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RESEARCH ARTICLE

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Land-use legacies influence tree water-use efficiency and nitrogen availability in recently established European forests

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Abstract

1. Forest regrowth following farmland (agriculture and pasture) abandonment has been positively associated with a number of processes including the regulation of hydrological cycling, the enhancement of soil functioning and an increase in forest productivity and carbon (C) sequestration. Although these changes in ecosystem functioning post-farmland abandonment have been observed in multiple locations and studies, the ecophysiological basis underpinning these patterns remains unclear. Here, we examine whether increased forest expansion following pastureland abandonment is associated with greater water-use efficiency (WUE) and legacies from previous land use in terms of nitrogen (N) availability.
2. We thus explored differences in leaf traits and N availability between recently established (post-1950) beech *Fagus sylvatica* (L.) forests on former pastureland and long-established beech forests (pre-1950). The investigated leaf traits were SLA, leaf N concentration (%N) and intrinsic WUE (iWUE, i.e. the ratio between photosynthesis and stomatal conductance); as well, leaf and soil stable N isotope composition (δ^{15}N) and total %N were used to assess changes in N availability. Finally, we compared the correlation strength between the above-mentioned parameters and those associated with tree productivity (wood density and basal area increment, BAI) and the richness of ectomycorrhizal fungi (ECM) in these two forest types.
3. Recent forests had greater iWUE than long-established forests, which was associated more with lower SLA than leaf %N. Leaf and soil δ^{15}N were more robust proxies than %N for detecting differences in N availability. Less negative leaf and soil δ^{15}N values in recent versus long-established forests suggest, on the one hand, greater N availability, probably due to higher historical N input originating from animal excreta on these former pasturelands, and, on the other hand, an increase in N loss pathways.

4. Our results point to greater correlations between leaf δ15N, tree iWUE and productivity in recent forests than in long-established forests, thereby suggesting a close link between C and N cycles. Our findings also highlight different N dynamics between the two forest types, with recent forests showing 'leaky' N cycling wherever lower N retention by trees and associated ECM fungi occurs as a legacy of previous land use.

KEYWORDS

beech forest, forest expansion, land-use change, nitrogen availability, water-use efficiency, δ13C, δ15N

1 | INTRODUCTION

Natural forest regeneration associated with climate and land-use changes such as the abandonment of farmland—the focus of this study—is having profound effects on the landscape and is leading globally to a shift towards increased forest cover (FAO, 2020). The greening of the planet observed in recent decades has been attributed to a combination of CO_2 fertilization, an alteration of nitrogen (N) cycles due to increasing N deposition, and climate and land-use changes (Song et al., 2018; Zhu et al., 2016). Of these drivers, the abandonment of farmland (including agricultural and pasture land) in both temperate (e.g. Buitenwerf et al., 2018) and tropical (e.g. Poorter et al., 2016) regions has notably favoured the spontaneous regrowth of secondary forests. This natural regeneration contrasts with ongoing forest loss due to deforestation (mainly in tropical ecosystems), intensive wildfires and forest dieback associated with drought (Allen et al., 2015; Song et al., 2018).

The regrowth of secondary forest has been shown to have a number of beneficial effects linked to carbon (C) regulation, nutrient and hydrological cycling, a move towards rewilding, and a reduction in soil erosion (Acharya et al., 2018; Navarro & Pereira, 2012). New forests established on former farmland may have higher rates of tree growth (Alfaro-Sánchez et al., 2019), above-ground biomass productivity (Poorter et al., 2016) and forest C storage (Vilà-Cabrera et al., 2017) than long-established forests. Global analyses have highlighted the importance of secondary forests as C sinks (Cook-Patton et al., 2020), which can be greater than that of long-established forests (Pugh et al., 2019).

These results could be related to the way in which the legacy of past land uses affects soil nutrient distribution and availability. Forests establishing themselves on former farmland may benefit from higher N stocks (Nadal-Romero et al., 2018) and mineralization rates (Compton & Boone, 2000), as well as greater microbial (i.e. decomposer) activity (Freschet et al., 2014). This is particularly true in former pastureland, where livestock excreta create patchiness in the distribution of N and other soil nutrients (Augustine, 2003; Fraterrigo et al., 2005). This heterogeneity in nutrient distribution may affect tree establishment (Fraterrigo et al., 2005) and lead to a patchy distribution of tree species. It may also cause microsite differences in soil N dynamics, with those areas with greater N input likely to experience N losses through volatilization, denitrification and nitrate (NO_3^-) leaching (Augustine, 2003). Higher N availability due to previous land use could also affect plant–microbe interactions, specifically root–ectomycorrhizal (ECM) fungi symbiosis. Several studies have revealed a loss in ECM fungi community composition and richness, mycelial production and root tip colonization as N availability increases (e.g. Franklin et al., 2014; Kjøller et al., 2012).

