Phenotypic Plasticity and Genetic Variation of Leaf Pigment Content in a Southern Beech

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Phenotypic plasticity and genetic variation of leaf pigment content in a Southern beech

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Abstract

Physiological, morphological and phenological attributes are potentially adaptive traits that determine functional responses to certain environmental conditions. They are crucial for understanding adaptations to environmental variation along a species natural range. In particular, leaf pigment content can be a good proxy to the physiological and phenological tree state. Our goal was to evaluate the variation in pigment traits among *Nothofagus alpina* populations in a common garden trial during two years to infer local adaptation and/or phenotypic plasticity. We also aim to analyse the correlation between pigment traits and phenological traits and climatic data from the geographic location of the populations. To comprise the entire range of the species in Argentina, we analysed 400 individuals from eight natural populations coming from four lake watersheds. Pigment traits were estimated using a spectrophotometer and analysed with linear mixed model (LMM). Significant differences among fixed factors (populations - years and watershed – years) were found in chlorophyll a, b, total, carotenoids and anthocyanins concentrations. Higher concentrations were found for 2018, year with the highest number of rainy days and accumulated precipitation. Two populations (Boquete and Tren Tren) were always the most contrasting ones. At population level, the correlation between the means of pigment concentrations and phenological traits

was significant in almost all cases. Conversely, the correlation between the means of population pigment concentrations and the main geographic, climatic and bioclimatic variables of the home range were not significant. The significance of the environmental factor (year) in the linear mixed models tested is evidence of phenotypic plasticity of pigment content, suggesting flexibility for acclimatization to moderate inter-annual changes in climatic conditions. The significance of population and watershed and the influence of the family factor on the variance of the pigment traits are evidence of the genetic control as well as the potential adaptive value of leaf pigment content in *N. alpina*, giving a base for adaptation to a long-lasting change in climate. High correlations between phenological and pigment traits indicate that, in *N. alpina*, the determination of only one pigment concentration could be used as a proxy of bud burst, senescence and growing degree days.

1. INTRODUCTION

Climate has long been identified as a key factor shaping the geographic distribution of plants (Major 1951). In present times, however, climate is changing rapidly (IPCC 2014) shifting species distribution (Lloyd et al. 2011; Fisichelli et al. 2014; Parmesan and Hanley 2015). Tree species are particularly affected by these changes, that occur faster than their long life-cycle, and could respond through migration, phenotypic plasticity and local adaptation (Parmesan and Hanley 2015). However, uncertainty remains about dispersal rates in pace with rapidly changing climate as trees may not be able to track favourable conditions through migration (Kremer et al. 2012). Therefore, phenotypic plasticity (i.e. the capacity of genotypes to adapt their phenotypes without genetic changes) could be a strategy to persist in situ under changes of local conditions (Aitken et al. 2008). Ultimately, species can adapt to the new environmental conditions by natural selection of the most adequate genotypes (i.e. change of the allele genetic frequencies within populations). Exploring patterns of phenotypic plasticity and intraspecific genetic variation at adaptive traits can help to understand how species respond to changes in their environments.

Potentially adaptive traits are physiological, morphological and phenological attributes that determine functional responses to certain environmental conditions (Violle et al. 2007). Variation in leaf pigment content can be a good proxy to the physiological tree state (Sims and Gamon 2002), and in particular photoprotective pigments play a relevant role in short-term responses to abiotic stress (García-Plazaola and Becerril 2000; Ramírez-Valiente et al. 2015). Chlorophyll a (Chla) and b (Chlb) are the main photosynthetic pigments, and photosynthetic capacity of plants is influenced by the total amount of leaf chlorophyll content (Chla+b) and the allocated ratio (Chla / Chlb) (Li et al. 2018a). Accessory pigments such as carotenoids participate in reaction centers and dissipate excess of energy avoiding damage to the photosynthetic system (Demmig-Adams et al. 1996; Taiz, L., & Zeiger 2002). Similarly, anthocyanins possess an antioxidant property preventing the production of free radicals and protecting the plant against UV light (Feild et al. 2001). High leaf anthocyanin concentrations have been determined at low temperatures or under other stressing conditions (Chalker-Scott 1999).

Environmental variation along a species natural range can promote local adaptations in leaf pigment concentrations (Kaluthota et al. 2015; Simões et al. 2020), causing variation among natural populations. Due to the difficulties of decoupling genotype by environment interactions, common garden trials are a good choice to study the genetic control of potential adaptive traits (de Villemereuil et al. 2018). On these trials, different genetic entities (typically families nested within populations) are evaluated under homogenous environmental conditions, and therefore the differences in potential adaptive traits are assumed to be mainly of genetic origin (Kawecki and Ebert 2004). To test phenotypic plasticity of potentially adaptive traits, the same genetic entities must be installed in different environments. If the ontogenetic state of the organism has low influence on the analysed traits, phenotypic plasticity could also be evaluated by measuring traits repeatedly in the same trial in climatically contrasting years. Information from common garden trials has revealed genetic variation among populations for leaf pigment traits (e.g. Hawkins et al., 2019; Kaluthota et al., 2015; Wang et al., 2021).

