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Contact CEH NORA team at
noraceh@ceh.ac.uk

Leaf age effects on the spectral predictability of leaf traits in Amazonian canopy trees

*Cecilia Chavana-Bryant[1,5,6], Yadvinder Malhi[1], Athanasios Anastasiou[2], Brian J. Enquist[3], Eric G. Cosio[4], Trevor F. Keenan[5,6] and France F. Gerard[7]

*Corresponding author: cecilia.chavana-bryant@ouce.ox.ac.uk

1 Environmental Change Institute, School of Geography and the Environment, University of Oxford, Oxford, OX1 3QY, UK
2 College of Medicine, Swansea University, Singleton Park, Swansea SA2 8PP, Wales, UK
3 Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona 85721, USA
4 Sección Química, Pontificia Universidad Católica del Perú, Avenida Universitaria 1801, San Miguel, Lima 32, Peru
5 Climate & Ecosystem Sciences Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, CA, 94720, USA
6 Department of Environmental Science, Policy and Management, UC Berkeley, Berkeley, CA, 94720, USA
7 Centre for Ecology and Hydrology, Wallingford, Oxfordshire OX10 8BB, UK

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Abstract

Recent work has shown that leaf traits and spectral properties change through time and/or seasonally as leaves age. Current field and hyperspectral methods used to estimate canopy leaf traits could, therefore, be significantly biased by variation in leaf age. To explore the magnitude of this effect, we used a phenological dataset comprised of leaves of different leaf age groups -developmental, mature, senescent and mixed-age- from canopy and emergent tropical trees in southern Peru. We tested the performance of partial least squares regression models developed from these different age groups when predicting traits for leaves of different ages on both a mass and area basis. Overall, area-based models outperformed mass-based models with a striking improvement in prediction observed for area-based leaf carbon (C_{area}) estimates. We observed trait-specific age effects in all mass-based models while area-based models displayed age effects in mixed-age leaf groups for P_{area} and N_{area}. Spectral coefficients and variable importance in projection (VIPs) also reflected age effects. Both mass- and area-based models for all five leaf traits displayed age/temporal sensitivity when we tested their ability to predict the traits of leaves of other age groups. Importantly, mass based mature models displayed the worst overall performance when predicting the traits of leaves from other age groups. These results indicate that the widely adopted approach of using fully expanded mature leaves to calibrate models that estimate remotely-sensed tree canopy traits introduces error that can bias results depending on the phenological stage of canopy

leaves. To achieve temporally stable models, spectroscopic studies should consider producing area-based estimates as well as calibrating models with leaves of different age groups as they present themselves through the growing season. We discuss the implications of this for surveys of canopies with synchronised and unsynchronised leaf phenology.

Key Words

Remote Sensing; Phenology; Age effects; Leaf physiology; Foliar chemistry; Mass- vs area-based PLSR models

1.1 Introduction

Morphological and biochemical leaf traits are important indicators of plant physiology and play a critical role in regulating plant growth, and energy and material cycling in terrestrial food webs (Mercado et al. 2011; Messier et al. 2010; Niinemets et al. 2015; Wright et al. 2004). The ability to characterise the variation of key functional leaf traits in space and time is therefore, central to improving our understanding of ecosystem function, seasonal productivity and global biogeochemical cycling (Chavana-Bryant et al., in review; Enquist et al. 2007; Reich et al. 1997; Violle et al. 2007)

In the last few decades, remote sensing – specifically, hyperspectral spectroscopy – has played an increasingly important role in the study of the diversity and functional ecology of leaves, canopy and ecosystem traits (Asner 2001; Asner et al. 2009; Asner et al. 2011a; Blackburn and Milton 1995; Curran 1989; Fourty et al. 1996; Kokaly et al. 2009; Peterson et al. 1988; Serbin et al. 2014; Townsend et al. 2008; Ustin et al. 2009; Wessman et al. 1988).

Hyperspectral spectroscopy uses measurements of reflected light in narrow sampling intervals (typically ⊡10-15 nm) over the entire reflected solar spectrum (i.e. 400-2500 nm), which together with chemometric partial least square regression (PLSR) techniques to link leaf and canopy spectroscopic measurements with their traits. From boreal to tropical environments, an extensive body of work has used spectroscopy and PLSR techniques to estimate vegetation traits associated with light capture and

growth (i.e. pigments, nitrogen, phosphorus, leaf mass per area (LMA), leaf water content (LWC) and soluble carbon) and structure and defense (i.e. lignin, cellulose and total carbon) (Asner et al. 2015; Asner et al. 2009; Asner et al. 2011a; Axelsson et al. 2013; Féret et al. 2011; Qi et al. 2014; Ramoelo et al. 2011; Richardson and Reeves 2005; Serbin et al. 2014; Singh et al. 2015). A major goal of these studies, whether used at the leaf level or embedded in a canopy model for upscaling purposes, is to develop generic PLSR models to predict leaf traits across space and time.

When developing chemometric PLSR models, spectroscopic studies have placed great importance in collecting leaf samples with high diversity of species and/or environmental growing conditions (Asner et al. 2009; Asner et al. 2011a; Asner et al. 2014; Axelsson et al. 2013; Peterson et al. 1988; Qi et al. 2014; Ramoelo et al. 2011; Richardson and Reeves 2005; Serbin et al. 2014) with much less importance given to capturing the temporal/seasonal diversity of leaf traits due to leaf age. However, seasonality is highly important in studies comparing plant communities found in different locations or covering large geographical areas, sampled at different times, or measured repeatedly between years (Grassi et al. 2005; Stokes et al. 2010; Wilson et al. 2001). The standard protocol in spectroscopic studies has been to use only fully expanded mature leaves (Asner et al. 2009; Asner et al. 2011a; Asner et al. 2014; Axelsson et al. 2013; Ramoelo et al. 2011; Richardson and Reeves 2005) or mature leaves of different growth seasons (Qi et al. 2014; Serbin et al. 2014) and to apply the resulting models across

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time/seasons. Even in studies that include leaf samples from "different developmental stages" such as Féret *et al.* (2011), the relative effects of including these samples in the development of PLSR models has not been addressed. To our knowledge, only a study by Yang *et al.* (2016) has assessed the potential of using leaf spectroscopy to predict leaf traits across their life cycle at two temperate deciduous forests but not the direct impact that leaf age can have on the performance of PLSR models.

Biochemical, morphological and physiological leaf traits change through time and/or seasonally as leaves age. Previous studies in both tropical (Chavana-Bryant et al. 2017; Kitajima et al. 2002; Kitajima et al. 1997; Wu et al. 2016) and non-tropical forests (Escudero and Mediavilla 2003; Field and Mooney 1983; Mediavilla et al. 2011; Niinemets et al. 2005; Niinemets and Kull 2003; Niinemets et al. 2004; Yang et al. 2016) have shown strong age effects on leaf traits of individual trees and that many of these age effects can have direct influence on the spectral reflectance behaviour of leaves (Datt 1999; Gausman et al. 1970; Knipling 1970; Roberts et al. 1998; Sims and Gamon 2003). Furthermore, two recent studies that detail the life-cycle evolution of spectral and physiochemical leaf traits in tropical canopy trees, have demonstrated that (1) age-related spectral changes in leaves are distinct enough to enable the prediction of leaf age across trees of different species from hyperspectral leaf reflectance measurements (Chavana-Bryant et al. 2017) and 2) the trait variation generated from sampling leaves of different leaf age groups across a relatively small sample of 12

Amazonian canopy trees can be as large as the variation found among the mature leaves of a large sample of almost 200 Pantropical trees from the GLOPNET database (Chavana-Bryant et al. In review). It is therefore, very likely that the combined effect of leaf aging and asynchrony in leaf life cycles will have an impact on the performance of current PLSR models used to estimate canopy leaf traits in tropical evergreen forests. In unsynchronised vegetation systems such as these, different proportions of leaves of different ages will be present throughout the annual cycle due to a range of species-specific leaf phenological behaviours, and seasonal trends due to intra- and interspecific phenological asynchrony (Borchert 1983; Borchert et al. 2002; Reich 1995; van Schaik et al. 1993). In forests with synchronised leaf phenology (e.g. dry tropical and temperate forests) there are times during the growing season when only developing or senescing leaves are present in the canopy (i.e. beginning and end) and even leaves at different stages of maturity (early, mid and late maturity) can display differences in traits (McKown et al. 2013). Furthermore, leaf trait and remote sensing sampling campaigns in tropical evergreen forests largely occur throughout the dry season, a time when their canopies are most in flux, while in dry tropical and temperate forests these can occur at different times of the growing season. A better understanding of how leaf age/seasonality affects the performance of PLSR models is therefore long overdue.

In this study, we address these issues using a phenological leaf dataset comprised of leaves of different leaf age groups –developmental (D), mature (M), senescent (S) and mixed-age (All)- from eight different canopy and emergent tropical trees. Chemometric PLSR models were developed using data subsets comprised of the different leaf age groups to answer the following questions: Does the robustness of PLSR models vary differently with leaf age when traits are measured on a mass and area basis? Does leaf age affect PLSR model performance differently for different leaf traits? How well do PLSR models, developed using leaves from a single leaf age group, predict the traits of leaves of other or mixed leaf age groups? And what are the implications for best practice when assessing leaf traits on a per mass basis versus per area basis using spectroscopy and PLSR techniques?

1.2 Materials and Methods

1.2.1 Field site

Data were collected in and around the borders of two lowland tropical rainforest plots located within the Tambopata National Reserve in the Madre de Dios region of Peru in southwestern Amazonia. The two study sites are part of the RAINFOR Amazon Forest Inventory Network (Malhi et al. 2002) and GEM intensive monitoring network, with RAINFOR codes TAM-06 (12°50'24"S, 69°17'59"W) and TAM-09 (12°49'48"S, 69°16'48"W). The sites are located between 215m and 220m above sea level within lowland closed-canopy primary tropical rain forest growing on Haplic alisol

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soils (Quesada et al. 2010). They are fairly seasonal sites with a four to five month-long dry season (number of months with <100mm rainfall) between June and October, mean annual rainfall over the period 2005-2012 of 1900mm (this may be lower than the long-term mean because of droughts in 2005 and 2010 (Lewis et al. 2011) and mean annual air temperature of 24.4°C. A detailed description of the geomorphology, climate and forest carbon cycle of this site can be found in Malhi *et al.* (2014).