Greater nutrient availability as a legacy of past land use enhances tree growth and productivity, with 25%–35% more plant biomass reported in forests established after land abandonment 50–100 years ago than in long-established pre-existing forests (Freschet et al., 2014; Vilà-Cabrera et al., 2017). However, whether this greater productivity comes at the expense of more water use and loss through transpiration is still not well-understood. Compared to trees in established, late-successional forests, trees in secondary forests—where resource-rich patches and high light conditions exist—may have more acquisitive ecological strategies that include higher SLA, photosynthetic capacity, hydraulic conductance and growth rates, along with lower wood density (e.g. McCulloh et al., 2011; Zhu et al., 2013).

The regrowth of secondary forests on abandoned farmland in Europe has steadily increased from the second half of the 20th century onwards, especially in the uplands of southern and eastern Europe (Améztegui et al., 2010; Buitenwerf et al., 2018; Vilà-Cabrera et al., 2017). Previous studies in the Mediterranean region have documented temporal and spatial changes in vegetation cover (e.g. Álvarez-Martínez et al., 2014; Malandra et al., 2019; Vicente-Serrano et al., 2005) and explored the ecological relevance of forest expansion in terms of changes in soil C and N stocks (Nadal-Romero et al., 2018; Pellis et al., 2019), and tree growth (Alfaro-Sánchez et al., 2019). Yet, the ecophysiological mechanisms underlying forest expansion in this region and whether they are affected by the legacy of prior land use (e.g. nutrient availability) and changes in environmental conditions along an altitudinal gradient are still not well-understood. In the Mediterranean region, higher precipitation and less vapour pressure deficit (VPD) at higher altitudes could alleviate the drought-induced reduction of stomatal conductance, photosynthesis and tree growth (e.g. Peñuelas et al., 2008), as well as any drought-induced limitation on tree N uptake related to both fine root biomass dieback or the lower diffusion and mass flow of N compounds (Kreuzwieser & Gessler, 2010). Moreover, greater soil moisture conditions at higher altitudes could have a positive effect on mineralization and nitrification but could also induce greater NO_3^- leaching (e.g. Cregger et al., 2014). The interacting influences of altitude, precipitation, VPD, soil moisture and soil N dynamics on forest regeneration are generally complex and poorly understood.

To assess some of these uncertainties, our study investigated differences along an altitudinal gradient in ecophysiological strategies and N dynamics between beech *Fagus sylvatica* (L.) forests that have recently become established (post-1950) on former pastureland and long-established beech forests (pre-1950). Specifically, we compared these two forest types in terms of the leaf traits associated with plant physiology (i.e. SLA, leaf area, LA, stable C isotope composition, $\delta^{13}C$) and N availability (i.e. leaf N concentration, %N, stable N isotope composition, $\delta^{15}N$). Leaf $\delta^{13}C$ was used to assess intrinsic water-use efficiency (iWUE, i.e. the ratio between photosynthesis, A, and stomatal conductance, g_s), which is a key physiological metric linking C and water cycling in forest ecosystems (e.g. Guerrieri et al., 2019; Keenan et al., 2013). We also measured soil $\delta^{15}N$ and N concentrations (either as total %N or inorganic N, i.e. ammonium NH_4^+ and NO_3^-), which—together with leaf $\delta^{15}N$—were used to describe variations in N availability (Craine et al., 2015) due to changes in land use, and its link to forest productivity (Ruiz-Navarro et al., 2016). A positive relationship between leaf %N and $\delta^{15}N$ is to be expected with an increase in N availability, which also reflects an increase in N loss pathways due to denitrification and NO_3^- leaching (e.g. Craine et al., 2015; Högberg et al., 1997). Finally, to elucidate differences between these two forest types in ecophysiological strategies, productivity and N availability, we correlated the leaf and soil parameters measured in this study with those from two related studies performed at the same site, one on wood traits (i.e. basal area increment and wood density; Alfaro-Sánchez et al., 2019) and the other on ECM fungi richness (Correia et al., 2021).

We hypothesized that (1) greater N availability due to N input from livestock excreta would lead to higher tree iWUE and to an increase in N loss pathways in recent compared to long-established forests. Additionally, we expected that (2) trees in recent forests would show greater coupling between C and N cycles, leading to (a) stronger correlations (i.e. greater coordination, Wright et al., 2004) between traits associated with physiology, tree productivity and N availability in recently established versus long-established forests, but also (b) to less N retention by trees and associated ECM fungi as a result of faster soil biogeochemical N processes. Finally, we hypothesized that (3) along the elevation gradient, a reduction in iWUE with increasing altitude would be observed in both recent and long-established forests, due to improvement in moisture conditions.