Subantarctic temperate forests develop in Chile and Argentina along the Andes and conform the southernmost temperate forests of the world (Marchelli et al., 2021a) . Climate change predictions for the region indicate increase in average temperature, decrease in average precipitation (Barros et al. 2015) and increase in the frequency and severity of extreme weather events (Alexander et al. 2006). These predicted changes would affect both thermal and rainfall gradients that characterized the region, and therefore the species growing along them. *Nothofagus* is the predominant genus in these forests. Among the native species, *Nothofagus alpina* (Poepp & Endl.) Oerst. (= *N. nervosa* (Phil.) Dim. et Mil.), locally known as raulí, is an ecological and economical important tree species. To the eastern side of the Andes, where climate change is altering in particular the deep rainfall gradient (Barros et al. 2015), the species has a narrow and fragmented distribution along different watersheds (Marchelli et al., 2021b). Its occurrence along environmental gradients of precipitation and temperature (i.e. altitude) suggest the existence of local adaptations. Genetic differentiation at neutral (Marchelli and Gallo 2006; Azpilicueta et al. 2013) and adaptive markers related with drought stress responses (Soliani et al. 2020) suggested the co-occurrence of demographic and selection forces. Moreover, genetic variation in shoot/root allometric traits, bud burst phenology and foliar senescence was reported (Duboscq-Carra 2018; Duboscq-Carra et al. 2020). However, studies on other adaptive traits are still needed. In particular, studies on traits useful as proxy of phenological variation could prompt common garden and in situ evaluations of forest tree species responses to stressful environmental conditions.

Our goal here was to evaluate the variation in pigment traits among *N. alpina* populations from different watersheds in a common garden trial during two years to infer local adaptation and/or phenotypic plasticity. Specifically, we aim to analyse: (i) the variation in chlorophyll *a*, chlorophyll *b*, total chlorophyll, carotenoids and anthocyanins (a) among natural populations and families within them and, (b) between two different years, (ii) the correlation between pigment traits and phenological traits in one of the years, and iii) the correlation between pigment traits and climatic data from the geographic location of the populations.

2. MATERIALS AND METHODS

2.1 Common garden trial and climatic information

For this study we used a provenance and progeny trial of *N. alpina* installed in 2011 in the INTA Forest station at Las Golondrinas (41° 59' 50" S, 71° 31' 31" W, 415 m asl) where we had previously studied phenological traits (Duboscq-Carra et al. 2020). The trial consisted in a randomized complete block design with lineal blocks in order to take into consideration the shading effect of a parallel forest windbreak. A total of 400 trees were used, corresponding to 65 open pollinated families (at least 25 individuals per block and four individuals per family) from 10 blocks. These individuals represent eight natural populations coming from four lake watersheds that comprise the entire range of the species in Argentina (Table 1, Fig. 1). This region is characterized by dry summers (Donoso 2004) and *N. alpina* grows along a west-east mean annual precipitation gradient from 3000 mm to 1200 mm (Sabatier et al. 2011). Concerning thermal conditions, populations of *N. alpina* develop in places with high thermal amplitude, where warmest month mean temperatures are between 9 - 14 ° C and in the coldest months between 0 - 2 ° C (Bianchi and Cravero, 2010). *Nothofagus alpina* most continuous forests are between 800–950 m asl, and can reach 1350 m asl scattered within other species (Sabatier et al. 2011).

Daily minimum, maximum and mean air temperature, daily total precipitation for both years, and for the periods 1968-1977 (ten years) and 2012-2017 (six years), were obtained from the station 87800 of the *Servicio Meteorológico Nacional* (41° 57' 0" S, 71° 32' 0" W, 337 m asl), located 5 km away from the common garden trial. These values were used to calculate monthly values of each variable (i.e. mean of daily values) for both years and mean monthly values for both periods (1968-1977 and 2012-2017). These periods were selected because they had almost complete data over each year (i.e. less than six missing values over the year). Climate data and bioclimatic variables from each geographic location of the *N. alpina* populations were also obtained, at 30 seconds spatial resolution (~1 km²), from global databases. Historical monthly climate data for mean temperature, solar radiation, wind speed, water vapour pressure, and total precipitation for the period 1970-2000 were obtained from

WorldClim v2.1 database (Fick and Hijmans 2017). Historical bioclimatic variables for the period 1981-2010 were obtained from Chelsa v2.1 database (Karger et al. 2017).