1.2.2 Tree sampling

As top-of-canopy leaves are expected to be the main contributors to the spectral response of forest canopies (Asner and Martin 2008; Clark et al. 2005), the tree sampling for this study focused on canopy and emergent trees (Table 1). A total of eight trees were sampled. According to the "Crown Illumination Index" (Clark and Clark 1992; Keeling and Phillips 2007), six of these were canopy trees and two were emergent trees. Diameter at breast height (1.3m; DBH) among sampled trees averaged 152cm (range: 117-237cm). The sampled trees averaged 32m in height (range: 29-35m), compared to a mean tree height of 27 ± 8m (± SE) for canopy trees ⧠ 40cm DBH in local plots. Among the sampled trees, canopy depth, the difference between tree height and height at the lowest branch of the canopy, averaged 10.3 m (range: 6.3-15.1m).

Table 1 **Trees sampled for leaf physiochemical and spectral traits.**

Species code	Species	Family	DBH (m)	Tree Canopy position	Height (m)	Canopy Depth (m)
1	*Licania brittoniana* Fritsch	Chrysobalanaceae	1.88	Canopy	29.50	10.50
3	*Simarouba amara* Aubl.	Simaroubaceae	1.62	Canopy	29.00	8.35
4	*Ruizodendron ovale* R.E. Fr. (Ruiz & Pav.)	Annonaceae	1.23	Canopy	32.62	10.07
6	*Couratari macrosperma* A.C. Sm.	Lecythidaceae	1.43	Emergent	33.74	6.27
8	*Bertholletia excelsa* Bonpl.	Lecythidaceae	2.30	Emergent	34.00	11.60
10	*Clarisia racemosa* Ruiz & Pav.	Moraceae	1.58	Canopy	33.75	9.25
12	*Gautteria boliviana* H. Winkl.	Annonaceae	1.17	Canopy	31.90	11.34
13	*Pouteria franciscana* Baehni	Sapotaceae	2.37	Canopy	35.39	15.09

DBH, diameter at breast height

Monitoring canopy and emergent tropical trees *in situ* presents considerable challenges. Canopy walkways and towers, where they exist, do not provide sufficient access across a large number of crowns. Canopy cranes are ideal for such sampling, but there are only a few across the tropics. Hence, we resorted to repeated climbing and sampling of a number of large trees. The total number of trees sampled in this study was limited by the need to sample structurally sound trees large enough to withstand the repeated climbing required to monitor their leaf life-cycle and sample leaves of different ages, and the difficulty involved in climbing large trees with ropes to minimise damage caused by repeated climbing. We therefore, chose to maximise variability within our leaf dataset by sampling 8 trees, each a different species, with a wide variety of leaf types

(e.g. compound and simple leaves), leaf sizes and shapes, and leaf thickness and waxiness. This sampling allowed us to examine the trait and spectral variability due to natural leaf aging in a tropical canopy tree community with diverse leaf properties.

Trees were monitored frequently for bud development, new leaf emergence and development, general leaf condition and signs of leaf senescence. We sampled the developmental, mature and senescent leaf life-cycle phases for all eight trees. During leaf development, individual trees were sampled on a weekly basis from early leaf development (after one week of active leaf expansion) until most leaf area expansion had been achieved (up to four consecutive weeks after active leaf expansion). During the mature leaf phase, individual trees were sampled on a monthly basis at different stages (i.e. newly matured, mid-maturity and end of maturity) for up to three months. Similarly, during the senescent leaf phase, individual trees were sampled at different stages (i.e. old leaves not yet abscising and senescent leaves starting abscission through to advanced senescence when most leaves had been abscised from the tree crown) for up to two months for old leaves and up to three months for senescent leaves. Example images and a detailed description of the leaf classification used in this study can be found in Figure 1.

Two top-of-canopy (fully exposed to direct sunlight) apical metre-long branches were collected each time trees were sampled. Sampled

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branches were cut, labelled, promptly lowered to the ground and recut under water to maintain hydraulic connectivity and minimise leaf desiccation during transport to the field lab, where leaf spectral and morphological (fresh leaf weight and area) measurements were carried out within two hours of the branches being cut. Demographic leaf counts were also collected for all sampled branches.
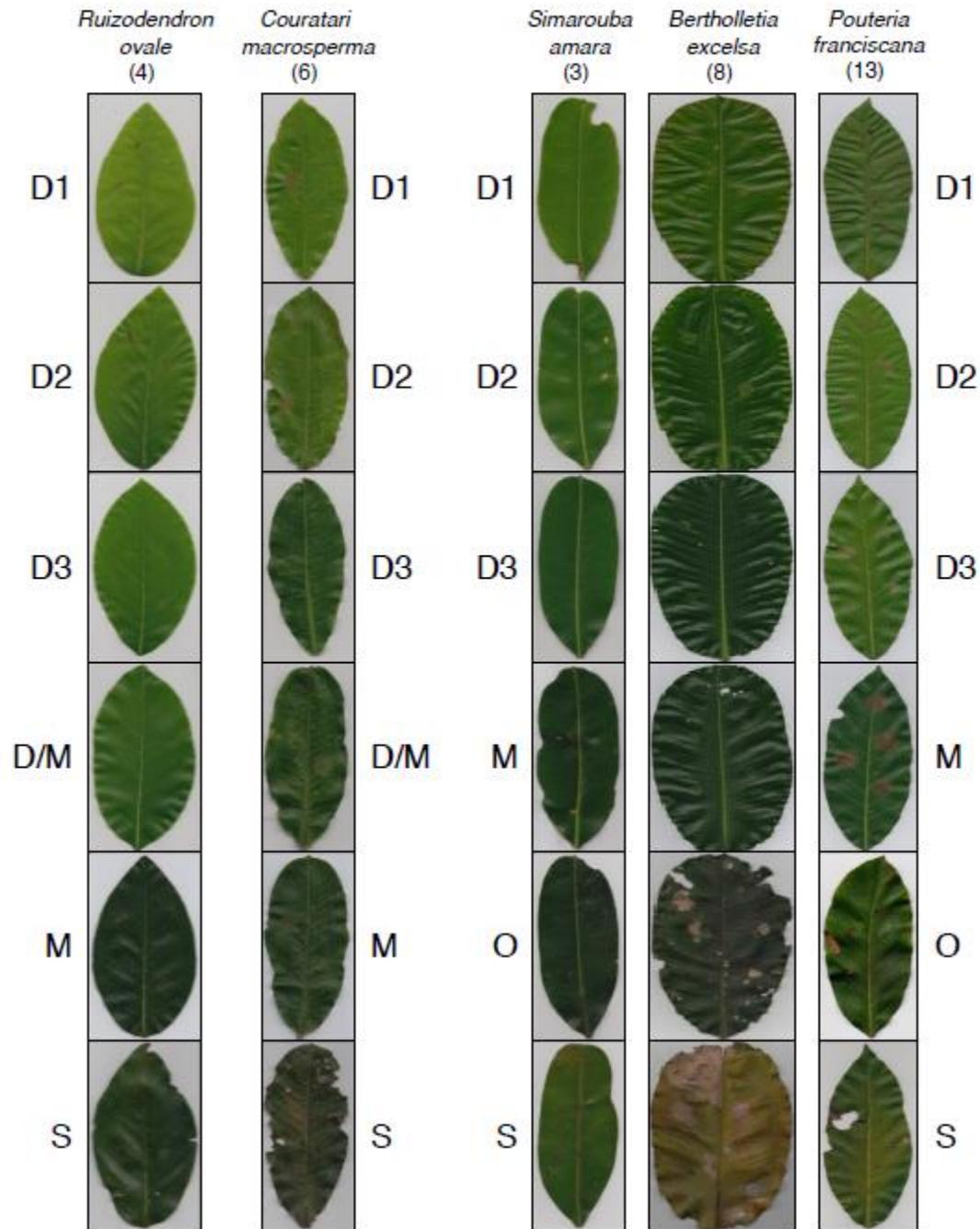
Figure 1 **Examples of leaves of different age classes, illustrating the leaf life-cycle of some of our sampled trees (tree codes in brackets). Leaf age classification: D1, D2, D3 and D4 are developing leaves in the process of gaining area, thickness and green colouration and collected after 1, 2, 3 and 4 weeks of active leaf expansion, respectively; M are fully-grown and structurally developed mature leaves that have achieved a fully darkened green colour; O are old leaves showing initial signs of epiphylls and/or senescence and near the end of their life cycle (~2 months before senescence and abscission) and;**

S are senescent leaves displaying loss of chlorophyll and/or onset of decomposition and in the process of dying and actively abscising.

1.2.3 Leaf spectroscopic and trait measurements

We randomly selected 15 to 30 leaves from each sampled leaf age class for spectral analysis. Leaves were cut (at base of petiole) from their branch just before reflectance measurements were collected. Measurements were collected using a field spectrometer (Analytical Spectral Devices FieldSpec Pro, Boulder, Colorado, USA) with a spectral range of 350-2500 nm and sampling intervals of 1.4 nm from 350-1000 nm and 2 nm from 1000-2500 nm. The spectrometer was fitted with a contact probe (Analytical Spectral Devices High Intensity Contact Probe, Boulder, Colorado, USA) which has its own calibrated light source designed to minimise measurement errors associated with stray light. To ensure measurement quality, the spectrometer was optimised every 5 minutes, spectra for every leaf was white referenced to a calibration panel (Spectralon, Lasphere, Durham, New Hampshire, USA) and an average of 25 spectrum, dark current and white reference measurements were used to improve the signal-to-noise ratio of the spectra. Furthermore, leaves were placed on a custom-designed measurement block covered in a low reflective (3%) material with a firm cushion fitted underneath to allow for the contact probe to be pressed against the leaf without causing damage, while ensuring that no light escaped the measurement. Four reflectance measurements from different sections of the leaf were collected and averaged for each leaf. This protocol delivered high-quality calibrated

spectra that did not require smoothing or other filters. The full-range leaf spectral data were trimmed at the far ends (<412nm and >2450nm).

In addition to classifying leaves by their age, we recorded the presence of epiphylls (which was always in very low abundance) and/or necrosis for each leaf surface we measured. To account for potential effects of leaf coatings and natural leaf damage on spectra, we only used reflectance measurements free of epiphylls and necrosis.

We chose LMA, LWC, P, N and C content as the ensemble of leaf traits to measure. Each of these traits is important to leaf physiological and ecological functioning (Evans and Poorter 2001; Field and Mooney 1986b; Kokaly et al. 2009; Poorter et al. 2009; Raaimakers et al. 1995; Reich et al. 2008; Westoby et al. 2002) and have demonstrated contributions to the spectral signatures of vegetation (Asner et al. 2009; Curran 1989). Carbon content (C) in leaves, as analysed in this and other spectroscopy studies, includes two functionally different components - non-structural and structural carbon. Non-structural carbon (i.e. sugars, starch and pectin) is produced and mainly stored in leaves and constitutes the mobile energy store of plants (Chapin et al. 1993.; Evans 1989), while structural carbon (i.e. lignin and cellulose) is generated to support strength and longevity, and to decrease palatability to herbivores (Melillo et al. 1982).