2 | MATERIALS AND METHODS

2.1 | Sites description and sampling

Twelve and six plots were established in recent and long-established beech-dominated forests, respectively, along an altitudinal gradient ranging from 831 to 1,452 m a.s.l. in Catalonia, NE Spain (Figure 1; see Alfaro-Sánchez et al., 2019 for further details). Plots were classified as either 'recent' or 'long-established' forests using aerial photographs taken in 1956–1957 and 2005 (http://www.creaf.uab.cat/mcsc). Briefly, patches that already had forest cover in the orthoimages from 1956 to 1957 were classified as 'long-established', while plots that had forest cover in the orthoimages from 2005 but pasture cover in 1956–1957 were classified as 'recent' (Figure 1; see Başnou et al., 2013; Vilà-Cabrera et al., 2017 for a similar approach). Although the timeline for recent forests can be traced back to the 1950s, it is extremely difficult to identify it for long-established forests as historically in this region the exploitation of natural resources has been intense, and significant changes in land use have continuously occurred over the last century. Nevertheless, bearing in mind that the long-established forests already appeared in the aerial photographs from the 1950s as mature forests, we can assume for them a timeline of at least 100 years. Hence, the two forest types were defined by the age of the land use and not by the stand age (see Mausolf et al., 2018 for similar criteria). This is important because long-established forests in the area may have been managed by selective thinning after single-tree or group selection since as far back as the 1950s; thus, they may not have the age structure of old-growth forests and their physiognomy may resemble that of recent forests (see Figure 1e,f). Indeed, the plots of the two forest types considered in this study did not differ in terms of their main structural characteristics (i.e. tree density, basal area and tree height; Table 1, Alfaro-Sánchez et al., 2019).

Within each plot, 30 beech trees with a DBH ≥ 7.5 cm were selected and mapped (540 trees in total). In summer 2017, one branch from the sun-exposed top of the canopy was collected from each tree using a pole pruner. Ten leaves were randomly selected from each branch, immediately placed in plastic bags and then stored in a portable freezer until transportation to the laboratory for subsequent measurements. For each sampled tree, the DBH was measured, and wood cores were sampled ($n = 2$) at 50 cm above-ground level using a Pressler core (5 mm) to determine tree age, growth, basal area increment (BAI) and wood density (Alfaro-Sánchez et al., 2019).

In November 2017, soil samples were collected within eight and six plots for recent and long-established forests, respectively, next to 10 trees randomly selected from among those used to sample leaves and wood cores. Three samples were extracted by digging $10 \times 10 \times 15$-cm holes at a distance of 1 m from the tree trunk in three directions (north, southeast and southwest). These soil samples were pooled to obtain a single composite sample for chemical analysis for each tree (total 128 samples, $n = 52$ for long-established forests and $n = 75$ for recent forests).