Precipitation and temperature data were used to calculate the monthly De Martonne aridity index (1926) following the equation:

$$\text{Aridity Index} = 12 * \text{monthly amount precipitation} / (\text{mean monthly air temperature} + 10)$$

Table 1. Geographic location and climatic information of the eight analysed populations of *Nothofagus alpina* with their corresponding watershed, number of families and individuals.

Lake watershed	Population	Location	Altitude (m asl)	MAP [mm.yr-1] ^a	Jan. T (°C) ^a	July T (°C) ^a	N° fam	N° ind
1 – Tromen	Tromen bajo (TRB)	39° 34' 10" S 71° 26' 28" W	1064	1492	12.99	0.95	8	44
1 – Tromen	Tromen alto (TRA)	39° 36' 18" S 71° 20' 57" W	1110	1270	13.5	1.01	13	69
2 – Curruhué – Huechulafquen	Paimún (P)	39° 42' 17" S 71° 33' 52" W	930	1800	12.1	0.93	6	39
2 – Curruhué – Huechulafquen	Curruhué (C)	39° 50' 04" S 71° 30' 00" W	1030	1400	13.2	0.89	9	54
3- Lolog	Boquete (B)	40° 01' 51" S 71° 34' 38" W	910	1600	13.1	1.37	5	34
3 – Lolog	Puerto Arturo (PA)	40° 01' 02" S 71° 22' 19" W	910	1200	14.6	1.93	13	70
4 – Lácar	Queñi (QE)	40° 09' 54" S 71° 45' 20" W	920	2400	9.7	0.00	8	53
4 – Lácar	Tren Tren (TT)	40° 11' 53" S 71° 25' 53" W	1040	1400	13.8	1.61	7	37

^a Climatic data obtained from Bianchi and Cravero, 2010. MAP: mean annual precipitation, Jan. T:

mean January and July T: mean July temperatures. **double column fitting table**

Fig. 1. *Nothofagus alpina* natural distribution in Argentina with the location of the studied populations and lake watersheds (modified from Duboscq-Carra et al., 2020). **Simple column fitting image.**

2.2 Pigment quantification

Fully expanded leaves (phase 5 according to Duboscq-Carra et al., 2020) were sampled in late spring (beginning of December) in 2018 and 2019. For each tree, we collected three not shaded leaves from different branches in different orientations. To avoid differences in environmental conditions (e.g. light intensity between days and within the canopy) that could bias phenotypic evaluation, each year we sampled the whole trial in a single day. Once collected, leaves were immersed in liquid nitrogen, transported to the laboratory and stored at -80 °C until pigment quantification.

Pigments were extracted following the protocol reported by Gould et al. (2000).

Chlorophylls and carotenoids were extracted using a solution of acetone: H₂O (4:1 v/v) while anthocyanins were extracted using a solution of 3 M HCl: H₂O: MeOH (1:3:16 v/v/v). For each tree, three foliar discs, 1 cm² total area, were cut and submerged in each solution during 24 hours in dark at 4 °C. After this period, centrifugation (1 min at 1000 rpm, 4°C) was used to separate solid residues. Pigment concentrations were estimated using a Pharmacia Biotech Ultrospec 1000 spectrophotometer. For the acetone extracts, absorbance was measured at 470, 647 and 663 nm (A₄₇₀, A₆₄₇ and A₆₆₃ respectively) to estimate the concentration of chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (Chl_{a+b}) and total carotenoids (Car) according to the equations given by Lichtenthaler, 1987:

$$\text{Chlorophyll a} = 12,25 * A_{663} - 2.79 * A_{647}$$

$$\text{Chlorophyll b} = 21.50 * A_{647} - 5.10 * A_{663}$$

$$\text{Chlorophyll total} = 7.15 * A_{663} + 18.71 * A_{647}$$

$$\text{Carotenoids} = \frac{(1000 * A_{470} - 1,82 * \text{Chlorophyll a} - 85,02 * \text{Chlorophyll b})}{198}$$

Chl_a / Chl_b and Chl_{a+b} / Car ratios were also calculated.

For the methanolic extracts, absorbance was measured at 530 and 653 nm (A₅₃₀ and A₆₅₃ respectively) to estimate the concentration of anthocyanins (Ant) as Gould et al. (2000):

$$\text{Anthocyanins} = A_{530} - 0.24 * A_{653}$$

Statistical analysis

Evaluation of environmental variation between both years at the common garden trial was based on temperature, precipitation and aridity index data. First, we tested the difference between years for monthly mean, minimum and maximum air temperature (T_{mean}, T_{min}, T_{max} respectively), and aridity index (AI) by t-tests. For monthly total precipitation (precip.) and rainy

days (rd) we used the Mann-Whitney test. Normality of the data distribution was assessed using a histogram and a Shapiro test for each variable. Homoscedasticity was checked with a Bartlett test. Second, we compared the distribution pattern of monthly mean air temperature, total precipitation and aridity index for both years and mean historical values for the period 1970-2000 (WorldClim) and 1981-2010 (Chelsa).