All sampled leaves were measured for LMA and LWC. For LMA and LWC calculations, leaf petioles were removed and each sampled leaf was then measured for fresh mass, using a high-precision balance with a 0.01g resolution (Durascale, My Weigh Europe, Erkelenz, Germany). Groups of individually identified leaves were then colour scanned at 300 dpi resolution. ImageJ software (NIH, New York City, NY, USA) used to calculate individual leaf areas. The scanned leaves were then individually placed in labeled paper bags and dried at 70°C for 72 hours before leaf dry mass was determined. LWC_{mass}, LWC_{area} (commonly known as "equivalent water content") and LMA were derived from these data as follows:

$$LWC_{mass} = \left(\frac{\text{fresh mass} - \text{dry mass}}{\text{fresh mass}} \right) \times 100$$

$$LWC_{area} = \left(\frac{\text{fresh mass} - \text{dry mass}}{\text{area}} \right)$$

$$LMA = \frac{\text{dry mass}}{\text{area}}$$

where leaf fresh and dry mass are in grams (g) and leaf area in square metres (m^{-2}).

For *Simarouba amara,* a species with compound leaves, leaf trait values were measured on independent leaflets rather than the entire compound leaf as the leaflet green leaf portion is comparable to that measured in whole leaves for other species.

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Leaf nutrient content was measured at the Department of Geosciences Environmental Isotope Laboratory at the University of Arizona. For each tree, five dry leaves out of each leaf age class were randomly selected for chemical analysis. All dry leaf samples were then homogenised and ground to a fine powder. Phosphorous (P), nitrogen (N) and carbon (C) contents were measured using a continuous-flow gas-ratio mass spectrometer (Finnigan Delta Plus XL, Thermo Fisher Scientific, Madison, WI, USA) coupled to an elemental analyzer (Costech Analytical Technologies, Valencia, CA, USA). Samples of 1.0 mg (± 0.1mg) were combusted in the elemental analyser. Precision is at least ±0.1mg based on repeated internal standards. Both mass based and area-based measurements were calculated for LWC, P, N and C content.

1.2.4 Experimental design

We used bootstrapping to produce 10 subsets of 60 leaf samples using leaves from each of four different leaf age groups (i.e. developmental (D), mature (M), senescent (S) and mixed-age (All)). These subsets were then used to develop both mass- and area-based PLSR models for the leaf traits under analysis (i.e., LMA, LWC_{mass}, LWC_{area}, P_{mass}, P_{area}, N_{mass}, N_{area}, C_{mass} and C_{area}). A schematic detailing how we developed different sets of 10 PLSR models per leaf age group-trait combination (i.e. 10 models for each of the sampled leaf traits per leaf age group) can be seen in Figure 2.
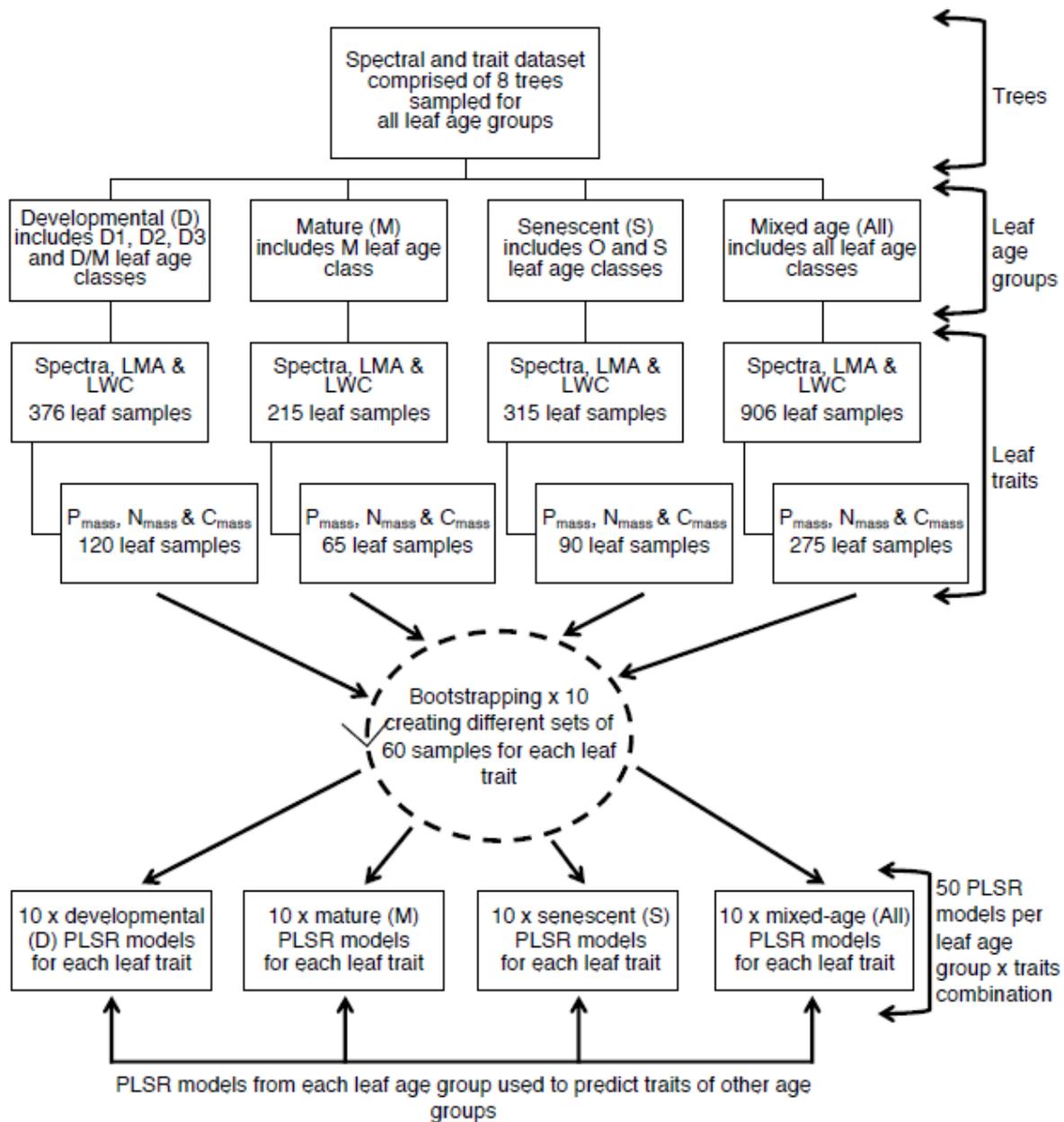
Figure 2 **Data subsets used to develop mass based partial least squares regression (PLSR) models for different leaf age groups. The same methodology was used to develop area-based PLSR models for LWC, P, N and C content. PLSR models "All" were developed using an equal number of samples from each of the three different leaf age groups.**

We used chemometric PLSR models to link leaf level reflectance spectra and trait measurements. PLSR is the standard statistical approach utilised in chemometric analyses and is designed to handle high predictor collinearity and situations where the number of predictor variables (2039 wavelengths in this study) is much higher than the number of observations. PLSR reduces the large predictor matrix down to a relatively few, uncorrelated latent factors. As in Asner *et al.* (2015; 2014; 2011b) and Serbin *et al.* (2014), we performed a one-time randomised 70/30 split of each data subset into calibration and independent testing sets. We adopted a 100 × permutation 10-fold cross validation (i.e. 1000 total permutations in which the calibration data set was further subdivided into 90% for calibration and 10% for validation) for each PLSR model. We evaluated the performance of PLSR models using two main metrics: R^2 (i.e. precision) and root mean square error (RMSE, i.e. accuracy) (Asner et al. 2011a; Hodgson and Bresnahan 2004; Lin and Torbeck 1998; Walther and Moore 2005). We identified the optimal number of latent factors for each of the 10 leaf trait per age class models by minimising RMSE and maximising R^2. The number of optimal factors varied between 4-6 for LMA models of different leaf age groups and between 6-8 and 5-8 factors for all other traits on a mass and area basis, respectively. We therefore, standardised to 6 latent factors for all LMA models and to 8 factors for all other leaf trait models.

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PLSR analyses were performed using custom functions along with the MCS function from LibPLS (http://www.libpls.net) and the PLSREGRESS function in Matlab (Version R2014a, Mathworks Inc., Natick, MA, USA).

We report the trait summary statistics for the four leaf age group data subsets used to develop these models, the mean results (R^2 and RMSE) of the 10 PLSR models for each leaf age group-trait combination and the standardised number of PLSR latent factors used to predict leaf traits across all leaf age groups. To evaluate the robustness of the PLSR models across leaf age groups, we used the 10 models from each group to predict the traits of leaves from the other three age groups. We report the R^2; RMSE as a percentage of the sample mean (i.e. %RMSE); mean spectral coefficients and their correlations between leaf age groups; and the mean relative importance of each variable (wavelength) in predicting leaf traits (VIPs) of the 10 mass based PLSR models for each leaf age group-trait combination. Wavelengths showing VIP values greater than one are considered important (Wold et al. 2001). Additionally, results of sensitivity analyses show stable R^2 and RMSE values beyond leaf sample sizes of 60 (Figure S1). Model accuracy (RMSE) showed less sensitivity to sample size than model precision (R^2).

Finally, we explored the leaf traits, spectral range and coefficients of variation (CV) of the different leaf age groups to further elucidate the

impact of leaf age on PLSR models. Tukey's HSD tests were used to compare the leaf trait means for the different leaf age groups.

1.3 Results

Both C_{mass} and P_{area} remained more or less stable throughout the leaf life cycle (i.e. showed similar trait range and means across leaf age groups), while other mass based traits displayed negative relationships and area-based traits displayed both negative (LWC_{area}) and positive (LMA, N_{area} and C_{area}) relationships with age (Figure 3, Tables 2 & 3). Mean LMA and C_{area} significantly increased between consecutive leaf age groups (p= <0.01 for both D-M and M-S leaf age group comparisons), while mean LWC, LWC_{area} and P_{mass} significantly decreased (p= <0.0001 for both D-M and M-S leaf age group comparisons). Interestingly, both leaf N and C content displayed different relationships with age depending on their measurement basis with LMA modulating area-based trait relationships with age. P_{area} and C_{mass} remained stable while P_{mass} and C_{area} showed negative and positive relationships with age, respectively; N_{mass} displayed a negative relationship while N_{area} showed a positive relationship with age. In terms of leaf age groups, the developmental (D) group displayed the largest trait ranges and covered most of the ranges shown by the mature (M) and senescent (S) leaf age groups while the M group had the smallest trait ranges for most leaf traits.