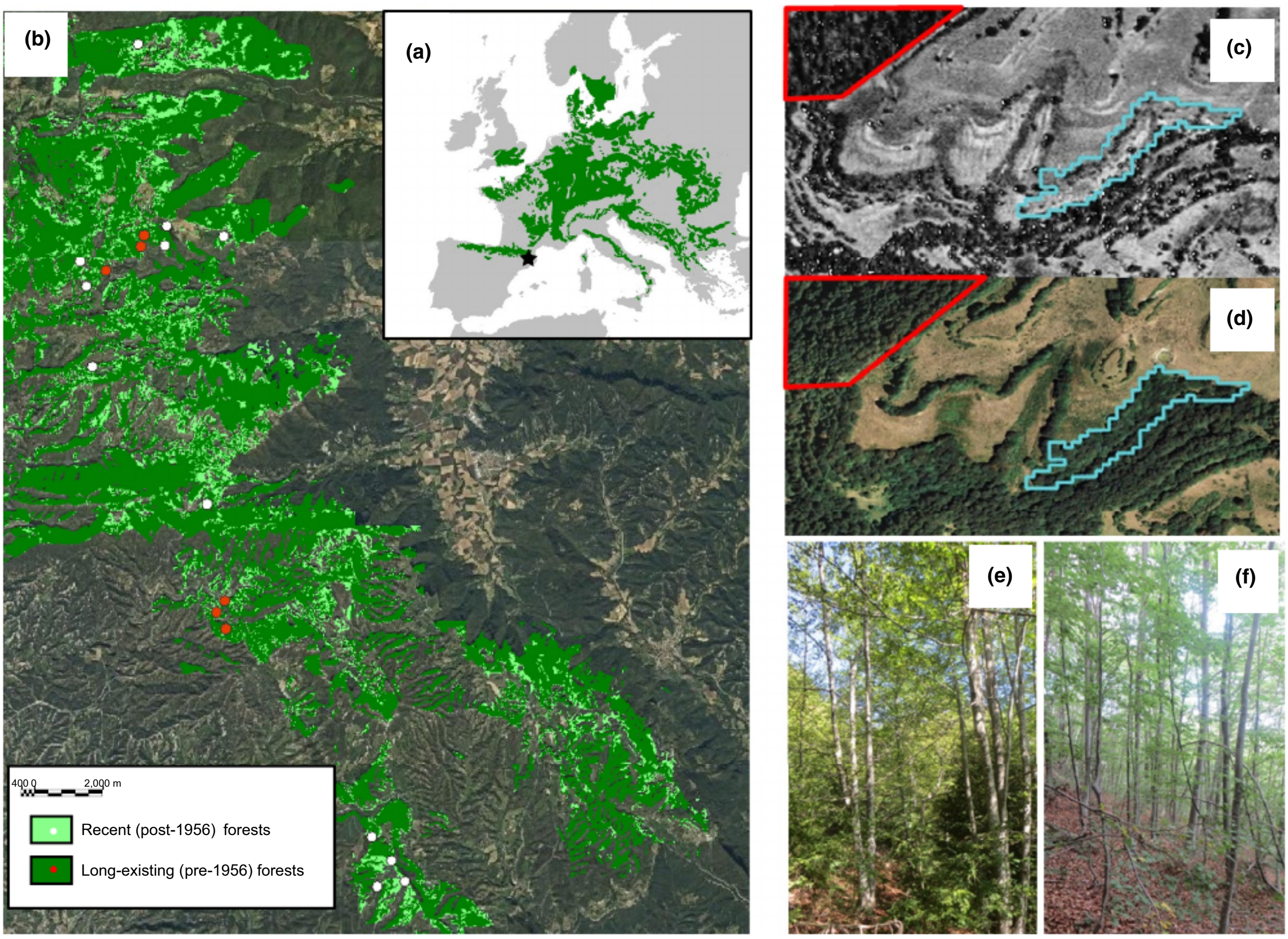

FIGURE 1 Map showing the studied forests. Distribution of European beech *Fagus sylvatica* in Europe and location of the study area (black star) in Catalonia (NE Spain, panel a). Presence of long-established (dark green) and recent (light green) beech forests in the study area, and location of the plots sampled in each forest type (red and white points, respectively, panel b). Orthoimages from 1956 (panel c) and 2005 (panel d) of part of the study area comprising a long-established (pre-1956) and a recent (post-1956) beech-forest patch (outlined in red and blue respectively). Note that the long-established (panel e) and recent (panel f) forests may have very similar physiognomies, and our study plots did not differ in tree density, basal area or tree height (Alfaro-Sánchez et al., 2019) owing to the management practices carried out in long-established forests

TABLE 1 Sites description. Altitude and structural characteristics (Mean and standard error, *SE*) for long-established and recent beech forests included in the study. DBH = Diameter at breast height, BA = Basal area, BAI = Basal area increment. Stars indicate significant differences between the two forests types for a given parameter (see Alfaro-Sánchez et al., 2019 for more details)

	Altitude (m)	Tree age (years)		Tree density (trees/ha)		DBH (cm)		BA (m^2/ha)		Tree height (m)		Ring width (mm)		BAI (mm^2/year)	
Forest type	Range	Mean	*SE*	Mean	*SE*	Mean	*SE*	Mean	*SE*	Mean	*SE*	Mean	*SE*	Mean	*SE*
Long-established	831–1,333	65*	1.3	1,413	36	19.6	0.5	29.7	0.4	16.4	0.2	1.50	0.010	558	6
Recently established	1,027–1,452	38	0.6	1,662	56	18.5	0.3	31.3	0.6	17.1	0.3	2.35*	0.013	806*	8

2.2 | Sample preparation in the laboratory and chemical analyses

All collected leaves were individually scanned with the ImageJ software at 1,600 d.p.i. on the day of the field sampling to calculate their one-sided LA surface (Schindelin et al., 2012). Then, leaves were oven-dried at 60°C for 72 hr and individually weighed with a precision scale to obtain their dry weight. The SLA was calculated as LA per unit of dry weight expressed in cm^2/g.

All soil samples were sieved (<2-mm mesh size) to remove stones, roots and undecomposed leaf litter. Samples were oven-dried at 80°C for 48 hr before chemical analyses. A subset of samples was considered for isotope analyses, which were randomly selected from the collected samples across the two forest types ($n = 57$ in total, 32 samples for recent forests and 25 samples for long-established forests).

