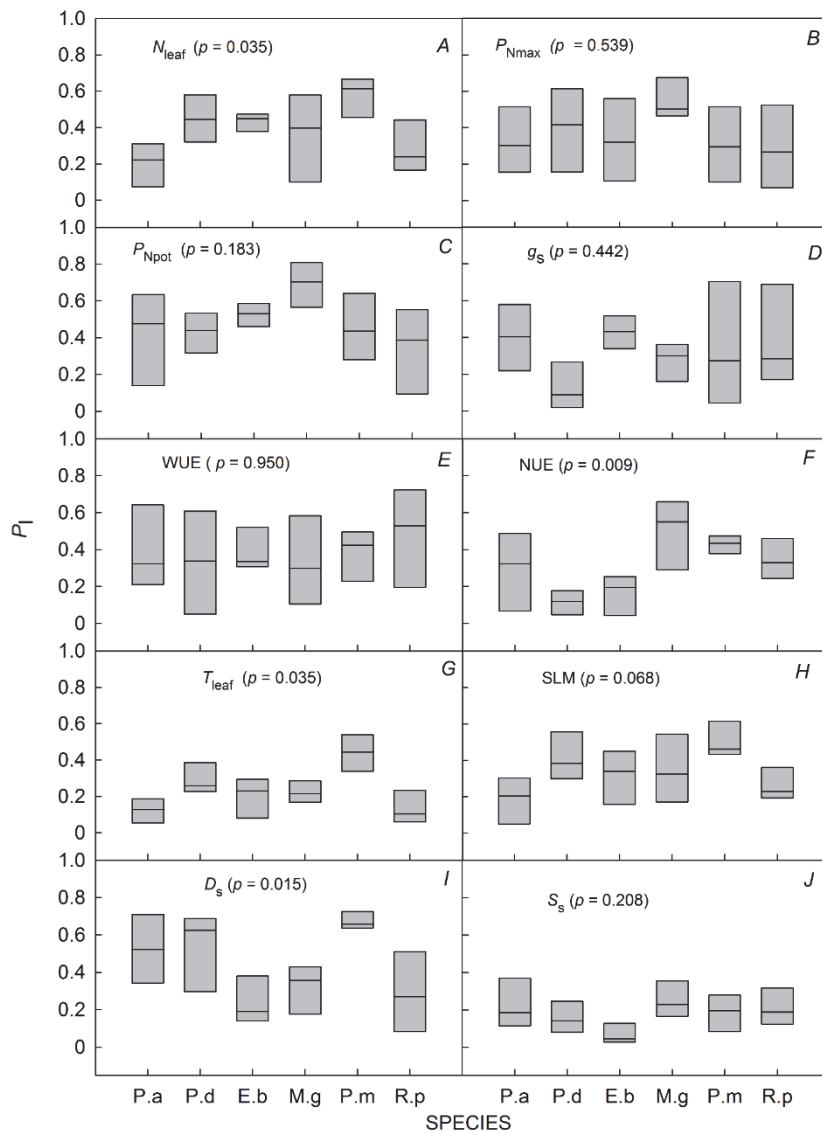
Fig. 2. Box plot plasticity index of leaf traits between species. The boundaries of the box indicate the 25th and 75th percentile. The *solid line* within the box denotes the median. P.a – *Protium apiculatum*; P.d – *Protium decandrum*; E.b – *Eschweilera bracteosa*; M.g – *Minquartia guianensis*; P.m – *Pouteria macrophylla*; R.p – *Rinorea paniculata*. Acronyms of leaf traits are as described in Fig. 1.