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Leaf reflectance spectra displayed differences in magnitude and shape between leaf age groups (Figure 4). Upper bounds of visible reflectance (VIS; spectral domain: 390-700 nm) where highest for the developmental leaf age group and closely followed by the senescent age group while lower bounds displayed similar values across leaf age groups. Age-induced reflectance differences in this spectral domain were especially pronounced at 550 nm (green peak). NIR reflectance (spectral domain: 700– 1400 nm) displayed opposite patterns to VIS. While upper bounds in NIR reflectance displayed similar values across leaf age groups, differences in lower bounds were pronounced with the senescent leaf age group showing a collapse between 700– 850 nm and a general flattening across the NIR spectral domain. Shortwave-infrared (SWIR; spectral domain: 1450– 2500 nm) reflectance displayed similar patterns to VIS (Figure 4). Upper bounds in SWIR displayed the largest differences between leaf age groups with the senescent age group showing significantly higher upper bounds. These were most pronounced around structural carbon absorption features around 2000–2250 nm. Finally, the Red-Edge (spectral domain: 650– 810 nm) also showed differences between leaf age groups with the widest amplitude displayed by the senescent age group.

We also observed age effects in terms of reflectance variability (measured as coefficients of variation or CVs; Figure 4). The developmental leaf age group displayed the highest variability across most of the spectrum (10-55%)

followed by the senescent group (8-41%) with the mature age group showing the lowest variability (7-33%). The largest differences in variability between leaf age groups were observed across the VIS, Red Edge, NIR and at the main water features around 1450 and 1940 nm.
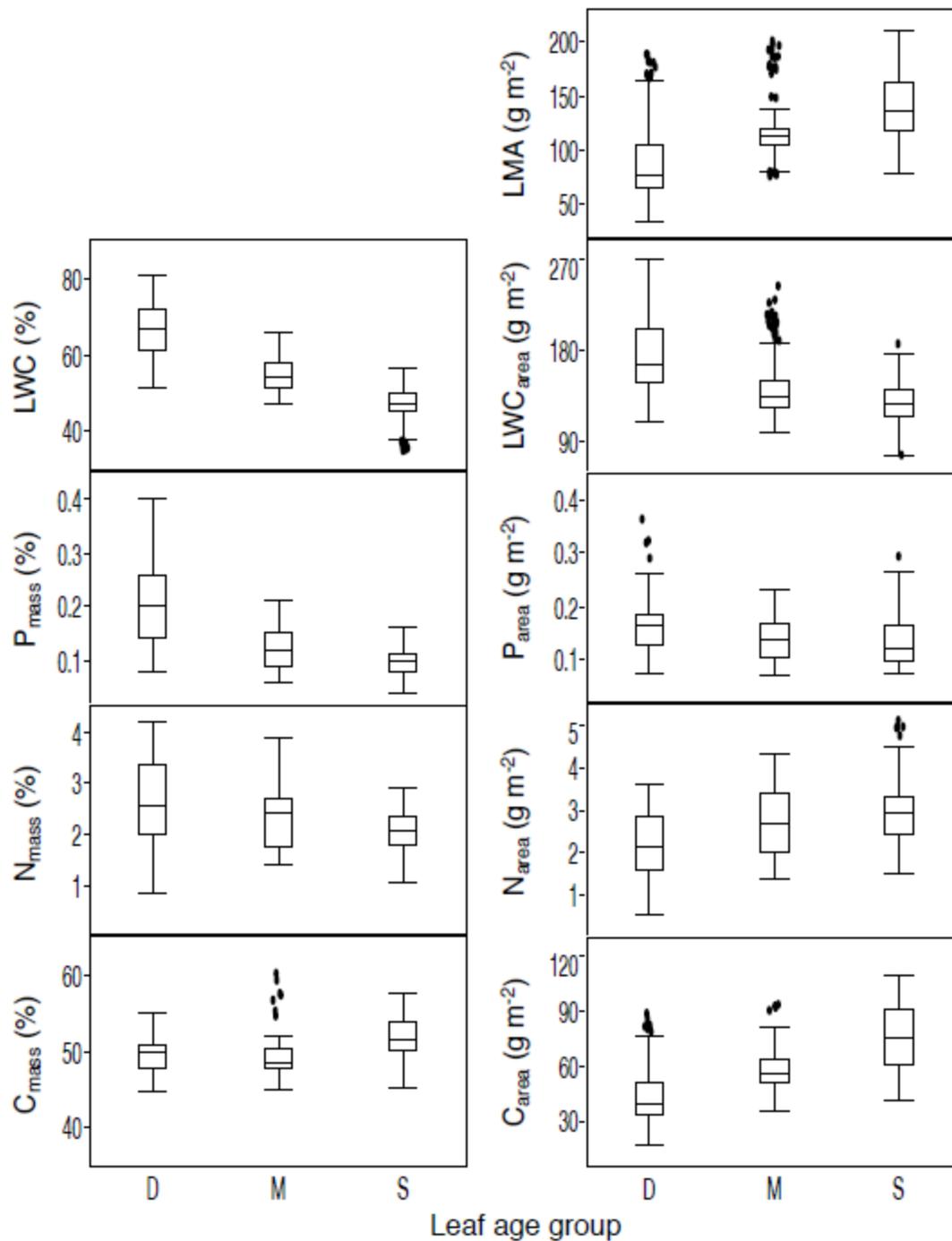
Figure 3   **Leaf age group summary trait statistics for LMA and LWC, P, N and C
content on both a mass and area basis. Leaf age groups: D= developmental,
includes leaves after one to four weeks of active expansion; M= mature,
includes newly matured, mid and end of maturity leaves; and S= senescent,
includes old leaves showing initial signs of senescence and senescent leaves
starting to abscise through to advanced senescence (most leaves abscised).
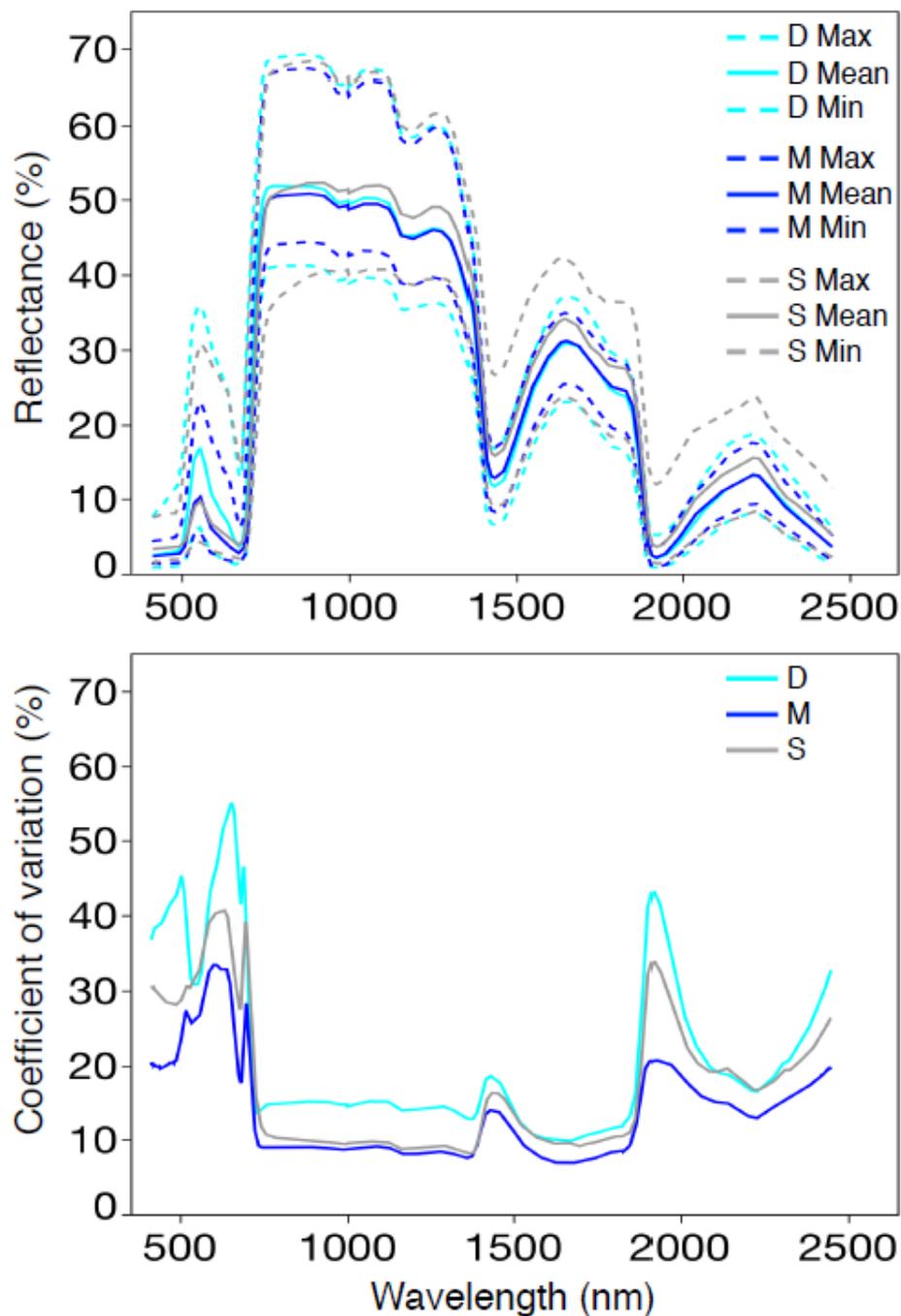Black dots are outliers.**

Figure 4   **Spectral summary statistics by leaf age group. Leaf age groups: D=
developmental, includes leaves after one to four weeks of active expansion; M=
mature, includes newly matured, mid and end of maturity leaves; and S=
senescent, includes old leaves showing initial signs of senescence and
senescent leaves starting to abscise through to advanced senescence (most
canopy leaves abscised). The coefficient of variation represents variation
between samples of the same age class.**

We examined the robustness of PLSR models developed on both a mass and area basis and when using leaves from different leaf age groups (developmental (D), mature (M), senescent (S) and mixed-age (All)) to predict (1) individual leaf traits across different leaf age groups and (2) individual leaf traits within leaf age groups.