2.2.1 | Leaf and soil N concentrations and stable isotope analyses

Leaf and soil samples were finely ground using a ball mill (MM300). Between 3 and 4 mg of the ground leaf and soil samples were weighed and placed in tin capsules to measure total C and N concentrations (expressed on a % mass basis), $\delta^{13}C$ (only for leaf samples) and $\delta^{15}N$ (for both soil and leaf samples). Analyses were carried out at the UC Davis Stable Isotope Facility using an elemental analyser (PDZ Europe ANCA-GSL) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer. Stable C and N isotope ratios were expressed in 'per mil' notation (‰) relative to international standards, that is, Vienna PeeDee belemnite (V-PDB) and atmospheric N_2 respectively. Leaf N concentrations were also expressed on a leaf area-based (N_{area}, g/m^2) by calculating the ratio between leaf N mass and SLA. We also calculated the difference between leaf and soil $\delta^{15}N$ values, that is, $\delta^{15}N_l$-$\delta^{15}N_s$, to account for spatial differences in the source of $\delta^{15}N_s$ and to improve the possibilities of detecting changes in N processes (Craine et al., 2015).

2.2.2 | Soil chemical analyses

The determination of organic matter (% OM), inorganic N (ammonium NH_4^+ and nitrate NO_3^-) and phosphorus (phosphate PO_4^{3-}) compounds was performed following standard protocols (Rodríguez et al., 2009). The soil organic matter was assessed after the combustion of samples at 550°C (Rosell et al., 2001). Inorganic N was obtained by extracting NH_4^+ and NO_3^- from soil samples with KCl and quantifying their concentration using colorimetry (indophenol blue method) in a microplate reader (Sims et al., 1995). Soil PO_4^{3-} was extracted from soil samples with acetic acid, and its concentration was calorimetrically determined using the molybdenum blue method (Allen et al., 1986).

2.3 | Calculation of intrinsic water-use efficiency from leaf $\delta^{13}C$

Intrinsic water-use efficiency, iWUE (Ehleringer et al., 1993), was calculated from leaf C isotope discrimination ($\Delta^{13}C_l$) based on the well-established theory that links $\Delta^{13}C$ and the leaf internal CO_2 (c_i) to atmospheric CO_2 (c_a) ratio, that is, c_i/c_a (Farquhar et al., 1989):

$$\Delta^{13} C_l = a + (b - a)\frac{c_i}{c_a}, \tag{1}$$

where a is the isotope fractionation during CO_2 diffusion through stomata (4.4‰) and b is the isotope fractionation during fixation by Rubisco (27‰). $\Delta^{13}C_l$ can be derived from the $\delta^{13}C$ measured in leaf samples as follows:

$$\Delta^{13}C_l = \frac{(\delta^{13}C_a - \delta^{13}C_l)}{\left(1 + \frac{\delta^{13}C_l}{1,000}\right)}, \tag{2}$$

where $\delta^{13}C_l$ and $\delta^{13}C_a$ are the C isotope compositions of leaf and atmospheric CO_2 respectively. The c_a and $\delta^{13}C_a$ values for the year of leaf samples collection were obtained from Mauna Loa records (Scripps, 2018). We calculated c_i by resolving from the $\Delta^{13}C_i$:

$$c_i = c_a \frac{\delta^{13}C_a - \delta^{13}C_l - a}{b - a}. \tag{3}$$

Finally, iWUE is obtained as follows:

$$\mathrm{iWUE} = \frac{A}{g_s} = \frac{c_a - c_i}{1.6} = \frac{c_a}{1.6}\left(\frac{\Delta^{13}C_l}{b - a}\right), \tag{4}$$

where 1.6 is the molar diffusivity ratio of CO_2 versus H_2O (i.e. $g_{CO_2} = g_{H_2O}/1.6$). For both c_a and $\delta^{13}C_a$, mean values during the growing season (May–August, given that our leaf samples were collected in August) were included in Equations 2–4.

2.4 | Statistical analyses

Independent sample t-test and nonparametric Kruskal–Wallis rank-sum tests were used to assess differences between recently and long-established forests in the raw measured parameters associated with physiology and N availability (Hypothesis 1). A nonparametric test was used for the parameters where the normality of data and homogeneity of variance conditions were not met, that is, SLA, leaf %N (%N_l) and $\delta^{15}N_l$.

The effects of forest type (Hypothesis 1) and altitude (Hypothesis 3) on leaf traits associated with physiology and N availability were assessed by employing linear mixed effects models (LMMs; package NLME, Pinheiro et al., 2017). Specifically, leaf traits (i.e. iWUE, SLA and %N_l) were included in the LMMs as dependent variables, while forest type (categorical variable, recent vs. long-established), altitude and tree-related parameters (tree age, wood density and DBH) were considered as fixed factors. Only in the case of iWUE did we also include SLA and %N_l as fixed factors. For the subset of samples for which soil %N (%N_s) and $\delta^{15}N_s$ were available, we also ran linear model (LM) and LMMs to investigate variations in %N_s, $\delta^{15}N_s$ and the difference $\delta^{15}N_l$-$\delta^{15}N_s$ (dependant variables) as a function of altitude, forest type and soil parameters (soil organic matter %, NH_4^+ and NO_3^- concentrations) as independent variables. In all the tested models, altitude was used as a proxy of environmental conditions, particularly in reference to moisture (i.e. greater precipitation, lower temperatures and VPD with altitude; e.g. Körner, 2007). An altitude × forest type interaction term was also included in the LMMs to assess differences between recent and long-established forests along the altitudinal gradient for iWUE.