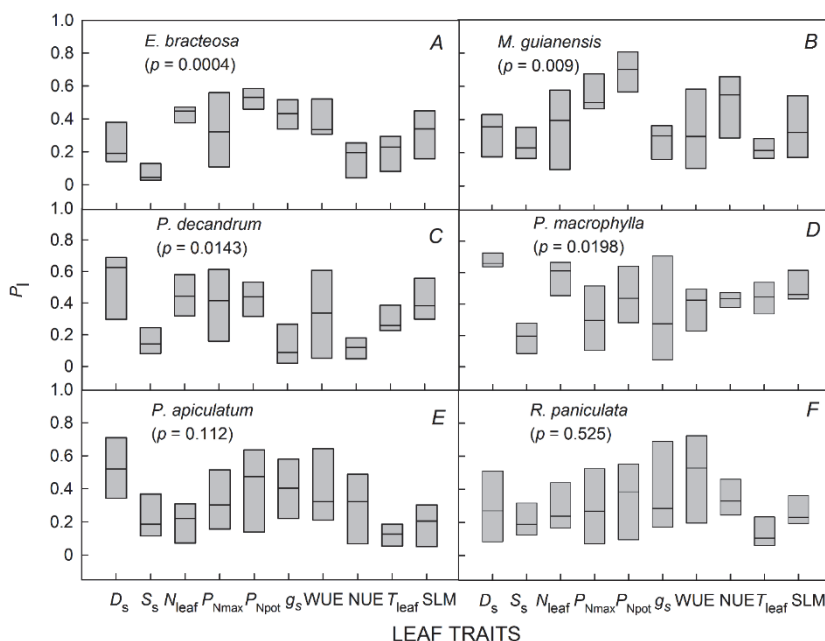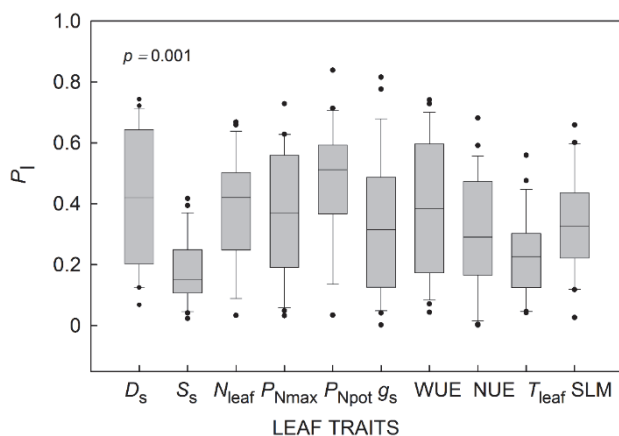

Fig. 3. Box plot plasticity index of leaf traits across species. The boundaries of the box indicate the 25th and 75th percentile and the whiskers indicate the 90th and 10th. *The solid line* within the box denotes the median, and the outliers are indicated by dots. Acronyms of leaf traits are as described in Fig. 1.

As sun leaves are thicker and have more protein per unit leaf area, we found the classic correlation between P_{Nmax} and N_{leaf} or SLM, which is consistent with the results reported elsewhere (Niinemets 1999, Valladares and Niinemets 2008, Niinemets 2016). The same is true for the positive correlation between P_{Npot} and T_{leaf}. Although *M. guianensis* had the lowest N_{leaf} content, it had high P_{Nmax} and P_{Npot} values in canopy leaves, which indicates that this species uses N parsimoniously. N_{leaf} was 75.3% higher in the canopy than that in the understory, which seems to be an acclimation strategy to increase photosynthetic rates of canopy leaves. Kenzo *et al.* (2015) also found that leaf nitrogen increases with increasing tree height. The absence of correlation between P_{Nmax} and leaf thickness can be attributed to low g_s, particularly in canopy leaves. In comparison with understory leaves, leaf thickness increased only 33.3% (Table 2) in canopy leaves, whereas P_{Npot} increased 103.6% (Table 3), which shows that a small increase in leaf thickness leads to a remarkable

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increase in potential photosynthesis. WUE did not differ between species nor between strata (Table 4). Despite the general lack of significance between strata on this parameter, in *R. paniculata* small differences in g_s and P_{Nmax} led to significant differences in WUE between strata. This shows that a subtle change in one leaf trait can produce a significant effect in another.

The plasticity index for D_s and T_{leaf}, N_{leaf}, and NUE varied between species, which shows that there are important interspecific differences in the acclimation potential in response to changes in conditions of the physical environment. D_s was the morphological leaf trait that showed the greatest variation (mean P_I of 0.430), which indicates that this character is highly responsive to changes in ambient conditions. Irrespective of the difference between species, S_s was the leaf trait that varied the least, in *E. bracteosa*, for example, the difference between strata was almost nil (5.2%), indicating that most of the change in total pore area of the leaf in this species depends on variation in D_s, a plastic trait that often increases with tree height (Kenzo *et al.* 2012). This suggests that high D_s plasticity is a strategic mechanism to ensure a tight control of water loss by transpiration. Our results agree with those reported by Sack *et al.* (2006) who found that D_s and SLM can increase 1.1–2 times across the canopy, without changes in stomatal size. It seems that in *P. apiculatum*, *P. decandrum*, and *P. macrophylla* (species with the highest P_I values for D_s) the strategy to optimize the use of resources was to increase D_s, as stomatal frequency more than double in canopy leaves of these species. Leaf thickness showed low plasticity, which suggests that understory leaves are rather thicker than one could expect from a photosynthetic perspective. This may occur because understory leaves tend to live longer to compensate construction costs, and to endure herbivory, long lifespan leaves often produced lignin-enriched tissues and increase the synthesis of defensive compounds such as tannins or saponins (Coley 1988, Bixenmann *et al.* 2016), which are not directly related to carbon assimilation.