We found that except for the senescent leaf age group, mass based traits were predicted in the same descending order of precision (i.e. R^2): $LWC_{mass}>P_{mass}>N_{mass}>C_{mass}$ (Table 2). However, area-based trait predictions showed no discernable pattern and generally displayed higher prediction precision and similar accuracy (i.e. RMSE) to mass based models (Tables 2 & 3). Among all traits, LMA and C_{area} displayed the highest prediction precisions across all leaf age groups (R^2= 0.94-0.96 and %RMSE= 5-8; 0.93-0.97 and %RMSE= 5-8, respectively; Table 3). LWC showed high and consistent prediction precision and accuracy between mass- and area-based models and although lower prediction precisions were observed for leaf N content, this trait also displayed consistent predictions between mass- and area-based models. However, striking contrasts in prediction precision between mass- and area-based models were observed for leaf C content. While C_{mass} displayed the lowest prediction precision across all traits and leaf age groups (Table 2), C_{area} showed some of the highest precisions (Table 3). Furthermore, age-specific differences in performance for both mass- and area-based models were observed. For example, P_{area} showed a drop in prediction precision

of 9-15% and 11-17% for developing and mixed-age leaf age groups, respectively, compared to the other three leaf age groups (Table 3) and LWC$_{mass}$ displayed a drop 11-12% for the senescent leaf age group (Table 2).

Table 2   **Leaf trait summary statistics on a mass basis and mean PLSR modelling results by leaf age group. PLSR modelling results include the standardised number of PLSR latent factors used to predict leaf traits across all leaf age groups and the standardised number of samples used to build these models. The total number of leaf samples available for each leaf age group is provided in brackets.**

Leaf trait		Leaf age groups			
		D (8 trees)	M (8 trees)	S (8 trees)	All (8 trees)
LWC_{mass} (%)	Mean (SD)	67 (6)	53 (4)	47 (4)	57 (10)
	Min-Max	51 - 81	44 - 62	35 - 57	35 - 81
	N°	60 (376)	60 (215)	60 (315)	60 (906)
	R^2	0.92	0.91	0.80	0.92
	RMSE	1.95	1.40	1.72	2.62
	% RMSE	3	3	4	5
	Factors	8	8	8	8
P_{mass} (%)	Mean (SD)	0.19 (0.07)	0.11 (0.03)	0.09 (0.03)	0.15 (0.07)
	Min-Max	0.08 – 0.40	0.06 – 0.18	0.04 – 0.16	0.04 – 0.40
	N°	60 (120)	60 (65)	60 (90)	60 (275)
	R^2	0.91	0.85	0.87	0.89
	RMSE	0.02	0.02	0.01	0.02
	% RMSE	10	12	9	13
	Factors	8	8	8	8
N_{mass} (%)	Mean (SD)	2.73 (0.84)	2.19 (0.55)	2.05 (0.41)	2.37 (0.72)
	Min-Max	0.85 – 4.19	1.40 – 3.68	1.07 – 2.89	0.85 – 4.19
	N°	60 (120)	60 (65)	60 (90)	60 (275)
	R^2	0.87	0.84	0.76	0.76
	RMSE	0.28	0.23	0.21	0.36
	% RMSE	10	9	10	14
	Factors	8	8	8	8
C_{mass} (%)	Mean (SD)	49.87 (2.07)	50.68 (4.18)	51.59 (3.24)	50.28 (2.91)
	Min-Max	44.85 – 55.13	45.04 – 60.14	45.27 – 57.71	44.85 – 60.14
	N°	60 (120)	60 (65)	60 (90)	60 (275)
	R^2	0.68	0.66	0.70	0.58
	RMSE	1.08	2.57	1.91	1.89
	% RMSE	2	5	3	4
	Factors	8	8	8	8

Table 3 **Leaf trait summary statistics on an area basis and mean PLSR modelling results by leaf age group. PLSR modelling results include the standardised number of PLSR latent factors used to predict leaf traits across all leaf age groups and the standardised number of samples used to build these models. The total number of leaf samples for each leaf age group is provided in brackets.**

Leaf trait		Leaf age groups			
		D (8 trees)	M (8 trees)	S (8 trees)	All (8 trees)
LMA $(g\ m^{-2})$	Mean (SD)	90.69 (33.52)	123.83 (26.03)	141.45 (31.78)	116.61 (38.39)
	Min-Max	34.60 – 187.39	76.07 – 199.67	79.42 – 210.49	34.60 – 210.49
	Nº	60 (376)	60 (215)	60 (315)	60 (906)
	R^2	0.96	0.94	0.96	0.94
	RMSE	5.89	6.09	6.52	9.15
	% RMSE	6	5	5	8
	Factors	6	6	6	6
LWC_{area} $(g\ m^{-2})$	Mean (SD)	173.76 (35.4)	143.39 (29.3)	127.46 (20.94)	150.56 (36.06)
	Min-Max	109.21 – 270.38	98.77 – 243.39	74.95 – 185.81	74.95 – 270.38
	Nº	60 (376)	65 (215)	60 (315)	60 (906)
	R^2	0.92	0.92	0.87	0.92
	RMSE	10.04	6.06	7.63	13.02
	% RMSE	6	4	6	9
	Factors	8	8	8	8
P_{area} $(g\ m^{-2})$	Mean (SD)	0.16 (0.05)	0.14 (0.04)	0.13 (0.05)	0.15 (0.05)
	Min-Max	0.07 – 0.36	0.07 – 0.23	0.07 – 0.29	0.07 – 0.36
	Nº	60 (120)	60 (65)	60 (90)	60 (275)
	R^2	0.73	0.81	0.87	0.70
	RMSE	0.02	0.02	0.02	0.03
	% RMSE	6	15	8	13
	Factors	8	8	8	8
N_{area} $(g\ m^{-2})$	Mean (SD)	2.20 (0.74)	2.73 (0.8)	2.96 (0.75)	2.56 (0.83)
	Min-Max	0.52 – 3.61	1.36 – 4.34	1.51 – 5.1	0.52 – 5.1
	Nº	60 (120)	60 (65)	60 (90)	60 (275)
	R^2	0.88	0.89	0.82	0.78
	RMSE	0.24	0.26	0.26	0.37
	% RMSE	10	9	9	12
	Factors	8	8	8	8
C_{area} $(g\ m^{-2})$	Mean (SD)	44.47 (16.96)	58.56 (13.35)	75.75 (17.93)	57.78 (21.45)
	Min-Max	16.53 – 88.18	36.21 – 92.98	41.08 – 108.55	16.53 – 108.55
	Nº	60 (120)	60 (65)	60 (90)	60 (275)
	R^2	0.97	0.93	0.96	0.94
	RMSE	3.25	4.28	3.64	5.24
	% RMSE	7	7	5	8
	Factors	8	8	8	8

When we analysed the performance of both mass- and area-based PLSR models developed using one leaf age group to predict the traits of the other three leaf age groups, we found that except for P_{area}, area-based models significantly and consistently outperformed mass based models (Figures 5 & 6). Overall, for both mass- and area-based traits, mixed-age models (All) displayed the best performance and mature models the worst performance when predicting the traits of other leaf age groups (Figures 5 & 6). Significantly, when predicting leaf traits for the mixed-age group (All; Figures 5 & 6) the developmental models (D) showed the best performance in terms of both precision and accuracy, however, precision still dropped by 24 and 26% when the D-model (instead of the S-model), was used to predict the chemical leaf traits P_{area} and C_{mass}, respectively. We also found that models displayed trait-specific age sensitivity. Our results show that only two traits -LMA and C_{area}- were predicted with consistently high precision (≥ 70 across leaf age groups) and accuracy (mostly ≤ 12 across leaf age groups) regardless of the age model used (Figure 6 a-d & q-t). In contrast, C_{mass} models displayed the worst overall performance with large decreases in prediction precision ($R^2 = 36$-53%) and some age models unable to predict C_{mass} for leaves of other leaf age groups (Figure 5 m-p). P_{area} models where the only area-based models that underperformed compared to mass based models, however, these results were mostly limited to the developmental and mixed-age leaf age groups. For developmental leaves, mass- and area-based PLSR models built using mature leaves (M) displayed the worst performance for four out of five leaf traits, and both M- and S-models unable to predict C_{mass} for this leaf age group (Figures 5

& 6 a, e, i, m, q). For mature leaves, S-models showed the worst overall performance with S-models unable to predict C$_{mass}$ **for this leaf age group (**Figures 5 **& 6 b, f, j, n, r). For senescent leaves, M-models displayed the worst overall performance with M-models unable to predict N**$_{mass}$ **and C**$_{mass}$ **for this leaf age group (**Figures 5 **& 6 c, g, k, o, s). For mixed-age leaves, M-models displayed the worst overall performance, however, S-models were unable to predict C**$_{mass}$ **for this leaf age group (**Figures 5 **& 6 d, h, i, p, t).**