There was no multicollinearity between fixed factors, as indicated by *R* values ≤0.7 in the correlation matrix of fixed effects. The model included the different plots within each forest type as a random effect. Marginal (only fixed factors) and conditional (fixed + random factors) proportions of the explained variance for each dependent variable (R^2_m and R^2_c respectively) were also calculated (package MuMIn, Bartoń, 2017). For each dependent variable, we first started with the simple LM and then increased the complexity (i.e. the random effect to account for variability between plots within each forest type). A final predictive model for each variable was selected based on the criteria of minimizing the Akaike information criterion (AIC), as well as on the quality of fitness assessed using residual distribution plots and quantile–quantile (*Q-Q*) plots (Zurr et al., 2009). In the case of the $\%N_s$ and the difference $\delta^{15}N_l$-$\delta^{15}N_s$, we found that including the random factor did not improve the model, that is, marginal and conditional R^2 were identical and AIC values were not different between LMM and LM. Hence, the results from the LM analyses were used for these variables. Finally, a log-transformation of the data was necessary to improve the model fitting for SLA and $\%N_s$. A description of the final models is given in Table S1.

To test for differences between the two forest types in resource-use strategies (Hypothesis 2), we assessed the strength and directionality of correlations between parameters associated with physiology, tree productivity and N availability in recently established versus long-established forests. Spearman rank correlation matrices (package GGCORRPLOT, Kassambara, 2019) were applied to account for non-normality and heteroscedasticity in some of the variables (i.e. SLA, $\%N_l$ and $\delta^{15}N_l$). Three analyses were carried out using (a) the full dataset (540 observations) including leaf traits as well as wood density; (b) the subset of trees for which tree productivity data, evaluated as BAI from 2016 (384 observations) and 2017 (334 observations), were available (from Alfaro-Sánchez et al., 2019) and (c) the subset of trees for which $\delta^{15}N_s$ data were available; in this latter case, we also included data on ECM fungal species richness taken from Correia et al. (2021) (54 observations). All variables included in the analyses are shown in Table S1. All statistical analyses and data visualization (GGPLOT2 package, Wickham, 2016) were carried out in R Studio Version 1.1.456 (RStudio Team, 2016) with R version 3.5.1 (R Core Team, 2018).

3 | RESULTS

3.1 | Leaf traits in recent and long-established beech forests

Nonparametric tests showed that recent forests had on average lower median SLAs and higher median leaf N concentrations (both $\%N_l$ or N_{area}) than long-established forests (Table 2; Figure 2a,b, inset panels). Recent forests had higher mean iWUE than long-established forests, with a difference of 1 µmol/mol (Table 2; Figure 2c, inset panel). Leaves of trees from recent forests were more enriched in the heavier isotope ^{15}N (i.e. had less negative $\delta^{15}N_l$ values) than those from long-established forests (Table 2).

The LMM analyses indicated that forest type had a significant effect on SLA, iWUE and $\delta^{15}N_l$ values but not on $\%N_l$ (Table 3). Indeed, SLA had a higher estimate in the long-established forests than in the recent forests; however, estimates were not different between the two forest types for $\%N_l$ or N_{area} (Table 3; Table S1). Conversely, we found higher estimates for iWUE and $\delta^{15}N_l$ in recent forests than in long-established forests (Table 3).

TABLE 2 Differences between long- and recently established forests for the investigated parameters. Results from the independent sample *t* test (for iWUE, $\Delta^{13}C_l$, soil %N and stable nitrogen isotope composition, $\delta^{15}N_s$, soil NH_4^+, soil NO_3^- and, the difference between leaf and soil $\delta^{15}N$, $\delta^{15}N_l$-$\delta^{15}N_s$) and nonparametric Kruskal–Wallis test (for SLA, leaf %N, leaf stable nitrogen isotope composition, $\delta^{15}N_l$) used to test for differences between recently and long-established forests for the investigated leaf traits and soil parameters. We report the mean (for parametric test) and median (for nonparametric test) and standard deviation (*SD*), degree of freedom (*df*), the statistic (*t*-values or Kruskal–Wallis chi-squared values, χ^2) and the level of significance (*p*-value). In the unit for soil NH_4^+ and NO_3^- dsw refers to dry soil weight