To evaluate the relative contribution of our sampling effects (i.e. population, year, and random variance within blocks and families) to variation in the pigment traits we used a linear mixed effects model (LMM). We considered the following LMM:

$$y_{ijkl} = \mu + \gamma_i + \rho_j + \gamma_i * \rho_j + \varphi_{k(\rho_j)} + \beta_l + \varepsilon_{ijkl}$$

where: y_{ijkl} is the pigment trait in the individual of the k^{th} family of the j^{th} population in the l^{th} block measured in the i^{th} year; μ is the general mean of the whole trial for the pigment trait; γ_i is the fixed effect of the i^{th} year; ρ_j is the fixed effect of the j^{th} population; $\varphi_{k(\rho_j)}$ is the random effect of the k^{th} family nested into the j^{th} population; β_l is the random effect of the l^{th} block; ε_{ijkl} is the residual error $\sim NID(0, \sigma^2)$.

We also evaluated the relative contribution of the watershed and year effect to variation in the pigment traits, considering the following LMM:

$$y_{imjl} = \mu + \gamma_i + \rho_m + \gamma_i * \rho_m + \varphi_{j(\rho_m)} + \beta_l + \varepsilon_{imjl}$$

where: y_{imjl} is the pigment trait in the individual of the j^{th} population of the m^{th} watershed in the l^{th} block measured in the i^{th} year; μ is the general mean of the whole trial for the pigment trait; γ_i is the fixed effect of the i^{th} year; ρ_m is the fixed effect of the m^{th} watershed; $\varphi_{j(\rho_m)}$ is the random effect of the j^{th} population nested into the m^{th} watershed; β_l is the random effect of the l^{th} block; ε_{imjl} is the residual error $\sim NID(0, \sigma^2)$.

For both LMMs, we used the "lme4" package (Bates et al., 2016) in R 3.3.0 software (RStudio Team 2020). For each pigment trait model, we used a "residuals versus fitted plot" to detect non-linearity, unequal error variances, and outliers. A likelihood ratio test (LRT) was used to analyse the significance of random effects, considering the complete model and a model without each factor.

Since the measurements of pigment content were made in consecutive years, the same development stage (juvenile trees) was evaluated. So, the traits are not expected to be influenced by the age of the trees, and the possible ontogenetic effect on the inter-annual variation was consequently dismissed.

Pearson correlation tests were performed between pigment traits and the main geographic and environmental variables of each natural population (Table 1). In addition, we tested the association between the estimated pigment concentrations and the phenological traits that showed significant differences among populations in Duboscq-Carra et al. (2020). Those traits were: day of the year to bud burst (DOY BB), growing degree days (GDD Tb 5 °C), day of the year when 10 % of the crown had autumnal leaves (DOY 10), and growing season length (GSL 10). This information was only available for the year 2018.

A principal component analysis (PCA) was performed considering mean values of Chla, Chlb, Chla+b, Car, Ant, Chla / Chlb and Chla+b / Car of each population as active quantitative variables. Mean climatic (5) and bioclimatic variables (35) from WorldClim and Chelsa, and the previously mentioned phenological variables (4) were used as supplementary quantitative variables in order to visualize potential relationships with pigment traits. Watershed and longitudinal location (i.e. west or east location) were used as qualitative variables for data visualization. We used the R package Factoshiny v2.2 for this analysis. The Wilks test was used to evaluate which qualitative variable better explain the distance between populations. Factoshiny was also used to test the correlation between pigment content and climatic and bioclimatic variables.

3. RESULTS

3.1 Climatic conditions of the evaluated years

Globally, a tendency of higher number of rainy days and accumulated precipitation for 2018 than for 2019 was observed (Table 2). This tendency was even stronger for spring months, with threefold total precipitation and almost double the

number of rainy days recorded in the spring of 2018 compared to the spring of 2019 (Table 2). However, no significant differences were found, by t-test or Mann-Whitney test, between years for the analysed variables (i.e. monthly rainy days, total precipitation, mean, minimum and maximum air temperatures, and aridity index, Table 2).

Table 2. Environmental conditions for the common garden trial located in Las Golondrinas for the evaluated years, including rainy days (rd), total precipitation (precip.), and mean values of monthly total precipitation (precip.), total rainy days (rd), mean, minimum and maximum air temperature (T_{mean} , T_{min} , T_{max} respectively) and aridity index (AI).