The P_I of P_{Npot} showed the highest value among the leaf traits (mean of 0.477), which suggests that all environmental changes that occur during the ontogeny of the tree (*e.g.*, changes in irradiance, temperature, and humidity conditions) ultimately lead to changes in the potential photosynthetic capacity of the leaf. Irrespective of variation in some morphological P_I, all species had similar P_I for P_{Nmax} (Fig. 2), which indicates that regardless of the acclimation strategy, almost all species ultimately tend to converge to similar photosynthetic rates, the exception was *P. apiculatum*, where P_{Nmax} was particularly low in canopy leaves (Table 3). It seems that in *P. macrophylla*, the high P_I of D_s, T_{leaf}, and N_{leaf} (0.440–0.674) contributed for canopy leaves to reach at least similar P_{Nmax} of understory leaves (Table 3), despite 39.9% reduction in stomatal conductance. Although the P_I of T_{leaf} was higher (mean of 0.44) in *P. macrophylla* than in the other species (P_I of 0.12–0.29, Fig. 2), P_{Nmax} values of canopy and understory

leaves were similar, which shows that in the other species the low plasticity of T_{leaf} can be compensated by increased plasticity in other leaf traits. This supports the hypothesis that as the sapling grows toward the canopy, the intrinsic (*e.g.,* decline in hydraulic conductivity along the xylem path) and extrinsic changes (*e.g.,* increase in irradiance and vapor pressure deficit along the forest profile) lead to increases in stomatal density, leaf thickness, leaf nitrogen content, and enzymes of carbon metabolism. One can argue that the P_I of P_{Nmax} is lower than that of P_{Npot} because hydraulic constraints along the xylem path prevent the stomata to reach their maximum potential aperture (Franks *et al.* 2009). The high plasticity index, found for P_{Npot} (Fig. 1), is in agreement with results reported by Valladares *et al.* (2000) who found that photosynthesis was three times greater in gaps than that under low light conditions. In contrast, we found only a 37.8% increase in P_{Nmax} in canopy leaves. Thus, although the P_I of P_{Npot} followed the trend observed by Valladares *et al.* (2000), the P_I of P_{Nmax} did not. The difference can be attributed to the size of plants (we used trees and saplings instead of small plants), as in canopy leaves g_s may decrease substantially. Scoffoni *et al.* (2015) found that in small plants g_s did not decrease under high irradiance, which allows the leaf to increase P_{Nmax} with increasing irradiance, and probably because of low g_s, the mean P_{Nmax} of canopy leaves was lower than that previously recorded in sun leaves of saplings (Marenco *et al.* 2001).

In canopy leaves, P_{Npot} more than doubled in *E. bracteosa* and *M. guianensis* (Table 3), and in *R. paniculata* WUE increased 103.8% (4.011 *vs.* 1.967 mmol mol^{-1}, Table 4), which indicates that their plasticity was based on improvement of the physiological performance. The plasticity of WUE observed in *R. paniculata* is consistent with the results reported by Kenzo *et al.* (2015), who found an increase of $\delta^{13}C$ (a proxy of WUE) with increasing tree height. It is remarkable that all the species converged to have similar plasticity indexes (mean of 0.343, Table 5), which indicates a similar adjustment in their biological machinery, in part because the physiological plasticity can be either equal (Rozendaal *et al.* 2006) or higher (Valladares *et al.* 2000) than the morphological plasticity.

It can be concluded that plasticity is a mechanism that integrates the effect of several factors which ultimately allow the plant to achieve its best performance in a given environment. The physical environment plays an important role, but the internal changes associated with ontogeny (*e.g.,* hydraulic changes of the xylem along the path and tree growth) may also contribute to modifications that occur in the leaves as the sapling grows toward the canopy. Our data support the hypothesis that predicts that leaf plasticity is greater for those leaf traits that ensure the best performance of the plant across a gradient in light availability (Valladares *et al.* 2002), and other factors of the physical environment. It seems that trees have developed mechanisms, which ultimately aim to maximize carbon uptake through their ontogenetic stages, as the

saplings grow toward the canopy. These strategies involve increasing stomatal density and leaf nitrogen, and decreasing stomatal size, as smaller stomata respond faster

to environmental cues and internal signals that drive stomatal movements, particularly to avoid water loss by transpiration.

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