Parameter (unit)	Mean/median (*SD*) Recent	Long-established	*df*	Statistic χ^2-value	*t*-value	*p*-value
SLA (cm^2/g)	181 (48.7)	240 (54.9)	1	127.84		<0.001
Leaf %N	2.59 (0.44)	2.47 (0.28)	1	13.88		<0.01
Leaf N_{area} (g/cm^2)	1.40 (0.40)	1.03 (0.26)	1	134.55		<0.001
$\delta^{15}N_l$ (‰)	−3.64 (1.24)	−5.78 (1.17)	1	215.05		<0.001
iWUE (µmol/mol)	63.64 (13.2)	53.81 (9.95)	456.5		9.66	<0.001
$\Delta^{13}C_l$ (‰)	22.00 (1.24)	22.92 (0.93)	455.8		−9.65	<0.001
Soil %N	0.95 (0.44)	1.20 (0.48)	49.53		−2.02	<0.05
Soil NH_4^+ (mg/kg dsw)	25.41 (14.9)	27.93 (16.3)	49.35		−0.60	0.55
Soil NO_3^- (mg/kg dsw)	29.05 (28.9)	25.65 (10.6)	41.06		0.61	0.54
$\delta^{15}N_s$ (‰)	−1.44 (0.81)	−2.99 (1.19)	40.4		5.57	<0.001
$\delta^{15}N_l$-$\delta^{15}N_s$ (‰)	−2.44 (1.22)	−2.83 (1.18)	52.49		1.24	0.22

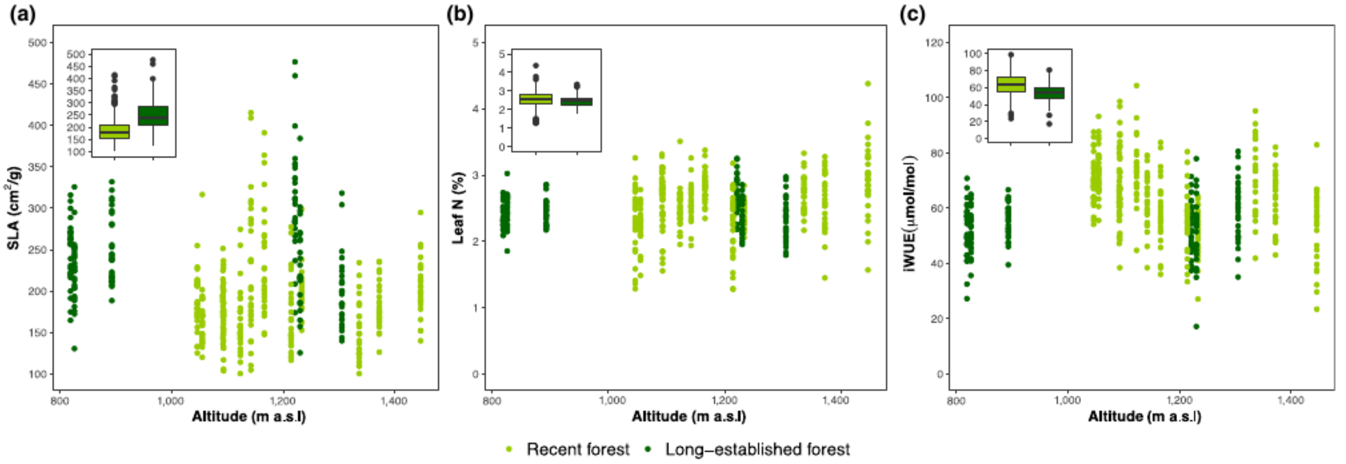

FIGURE 2 Changes in leaf traits along an altitudinal gradient. Changes in SLA (panel a), leaf N concentrations (%N, panel b) and intrinsic water-use efficiency (iWUE, panel c) in recent and long-established beech forests along an altitudinal gradient. Boxplots in the inset panels show changes in SLA, leaf N % and iWUE for the two forest types

Altitude was not always a significant predictor in the LMMs. Neither the SLA, the $\%N_l$ nor $\delta^{15}N_l$ (Table 3; Figure 2a,b) changed with altitude, nor the interaction between forest type × altitude was a significant predictor in the model (Supporting Information Text S1). However, we did note a significant negative effect for altitude in the case of iWUE, though slopes of the relationship between iWUE and altitude were different for the two forest types, as suggested by the significant effect for the altitude × forest type interaction (Figure S1; Table 3).

In the studied forests, SLA increased with $\%N_l$ and tree age but decreased with DBH (Table 3); no significant relationship was found between SLA and wood density (Supporting Information Text S1). Only in the case of N_{area}, a positive relationship with DBH was found (Table S2). iWUE was negatively correlated with SLA but positively correlated with $\%N_l$; neither tree age nor DBH were significant predictors in the model (Table 3). Including wood density did not improve the LMM (Supporting Information Text S1). Finally, $\delta^{15}N_l$ was positively correlated with $\%N_l$ (Figure 3a) and DBH, but negatively correlated with tree age.

3.2 | Soil N concentrations and δ^{15}N in recent and long-established forests

An independent *t*-test showed that recent forests had lower mean $\%N_s$ but less negative $\delta^{15}N_s$ than long-established forests. No differences were observed in the mean NH_4^- and NO_3^- values between the two forest types (Table 2).