Year	Annual rd (days) ^a	Spring rd (days) ^b	Annual precip. (mm) ^a	Spring precip. (mm) ^b	Mean precip. (mm) ^c	Mean rd (days) ^c	Mean T_{mean} (°C) ^c	Mean T_{min} . (°C) ^c	Mean T_{max} . (°C) ^c	Mean AI ^c
2018	122	36	987.8	332.5	83.0	10.1	10.94	3.58	17.08	56
2019	105	22	813.6	112.5	67.7	8.75	11.16	3.39	17.64	48

^a January to December. ^b September to November. ^c Mean: correspond to monthly mean value of the reported variable.

Considering variation over the year, monthly, mean air temperature, total precipitation and aridity index were not equally distributed for both years (Fig. 2). Total precipitation for 2018 was globally higher than the historical reference, except during late autumn and early winter; while total precipitation for 2019 was globally lower than the historical reference (Fig. 2). Mean temperatures were higher than those of the historical periods for almost all months, and for 2019 it was globally higher than for 2018. Changes on total precipitation showed a higher impact on aridity index (Fig. 2). The year 2018 was less arid than the year 2019 according to the aridity index, showing a lower number of months classified as extremely arid (Fig. 2). Furthermore, during growing seasons, as a consequence of the difference in accumulated precipitation, 2018 presented better conditions for tree growth, showing higher values of aridity index than 2019.

Fig. 2. Monthly mean temperature, total precipitation and aridity index of the common garden trial located in Las Golondrinas, for 2018, 2019 compared to mean values from the periods 1968-1977 and 2012-2017. Dashed lines in the y axis represents levels in climatic classification according to aridity index: 0-5: extremely arid; 5-10: arid; 10-20: semiarid; 20-30: subhumid; 30-60: humid; > 60 perhumid. Arrow indicates collecting date. **Double column fitting image**

3.2 Leaf pigment variation

Fixed factors were significant for both LMMs, while interaction between both fixed factors (i.e. population and year, or watershed and year) was not detected for any pigment trait (Table 3). Both for populations and for watersheds, the concentration of Chla, Chlb, Chla+b, Car and Ant all presented significant differences between years ($p < 0.05$), with higher concentrations in 2018 than in 2019 (Fig. 3 and Fig. 4). The difference was stronger for anthocyanin

concentration. For example, among populations mean value of Ant concentration was 1.8 times higher in 2018 than in 2019, while the other pigments showed a slightly higher concentration in 2018 than in 2019 (~ 1.1 times).

Table 3. Significance of the fixed factor (population and year or watershed and year) and its interaction in LMMs for Chla, Chlb Chla+b, Chla/Chlb, Car, Chla+b/Car and Ant.

Fixed Factor ^a	Chla	Chlb	Chla+b	Chla / Chlb	Car	Chla+b/Car	Ant
P	2.6 e-04	7.4 e-04	3.6 e-04	0.1571	0.0343	0.8720	3.3 e-04
Y	6.6 e-13	1.9 e-10	1.2 e-12	0.5579	5.1 e-05	0.0373	<2.2 e-16
P * Y	0.8634	0.8883	0.8835	0.8331	0.7341	0.6821	0.1630
W	1.5 e-09	1.2 e-08	1.0 e-09	0.1257	1.4 e-06	0.5760	0.0436
Y	3.6 e-12	8.6 e-10	8.1 e-12	0.5570	1.2 e-04	0.0498	<2.2 e-16
W * Y	0.5308	0.5077	0.5542	0.5599	0.8024	0.3356	0.4086

^a P:population, Y:year, W: watershed. *: interaction between fixed factors.

Differences among populations were detected for all pigments (Fig. 3). Boquete and Tren Tren were globally identified as the most contrasting populations showing the highest and the lowest concentration respectively. In particular, for Chla+b and Chla, Boquete and Puerto Arturo showed the highest concentrations, while Tren Tren and Queñi the lowest ($p < 0.05$). Regarding Chlb, differences were significant ($p < 0.05$) between Boquete and Tren Tren, while the other populations were in the middle of these extremes. The family and block factors had a significant effect ($p_{LRT} < 0.001$), although they only explained around 6 % and 10 % respectively in each variable. Carotenoids showed the same pattern as Chlb with Boquete showing the highest concentrations while Tren Tren the lowest. The random factors had significant effects ($p_{LRT} < 0.001$), with family and block explaining 9 % and 11 % of the variability respectively. Finally, for anthocyanins, Boquete and Paimun had the highest concentrations while Tren Tren and Tremen Alto the lowest. Both random factors (family and block) had significant effect ($p_{LRT} < 0.001$) although they only explained around 4% of the total variance.