We also found differences in spectral PLSR coefficients and VIPs due to leaf age (Figure 7). Only correlations between coefficients for LMA models were found to be consistently high across leaf age groups, with correlations also high between all leaf age groups for the four other traits on both a mass and area basis (Figure S2).
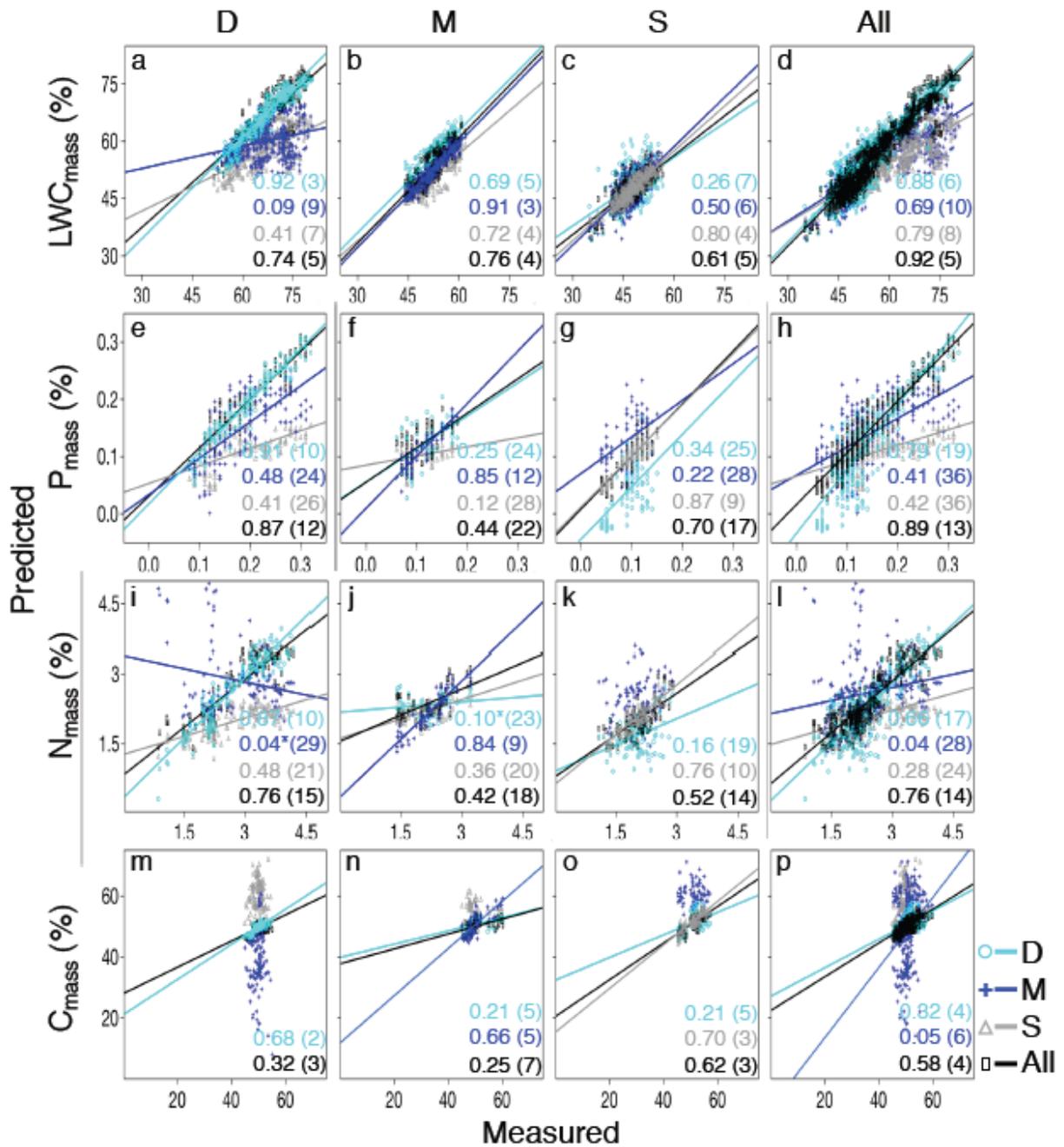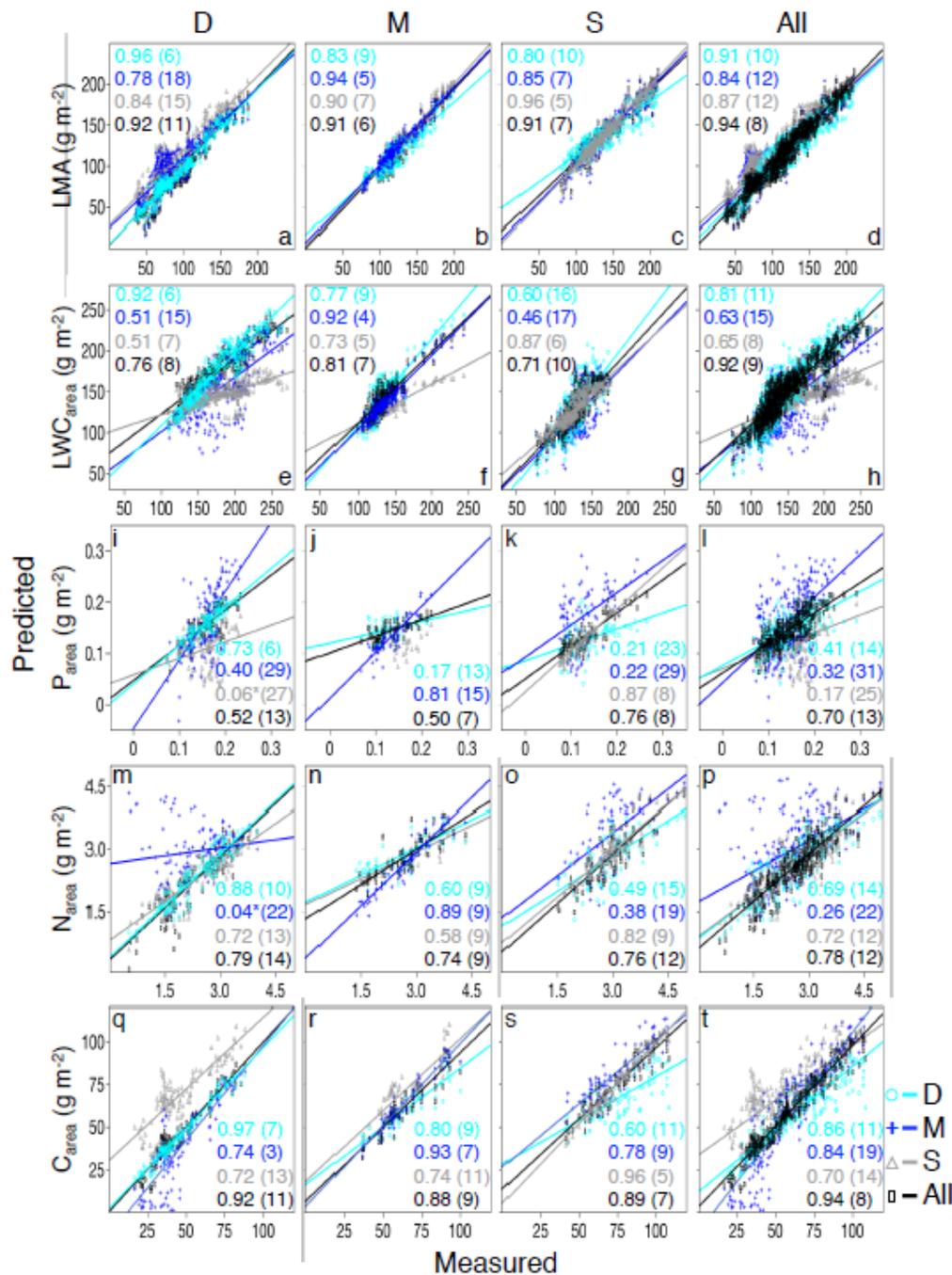
Figure 5   **Partial least squares regression (PLSR) results for measured vs spectrally predicted leaf traits on a mass basis. Leaf traits (LWC$_{mass}$, P$_{mass}$, N$_{mass}$ and C$_{mass}$) for the four different leaf age groups (columns left to right: D= developmental, M= mature, S= senescent and All= mixed-age) were spectrally predicted using PLSR models calibrated with leaves of the different leaf age groups. For leaves of each leaf age group, we report R^2 and (%RMSE) for the four different PLSR leaf age models: Developmental model= light blue, Mature model= dark blue, Senescent model= grey and Mixed-age model= black. Non-significant regressions are not reported, all significant regression *P* values= <0.0001, except for * were *P* value= 0.03.**

Figure 6 **Partial least squares regression (PLSR) results for measured vs spectrally predicted leaf traits on an area basis. Leaf traits (LMA, LWC_{area}, P_{area}, N_{area} and C_{area}) were spectrally predicted for the four different leaf age groups (columns left to right: D= developmental, M= mature, S= senescent and All= mixed-age) using PLSR models calibrated with leaves of the different leaf age groups. For leaves of each leaf age group, we report R^2 and (%RMSE) for the four different PLSR leaf age models: Developmental model= light blue, Mature model= dark blue, Senescent model= grey and Mixed-age model= black. Non-significant regressions are not reported, all significant regression P values= <0.0001, except for * were P value= 0.03.**
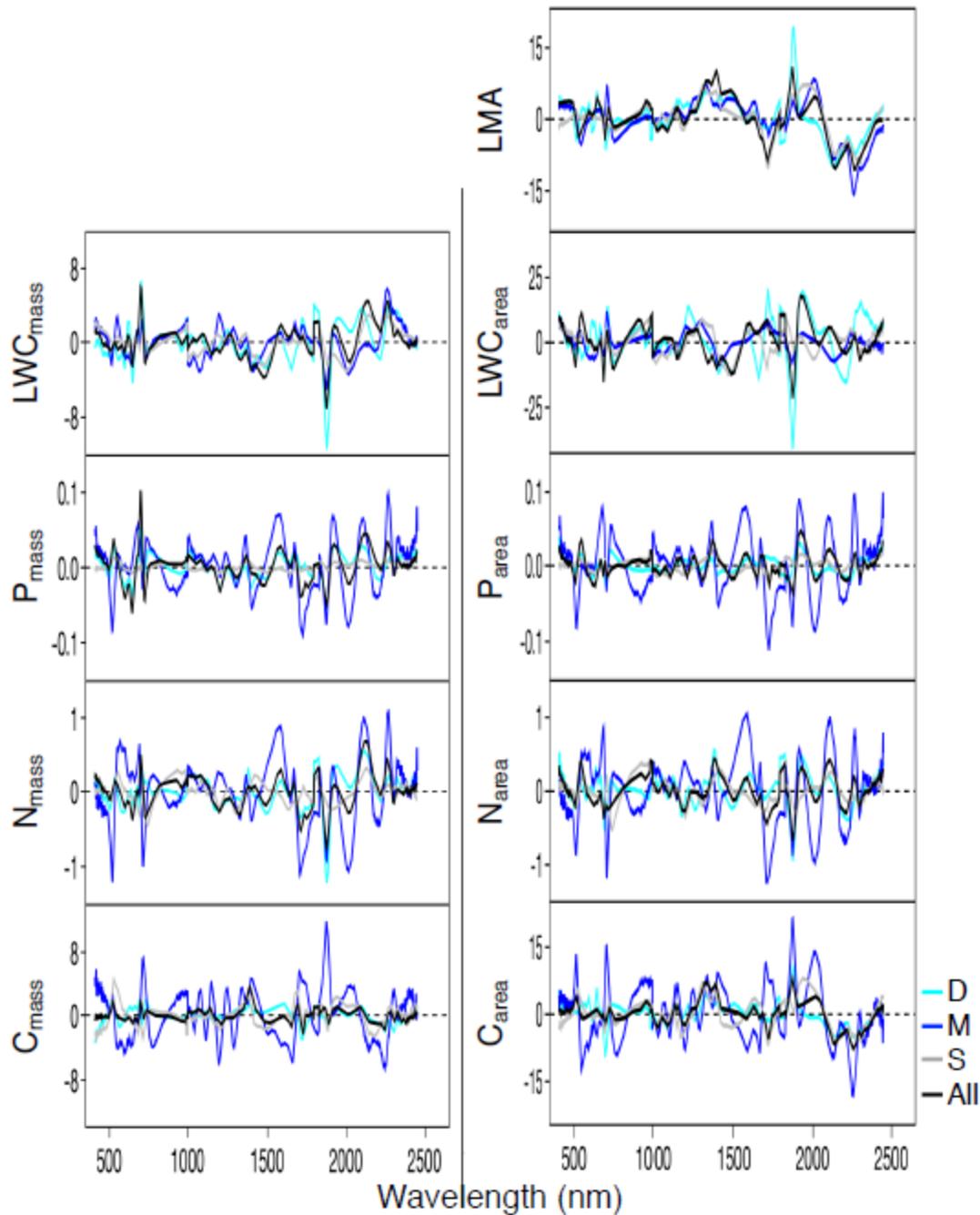
Figure 7   **Partial least squares regression (PLSR) coefficients by leaf age group. Leaf traits: leaf mass per unit area (LMA), leaf water content (LWC$_{mass}$; LWC$_{area}$), phosphorous (P$_{mass}$; P$_{area}$), nitrogen (N$_{mass}$; N$_{area}$) and carbon (C$_{mass}$; C$_{area}$) content. Leaf age groups: D= developmental (light blue), M= mature (dark blue), S= senescent (grey) and All= mixed-age (black).**