Regarding watersheds, significant differences were detected for all pigment concentrations. Lolog and Lácar were globally identified as the most contrasting watersheds showing the highest and lowest concentration respectively; while the other watersheds were in the middle of these extremes. The block factor had a significant effect ($p_{LRT} < 0.001$), although it only explained around 5% in Chla and Ant and around 10 % in Chla+b, Chlb and Car of the total variance. The population nested into watershed factor did not have a significant effect in the model.

For both populations and watersheds, anthocyanins showed the highest level of intra-specific variation. For example, for populations, pigment content was 35%, 20%, 17.5%, 17%, and 16% higher for Boquete than for Tren Tren, for Ant, Chlb, Car, Chla and Chla+b respectively. Considering watersheds, pigment content was 17%, 14%, 13%, 13% and 12% higher for Lolog than for Lacar, for Ant, Chlb, Car, Chla+b and Chla *respectively*.

On the contrary, Chla / Chlb ratio and Chla+b / Car did not present significant differences among populations, watersheds or years, with the exception of Chla+b / Car for which the difference between 2018 and 2019 was significant (Table 3).

Fig. 3. Year by Population interactions for leaf pigment content in *Nothofagus alpina*.

Represented values are the mean effects on the LMM, with standard errors represented by whiskers. Icon shape indicates watersheds: circles for Tromen, triangles for Curruhue-Huechulafquen, squares for Lolog, diamond for Lácar.

Fig. 4. Year by Watershed interactions for leaf pigment content in *Nothofagus alpina*.

Represented values are the mean effects on the LMM, with standard errors represented by whiskers. **double column fitting image.**

3.3 Correlation and principal component analyses

At population level, the correlation between the means of pigment concentrations and phenological traits was significant in almost all cases, being high and negative for day of the year to bud burst (DOY BB) and growing degree days (basal temperature = 5 °C; GDD Tb 5° C), and high and positive for beginning of senescence (DOY10) and growing season length (GSL10) (Fig. 5). Conversely, the correlation between the means of population pigment

concentrations and the main geographic and environmental variables of the home range (Table 1) were not significant. Moreover, significant correlations were not detected between pigment content and climatic and bioclimatic variables.

Fig. 5. Pearson correlation of pigments concentrations and phenological traits for *Nothofagus alpina* populations in 2018. Square colours identify significant correlations: red for negative, blue for positive. White squares mean no significant correlation (p value>0.05).

The first two dimensions of PCA analysis express 93.55% of the total variation (Fig. 6). This value was greater than the reference value (i.e. the 0.95-quantile of the inertia percentages distribution obtained by simulating 1350 data tables of equivalent size on the basis of a normal distribution) that equals 74.1%. The Wilks test p-value indicated that separation of the populations was better explained by watershed (p=0.070) than by longitudinal location (p=0.403).

The first dimension separate Boquete and Puerto Arturo (to the right of the graph, characterized by a strongly positive coordinate on the axis) and Tren Tren and Queñi (to the left of the graph, characterized by a strongly negative coordinate on the axis) populations (Fig. 6a). Boquete and Puerto Arturo had high values of Chla, Chla+b, Chlb, and Car and low values of Chla / Chlb; while Tren Tren and Queñi had an opposite trend of pigment traits. High

correlation with this dimension was detected for $Chla+b$, $Chla$, $Chlb$, Car and $Chla / Chlb$ (correlation = 0.99, 0.99, 0.98, 0.87 and -0.71 respectively) and $GSL10$, $DOY10$ and $DOYBB$ (correlation = 0.87, 0.84 and -0.72 respectively) (Fig. 6b). Regarding watersheds, Lolog and Lácar showed a high positive and negative correlation with this dimension ($\cos^2 = 0.99$ and 0.98 respectively).

The second dimension separate Tromen Alto (to the bottom of the graph, characterized by a strongly negative coordinate on the axis) and Paimún (to the top of the graph, characterized by a strongly positive coordinate on the axis) populations (Fig. 6a). Tromen Alto had a low value of $Chla / Chlb$ and a high value of $Chla+b / Car$; while Paimún had an opposite trend. $Chla+b / Car$ showed a high negative correlation with this dimension (correlation = -0.98). Regarding watersheds, Tromen and Curruhue - Huechulafquen showed a negative and positive correlation with this dimension ($\cos^2 = 0.93$ and 0.7 respectively).

Climate data and bioclimatic variables were not correlated with first nor second dimensions.

Fig. 6. First plane of Principal Component Analysis (PCA) of a) populations and b) active (black) and supplementary (blue) quantitative variables. Variable labels are only showed for variables with good representation in the plane (i.e. $\cos^2$ greater than 0.6).