1.4 Discussion

To our knowledge, our study is the first to investigate the relative effect of leaf age on the PLSR models used to spectrally predict traits important to leaf physiological and ecological functioning. PLSR models have been traditionally developed on a mass basis as this is the standard unit used in studies of leaf traits, plant physiology and economy, and ecosystem function. However, spectral reflectance is inherently an area-based measurement. Furthermore, as contrasting and combining leaves of different age groups (i.e., developmental, mature, and senescent) could result in some leaf samples sharing similar values of constituent content per mass unit while having very different morphological and optical properties, we also developed and tested the effect of leaf age on the performance of area-based PLSR models.

1.4.1 Does the robustness of spectral PLSR models vary with leaf age and does leaf age affect model performance when predicting different leaf traits?

We found that within each leaf age group, the four individual leaf traits we analysed on a mass basis were largely predicted in the same descending order of precision: $LWC>P_{mass}>N_{mass}>C_{mass}$. These results are comparable with previous studies using PLSR approaches (Asner et al. 2011a; Asner et al. 2011b; Richardson and Reeves 2005; Serbin et al. 2014), and more importantly, indicate that mass based leaf trait-spectra relationships are stable within the different leaf life-cycle stages (i.e. developmental, mature

and senescent). Area-based models displayed no obvious pattern and mostly outperformed mass based models with LMA and C_{area} models displaying the highest prediction precision (>90%) among all traits analysed and also excellent accuracy ($\leq$8) (Table 3),

We also observed interesting differences in trait-specific age effects between mass- vs area-based PLSR models. Significantly, our results show that area-based models may be less sensitive to age effects. We found that three out of the five traits we analysed on an area basis -LMA, C_{area}, LWC_{area}- displayed no age effects, while only one out of four mass based traits -P_{mass}- showed no such effects. LMA and C_{area} were not only predicted with the highest precision among all traits but also consistently across all leaf age groups (R^2= 0.94-0.96 and 0.93-0.97 and %RMSE= 5-8 for both traits, respectively; Table 2). Although LWC_{area} and P_{mass} models displayed lower prediction precisions than LMA and C_{area} across leaf age groups (R^2= 0.87-0.92 and 0.85-0.92 and %RMSE= 4-9 and 9-13, respectively; Table 2), these were also consistent across age groups (5 and 2-3% differences in R^2 and %RMSE between age groups for LWC_{area}, and 2-6 and 1-4% for P_{mass}, respectively).

The contrast in our results between C_{mass} and C_{area} models are particularly striking as leaf C content is a trait that has been shown within the PLSR literature to be difficult to predict on a mass basis (see Asner et al., 2014, 2011a, 2009; Serbin et al., 2014). C_{mass} may be a particularly challenging

trait to predict as total C_{mass} remains more or less stable throughout the leaf life-cycle (see Figure 3) while the ratio of the two different carbon constituents (non-structural and structural carbohydrates) changes: at the beginning of the leaf life-cycle, leaf C content is mostly comprised of non-structural carbon (sugars and starch) used to fuel leaf development, while in senescent leaves, it is mostly comprised of structural C (lignin and cellulose). Correspondingly, the significant loss in prediction precision in C_{mass} models for leaves of mixed age (All) is likely the result of (1) the anti-correlation of spectral features of non-structural and structural carbon (Asner et al. 2015) and (2) a predicted variable (C_{mass}) that remains more or less stable while the predictor variables (leaf spectra) change significantly with age. However, C content measured on an area basis (C_{area}) captures the age-related changes in this trait (which shows a positive relationship with age, see Figures 3), and therefore, offers an alternative approach for estimating leaf C content with a high degree of precision from spectroscopic data. Similarly, the lower prediction precisions we observed for developmental and mixed-age (All) P_{area} models are likely to be related to the lack of change displayed in this trait with age on an area basis.

Models for LWC_{mass} displayed a significant decrease in precision of 11-12 % for the senescent leaf age group (S; Table 2) while a less pronounced decrease was observed for LWC_{area} (4-5%). This decrease in model precision may be due to a diminished contribution of LWC (relative to the

contribution of intercellular leaf structure and biochemical leaf traits) to the optical signal in the NIR (700-1400nm) and SWIR (1400-2500nm) spectral regions as a result of the significantly lower mean LWC found in senescent leaves, whether expressed on a mass or area basis. Mixed age effects in both of these spectral regions due to intercellular leaf structure and biochemical features could have also contributed to the observed decrease in prediction precision. NIR and SWIR are directly correlated to LWC with strong water absorption features centred around 970, 1200, 1450 and 1940nm (Figure 4). However, NIR is also indirectly correlated to LMA as reflectance in this spectral region is determined by the complex intercellular structure of leaves (i.e. the number of air-cell wall-water interfaces within leaves) for which LMA is a proxy, while SWIR is both directly and indirectly correlated to biochemical features of nitrogen, phosphorus, proteins and non-structural and structural carbon constituents. The age effects we observed for N_{mass} and N_{area} models are more difficult to unravel. N_{mass} models showed lower prediction precisions for both senescent (S) and mixed-age (All) leaf age groups while N_{area} models displayed lower precisions for the mixed-age leaf age group. These results could be the result of tree-specific relationships with leaf age displayed by these traits as previously reported in Chavana-Bryant *et al.* (2017).

Our findings suggest that using area-based PLSR models to predict leaf LWC, N and C content may avoid the trait-specific age effects we

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observed for these traits on a mass basis. However, the prediction decreases we observed in both mass- and area-based models of mixed-age (All) leaf age groups point to the challenge of consolidating the combined age-related changes in leaf properties that are captured by spectral signatures (Figure 4) with trait specific behaviours, in particular, when leaves of different ages display similar values of constituent content. This effect is revealed by the lack of change displayed in P_{area} and C_{mass} with age (Figure 3) resulting in lower prediction precisions for P_{area} models and C_{mass} models, respectively. This effect is further highlighted in the next section of our discussion when we use PLSR models developed using leaves from one leaf age group to predict traits of leaves of another leaf age group.

1.4.2 What is the performance of chemometric PLSR models developed using leaves from one leaf age group when predicting the traits of leaves of other leaf age groups?

Chemometric approaches such as PLSR are known to be subject to instability when models developed with one dataset are used to predict chemicals from spectra in another dataset (Boulesteix and Strimmer 2006; Martens 2001). When we tested the robustness of chemometric PLSR models across time (i.e. using models developed with one leaf age group to predict traits of leaves of other leaf age groups), we found that models for all leaf traits displayed leaf age/temporal sensitivity. However, as per our previous results, except for P_{area} models, area-based models consistently and significantly outperformed mass based models (Figures 5 and 6).

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Furthermore, mass based models of different leaf age groups were sometimes unable to predict N_{mass} and C_{mass} (Figure 5), while area-based models only failed to do so on one occasion (i.e. the senescent area-based model for P_{area} was unable to predict this trait for the mature leaf age group).

Our results also show that on both mass and area basis, mixed-age models (All) displayed the best performance (Figure 5 a-t). However, even models developed using mixed-age leaves (All) sometimes displayed significant decreases in precision (11-41% and 11-31% for mass- and area-based, respectively) and accuracy (8-10% and 8% for mass- and area-based, respectively) when predicting the traits of leaves from single age groups. It is also important to highlight that, among the models of the individual leaf age groups, the developmental (D) models displayed the best performance when predicting the leaf traits for the mixed-age (All) group for both mass- and area-based models. Considering that the developmental leaf age group displayed the largest trait ranges compared to the mature and senescent age groups (see Figure 3), these results provide evidence that when developing PLSR models capturing the temporal/seasonal diversity of leaf traits due to leaf age can be as important as capturing trait diversity generated by species diversity and/or environmental growing conditions. This is further supported by several studies which have highlighted that up to 30% of trait variation found "within-sites" or species can be attributed to plant asynchrony (Albert et al. 2011; Albert et al. 2010; Bolnick et al. 2011;

Cornwell and Ackerly 2009; McKown et al. 2013; Violle et al. 2012) and that the trait variation generated from sampling leaves of different leaf age groups across a relatively small sample of 12 Amazonian canopy trees can be as large as the variation found among the mature leaves of a large sample of almost 200 Pantropical trees from the GLOPNET database (Chavana-Bryant et al. In review).

LMA was the trait that displayed the least age/temporal sensitivity (i.e. models performed well across leaf age groups) followed by C_{area} and LWC_{area}, respectively. Although LMA could be predicted by models developed from the different leaf age group datasets, significant losses in prediction precision (and accuracy) were observed in some cases (10-18%; Figures 5 & 6) and much larger losses were observed for other traits. For example, C_{area} and LWC_{area} models displayed losses in prediction precision of 19-36% and 19-41% when used to predict the traits of other leaf age groups.

These results were also reflected in the spectral coefficients' differences we observed between models for individual leaf traits developed from different leaf age groups (Figure 7). When we analysed the correlations between spectral coefficients of the different leaf age group models within individual mass-based traits and LMA, we observed that only the spectral coefficients of LMA models displayed consistently strong correlations between different leaf age groups (i.e. single leaf age group models can

predict LMA for other leaf age groups), while the coefficients for models of different leaf age groups within the other four leaf traits displayed correlations of varying strength (i.e. single leaf age group models for these traits display age sensitivity and cannot consistently predict the leaf traits of other age groups) (Figure 5). Although previous chemometric studies using PLSR approaches have reported differences in spectral coefficients and VIPs for different leaf traits, our study is the first to report differences in both of these metrics within leaf traits due to leaf age (Figure 7 and Figure S1).