4. Discussion

4.1 Phenotypic plasticity of *N. alpina* pigment content

The two analysed years varied particularly in the distribution of total precipitation and, consequently, aridity index over months. This inter-annual variation constitutes a good scenario to test temporal phenotypic plasticity. The significance of the environmental factor (year) in the linear mixed models tested is evidence of phenotypic plasticity of pigment content, since their concentration varied when the same genotypes were exposed to different environmental conditions. Additionally, the lack of significance of the interaction between year and population or watershed (environmental and genetic factors, respectively) should be interpreted as the lack of genetic control of the detected plasticity itself (Whitman and Agrawal 2009), which can be clearly visualized in the parallel dotted lines of Fig 3. and Fig. 4.

All pigment concentrations were significantly higher in 2018. However, inter-annual variation was higher for anthocyanins (i.e twice for 2018) than for the other pigments. Regarding pigment ratios, only $Chla+b / Car$ was significantly higher in 2018 than in 2019, with populations Tremen Alto and Tren Tren showing the highest values. The year 2018 showed higher and lower values of aridity index than 2019 during summer/spring and autumn/winter, respectively. Moreover, 2018 showed less stressful conditions during the last and the first part of the growing seasons 2017-2018 and 2018-2019, respectively, than those of the historical periods. Conversely, 2019 showed more stressful conditions than those of the historical periods during growing seasons. Inter-annual leaf pigment variation has been also reported in other species under contrasting environmental conditions, although specific patterns depend on pigments, species and variation of environmental conditions between years (Mészáros et al. 2007; Uvalle Saucedo et al. 2007; Szollosi et al. 2011). For example, in a comparative study, over two growing seasons, *Quercus petraea* showed lower concentrations of total chlorophyll and carotenoids in the driest year; while *Quercus cerris* showed similar and lower content of total chlorophyll and carotenoids for the same year respectively (Mészáros et al. 2007). In other study, however, both species showed lower total chlorophyll and higher total carotenoids during the growing season with higher annual mean temperature and summer

heat waves (Szollosi et al. 2011). In our study, aridity index during growing seasons was lower for 2019 than mean values of historical periods. Conversely, for 2018 values were higher and, particularly in the growing season were globally within the per-humid class. Although pigment content variation could be determined by stressful conditions of 2019, the detected pattern suggests it is more probable related with the unusual benign environmental conditions of 2018. Our results in *N. alpina* also suggest high sensitivity of pigment content to the distribution of total precipitation over the year. However, we could not rule out that variation in other non-evaluated environmental conditions (e.g. wind speed, solar radiation) also influenced pigment content.

4.2 Genetic variation of *N. alpina* pigment content

Population and watershed were significant factors conditioning the concentration of chlorophyll *a*, chlorophyll *b*, total chlorophyll, carotenoids and anthocyanins, according to the linear mixed models tested. Moreover, the family factor was shown to have an influence on the variance of the pigment traits. These results are evidence of the genetic control as well as the potential adaptive value of leaf pigment content in *N. alpina*. Interspecific variation might explain more than 80% of the total variation of chlorophylls, with coefficient of variations around 0.4 for Chl*a*, Chl*b* and Chl*a+b* (Li et al. 2018 a). High interspecific variation has been also reported for carotenoids (e.g. Asner et al., 2009; Bündchen et al., 2016). In turn, intraspecific variation has been also reported for leaf pigments (e.g. Kaluthota et al., 2015; Martin et al., 2006; Ramírez-Valiente et al., 2015; Simões et al., 2020; Wang et al., 2020). In *N. alpina*, intraspecific variation for total chlorophyll has been previously reported based on non-destructive measurements by a portable chlorophyll meter (Duboscq-Carra 2018). As far as we know, reports on intraspecific variation of other pigments are not available for the species.

In our study, for all pigments, higher values were detected in population Boquete, located at the western edge, near the Andes, while the lowest values correspond always to Tren Tren population, at an eastern site. However, as for inter-annual variation, differences were higher for anthocyanins. High pigment content is usually characteristic of shade tolerant leaves (García-Plazaola and Becerril 2000; Wang et al. 2020), which coincides with denser

forests at western sites. This pattern could be also related with the west-east decrease on precipitation, as negative impact of drought in pigment concentrations has been previously reported (García-Plazaola and Becerril 2000; Esteban et al. 2015). However, the differentiation among watersheds indicates that, besides longitudinal location, other environmental factors would be conditioning pigment content. Lolog and Lácar were the most differentiated watersheds, with the highest and the lowest pigment content respectively. This pattern was also not related to the latitudinal location. Moreover, no correlation was detected between pigment content and i) altitude, mean annual precipitation or mean January and July temperatures of geographic locations (data from local databases), or ii) climatic or bioclimatic data (from WorldClim and Chelsa databases). Results of principal component analysis also agree with this trend, as pigment content separate populations, and this separation was better associated with watershed than with longitudinal location; and climatic and bioclimatic variables were not associated with population separation. Intraspecific differences in anthocyanins were also detected in *Quercus* spp. (Ramírez-Valiente et al. 2015), where an association between freezing tolerance and lower anthocyanin content was observed. In our case, although correlations between pigment traits and climatic variables at the home environments were not significant, the populations with the lowest anthocyanin content (Tren Tren and Tromen Alto) correspond to open forests towards eastern locations where the impact of frosts might be higher. This could suggest an adaptation to cold temperatures.

Regarding the pigment ratios, $Chla / Chlb$ and $Chla+b / Car$, were important for population separation in the principal component analysis. Populations from Lolog and Lácar watersheds were separated according to chlorophyll *a*, *b*, total chlorophyll, carotenoids and $Chla / Chlb$; while Tromen Alto (Tromen watershed) and Paimún (Curruhue-Huechulafquen watershed) were separated according to $Chla / Chlb$ and $Chla+b / Car$. Genetic variation was globally not detected by linear mixed models (neither for population nor watershed). However, a tendency of higher values for Tromen Alto and Tren Tren and lower values for Tromen Bajo and Paimún was detected. This tendency partially agrees with results from multivariate analysis.

Chla / Chlb ratio has been reported as a stable parameter irrespective of the stress factor considered (Esteban et al. 2015), with a small coefficient of variation among different species, life forms, and communities (Li et al., 2018a). However, Chla and Chlb differ in their absorption peaks and are mainly associated to different photosystems (Li et al. 2018b). Therefore, the lower value of Chla / Chlb associated with Boquete and Puerto Arturo (Lolog watershed) and Tromen Alto (Tromen watershed) could be related to a better light availability, and consequently lower allocation to photosystem II. Carotenoids participate in light harvesting but are also involved in photoprotection (Esteban et al. 2015). Therefore, Chla+b / Car depends mainly on light conditions (Demmig-Adams et al. 1996). In addition, high values of Chla+b / Car are associated with a better cold acclimation (Rapacz et al. 2007). Among *N. alpina* populations, results suggest that environmental conditions determine a prioritization of light capture and a higher tolerance to cold in Tromen Alto (high value) while excess energy dissipation could be important in Paimún (low value). Although mean temperatures are similar between these two populations, Tromen Alto is a more open forest located at a higher altitude, with a lowest mean annual precipitation than Paimún, and this micro-site conditions might promote more intense frosts.

High correlations between phenological and pigment traits indicate that, in *N. alpina*, the determination of only one pigment concentration could be used as a proxy of bud burst, senescence and growing degree days. Correlations were expected as genetic variation patterns (e.g. more differentiated populations) were similar for both types of traits, suggesting that similar environmental factors are influencing their variation. Phenological evaluations are very time-consuming, therefore using pigment quantification to estimate phenological traits could prompt progeny and provenance trial evaluations. Correlations between both traits and promising results on pigment based vegetation indices have been previously reported in other forest tree species (e.g. Anderson and Ryser, 2015; Wong et al., 2019; Yang et al., 2017).

Globally, the phenotypic plasticity detected in the analysed populations of *N. alpina* suggest flexibility for acclimatization to moderate inter-annual changes in climatic conditions. On the other hand, the genetic control of pigments content and its variation among

populations and watersheds gives a base for adaptation to a long-lasting change in climate.

Future studies must focus on identifying the factors that determine the genetic variation.

Abbreviations: Chlorophyll a (Chla); Chlorophyll b (Chlb); Total chlorophyll (Chla+b); Chlorophyll allocated ratio (Chla / Chlb); Total carotenoids (Car); Anthocyanins (Ant); Monthly mean air temperature (Tmean); Monthly minimum air temperature (Tmin); Monthly maximum air temperature (Tmax); Aridity index (AI); Total precipitation (precip); Rainy days (rd); Linear mixed effects model (LMM); Likelihood ratio test (LRT); Principal component analysis (PCA) Day of the year to bud burst (DOY BB); Growing degree days (basal temperature = 5 °C) (GDD Tb 5° C); Day of the year when 10 % of the crown had autumnal leaves (DOY 10) Growing season length (GSL 10)

5. Ethics approval and consent to participate

Not applicable

6. Consent for publication

Not applicable

7. Availability of data and material

The data will be available upon acceptance in the public institutional repository INTA Digital (www.repositorio.inta.gob.ar)

8. Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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10. Author contributions

PM and VEM conceptualized the study. JAR, PM, VEM performed sample collections. JAR performed pigment quantification and statistical analyses and wrote the manuscript with the contributions of PM and VE. MP installed and maintained the common garden trial. All authors were involved in the final revision and editing process of the manuscript.

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