As developing mass-based chemometric PLSR models using only "fully expanded mature leaves" is the standard practice in most spectroscopic studies, it is important to highlight two of our main findings: (1) that the mature (M) models displayed the worst overall performance when predicting the traits of leaves from other leaf age groups on both mass- and area-basis (Figure 5) and (2) current models developed using only mature leaves are likely to display leaf age/temporal sensitivity. The model uncertainty introduced by leaf age effects is a reduction in precision for all traits. For example, even for LMA and C_{area} models, which show good performance across all leaf age groups, in vegetation systems with synchronised leaf phenology such as temperate and deciduous tropical forests, prediction precision would be significantly decreased if PLSR models were used to predict the leaf traits of leaves collected at different times in the growing season (i.e. early or late in the growing season).

Therefore, to achieve temporally stable PLSR models, it is important to ensure that PLSR models are calibrated using samples representing all leaf age groups (i.e. collected at different times of the growing season).

Our findings can have important implications for canopy and landscape level trait estimation for three main reasons. First, the sampling of mature leaves, even when chosen to represent the species diversity of a site or area, are unlikely to cover the full range of trait variation introduced by leaf age. Second, leaf reflectance represents a composite measure of morphological, structural and biochemical leaf traits, with some of these traits displaying different age-related changes and some sharing similar values of constituent content across different leaf ages. Lastly, current PLSR models calibrated with only mature leaves are embedded for upscaling purposes into canopy models and used to predict canopy traits across time/seasons and across the canopy/landscape, however, these canopies are likely to contain leaves of different leaf ages. This is because leaves of both tropical and temperate trees can spend a significant part of their life-cycle (~27 to 55% and ~50%, respectively) in non-mature life phases. For example, the leaf life span of trees included in this study ranged from ~10 months to ~two years. These leaf life spans are similar to those found by Reich *et al.* (2004) for a large sample of well-lit trees in an Amazonian terra firme mature forest canopy. Within these life-cycles, leaves spent on average ~1.5 months in the developmental stage (which does not include budding period), ~2 months in old stage and/or up to 3

months in the senescent stage. The duration of the developmental stage for our trees are similar to those exhibited by other tropical trees (Cai et al., 2005; Ogawa et al., 1995), temperate-deciduous and tropical evergreen trees in montane cloud forest sites (Williams-Linera, 2000) and a wide range of species from a macro-analysis by Pantin *et al.* (2012). However, the old/senescent phase tends to be shorter (1-2 months) in deciduous trees (McKown et al., 2013). It is therefore, very likely that the combined effect of leaf aging and asynchrony in leaf life cycles will have an impact on the performance of current PLSR models used to estimate canopy leaf traits. For example, in evergreen tropical forests most field and aerial spectranomic campaigns are conducted during the dry season, a time when field-based studies (Brando et al. 2010; Chavana-Bryant et al. 2017; Chave et al. 2010; Doughty and Goulden 2008; Malhi et al. 2014 among many others) have shown that canopy leaf turnover is at its highest with many trees exchanging their leaves through a wide variety of phenological behaviours and intra- and interspecific phenological asynchrony (Borchert 1983; Borchert et al. 2002; Reich 1995; van Schaik et al. 1993). This results in different proportions of leaves of different ages being present in tropical evergreen canopies not only during the dry season but throughout the annual cycle. Furthermore, in forests with synchronised leaf phenology (e.g. dry tropical and temperate forests) there are times during the growing season (i.e. beginning and end) when only developing or senescing leaves are present in the canopy and even leaves at different stages of maturity (early, mid and late maturity) can display differences in

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traits (McKown et al. 2013). Temperate trees can also display a second or third flush of leaves during the growing season which can substantially reduce the time window when mature leaves dominate their canopy. Chemometric PLSR models calibrated with only mature leaves are, therefore, likely to experience different degrees of sensitivity to leaf age throughout the annual cycle in tropical evergreen forests or throughout growing season in deciduous forests, which could explain some of the model residuals in published studies (Asner et al. 2015; Asner et al. 2009; Asner et al. 2011a; Axelsson et al. 2013; Féret et al. 2011; Qi et al. 2014). Furthermore, Chavana-Bryant *et al.* (2017), provides evidence of how **some widely used** spectral vegetation indices, such as NDVI, EVI, PRI, etc. are affected by leaf age at the leaf level and in Chavana-Bryant *et al.* (in preparation) also at the canopy level. **Spectroscopic studies may therefore benefit from moving away from standard practice (sampling only fully expanded mature leaves) and instead collect samples that represent the different seasonal phases and/or leaf ages encountered at the time of sampling. In the supplementary materials section, we provide practical protocols (from Chavana-*Bryant et al.* in review) that can be used to keep track of leaf age differences during field collection campaigns.**

Finally, although further work comparing the performance of mass- versus area-based PLSR models for a wider range of traits (e.g. leaf pigments, etc.) and leaf samples will be required. our results provide a valid case for incorporating the development of area-based PLSR models into common

practice. In the case of photosynthetic related traits, area-based PLSR models may prove to be more relevant than mass-based models.

1.5 Conclusions

This study is the first to demonstrated the importance of leaf age in the development of chemometric PLSR models used to estimate leaf and canopy traits from hyperspectral radiometry. It is also the first study to develop area-based PLSR models for several traits that are important to leaf physiological and ecological functioning and that have demonstrated contributions to the spectral signatures of vegetation. The results of this study reveal that leaf age affects the precision and accuracy of trait estimates of both mass- and area-based PLSR models, with area-based models showing significantly less sensitivity to leaf age effects. The latter was particularly striking for leaf carbon content. Age effects vary across traits, and precision is more widely affected than accuracy. To achieve temporally stable models (i.e. models that can be applied on airborne acquisitions obtained at any time during the growing season and are not affected by canopies containing a mix of leaf age classes), spectroscopic studies would benefit from moving away from the standard practice of focusing on mature leaves and instead develop both mass- and area-based models calibrated with leaves representing different leaf age groups. In regions where leaf phenology is strongly synchronised (e.g. temperate and deciduous tropical forests) this would involve collecting leaves at different times of the growing season, while in regions with

unsynchronised leaf phenology (e.g. evergreen tropical forests) it would involve sampling leaves representative of the different ages encountered in the field at the time of year of interest or across the year. Finally, the use of area-based PLSR models, which are less sensitive to leaf age, may prove to be more appropriate for estimating canopy traits associated to photosynthesis.

1.6 Acknowledgements

1.7 References

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List of Figure Captions

Figure 1   Examples of leaves of different age classes, illustrating the leaf life-cycle of some of our sampled trees (tree codes in brackets).

Figure 2   Data subsets used to develop mass-based partial least squares regression (PLSR) models for different leaf age groups.

Figure 3   Leaf age group summary trait statistics for LMA and LWC, P, N and C content on both a mass and area basis.

Figure 4   Spectral summary statistics by leaf age group.

Figure 5   Partial least squares regression (PLSR) results for measured vs spectrally predicted leaf traits on a mass basis.

Figure 6   Partial least squares regression (PLSR) results for measured vs spectrally predicted leaf traits on an area basis.

Figure 7   Partial least squares regression (PLSR) coefficients by leaf age group.

Author Contribution Statement

Cecilia Chavana-Bryant: Research Plan and Design, Field methodology, Field data collection, Data Integration and Quality Assurance, Data analysis, Writing- Original draft preparation, Reviewing and Editing. Yadvinder Malhi: Research Plan and Design Supervision, Writing- Supervision, Reviewing and Editing. France F. Gerard: Research Plan and Design Supervision, Writing- Supervision, Reviewing and Editing. Eric G. Cosio: Field data collection logistical support. Athanasios Anastasiou: Data Integration and Quality Assurance, Data analysis. Brian J. Enquist: Leaf chemical analyses at the University of Arizona, Writing- Reviewing. Trevor Keenan: Writing- Reviewing and Editing
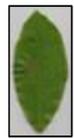
D = includes developing leaves in the process of gaining area, thickness and green colouration and collected after 1, 2, 3 and 4 weeks of active leaf expansion, respectively.

M = includes fully-grown and structurally developed mature leaves that have achieved a fully darkened green colour and includes newly matured, mid and end of maturity leaves

S = includes old leaves showing initial signs of senescence and senescent leaves starting to abscise through to advanced senescence (most canopy leaves abscised).
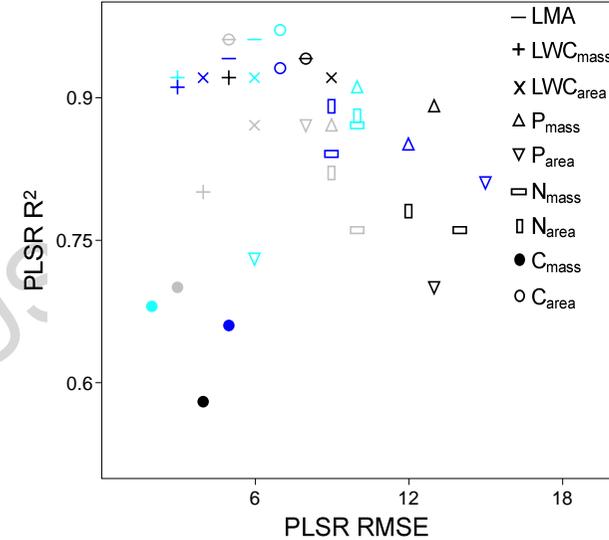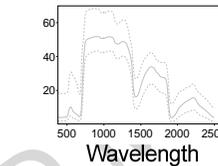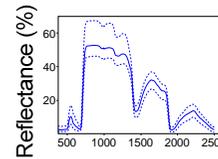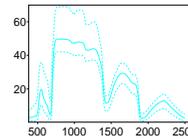
Leaf physiochemical traits change with age

Leaf reflectance also changes with age

PLSR models calibrated with leaves of different ages and developed using area based trait measurements are more temporally stable then models developed with only mature leaves on a mass basis

Graphical abstract

Highlights

- **Sample bootstrapping tested for leaf age effects on mass and area based PLSR models**

- **Trait estimates of area based models outperformed mass based models**

- **Models calibrated with only mature leaves showed the worst performance**

- **Results show temporally stable models need calibration with leaves of different ages**