Results from LMMs indicated that forest type had only a significant effect for $\delta^{15}N_s$, as estimates in recent compared to long-established forests were less negative (Table 4). There was no significant effect of altitude on either $\%N_s$ or $\delta^{15}N_s$ (Table 4). No significant relationships were found between $\%N_s$ and NH_4^+ or NO_3^-, although changes in $\%N_s$ were positively related to changes in the percentage of organic matter (%OM) in the sampled top soil (Table 4). Negative relationships between $\delta^{15}N_s$ and $\%N_s$ were observed; changes in $\delta^{15}N_s$ were not significantly associated with either changes in NH_4^+, NO_3^- or % OM (Table 4).

A positive relationship ($p < 0.001$, $R^2 = 0.42$) was found between leaf and soil δ^{15}N (Figure 3b). In general, $\delta^{15}N_l$ values were more negative than $\delta^{15}N_s$, with differences between the two (i.e. $\delta^{15}N_l$-$\delta^{15}N_s$) ranging between −2.44‰ and +0.48‰ in recent forests, and −2.83‰ and −0.89‰ in long-established forests, though difference in the mean values was not significant (Table 2). We found a significant effect for forest type on the difference $\delta^{15}N_l$-$\delta^{15}N_s$, with significantly lower estimate in recent than in long-established forests (Table 4). $\delta^{15}N_l$-$\delta^{15}N_s$ significantly increased with $\%N_s$; no significant relationships were found between $\delta^{15}N_l$-$\delta^{15}N_s$ and inorganic N forms or % OM across the studied forests (Table 4).

3.3 | Differences between forest types in correlations between leaf and tree traits and N availability

The strength and directionality of correlations between leaf and tree parameters and proxies of N availability for each forest type were assessed using Spearman rank correlation analyses (Figure 4; Table S1, Supporting Information Text S2). Both recent and long-established forests showed a negative correlation between iWUE and SLA, and positive correlations for SLA with $\%N_l$ and $\Delta^{13}C_l$, as well as positive correlations for $\delta^{15}N_l$ with $\%N_l$ and LA with $\delta^{15}N_l$ and $\%N_l$. Positive correlations for iWUE with wood density and of $\delta^{15}N_l$ with altitude only existed in recent forests. Similarly, negative correlations for SLA with wood density, for wood density with $\Delta^{13}C_l$ and $\delta^{15}N_l$, and for iWUE with $\delta^{15}N_l$ were only found in recent forests (Figure 4). We only detected significant negative correlations between iWUE and BAI2016 in recent forests. Both forest types showed significant positive correlations for $\delta^{15}N_l$ with BAI (both in 2016 and 2017; Figure S2).

TABLE 3 Effects of forest type, altitude and tree parameters on leaf traits. Results of linear mixed effect models for SLA, leaf nitrogen (%N_l), intrinsic water-use efficiency (iWUE) and leaf stable nitrogen isotope composition ($\delta^{15}N_l$). Forest type (long-established vs. recent), altitude and tree parameters (age and DBH) were included as fixed factors. SLA and %N_l were moreover included as fixed factors in the model for iWUE. Different plots for each forest type were included as random factor in the model. Note that log-transformation was required for SLA in order to improve the quality of the fit (particularly for the normality of residuals). For each model we also provide the proportion of variance explained by fixed factors only (marginal R^2, R^2_m) and fixed + random factors (conditional R^2, R^2_c). *SD* in the random factor indicates standard deviation

					Random effect (*SD*)			
Fixed effect	**Estimate**	***SE***	***t*-value**	***p*-value**	**Plots**	**Residual**	R^2_m	R^2_c
SLA								
Intercept	5.05	0.24	21.14	<0.001	0.12	0.21	0.25	0.44
Long-established versus recent	0.23	0.07	3.38	<0.01				
Altitude	0.000014	0.0002	0.07	0.07				
DBH	−0.007	0.0016	−4.23	<0.001				
Tree age	0.0022	0.0007	2.95	<0.01				
%N_l	0.066	0.027	2.42	<0.05				
%N_l								
Intercept	2.01	0.35	5.76	<0.001	0.17	0.34	0.06	0.25
Long-established versus recent	−0.04	0.10	−0.37	0.71				
Altitude	0.0004	0.0003	1.42	0.18				
DBH	0.0008	0.003	0.29	0.76				
Tree age	−0.002	0.001	−1.34	0.18				
SLA	0.0007	0.0003	2.08	<0.05				
iWUE								
Intercept	113.37	13.41	8.46	<0.001	4.25	9.51	0.36	0.47
Long-established versus recent	−49.37	16.27	−3.03	<0.01				
Altitude	−0.04	0.01	−3.29	<0.01				
DBH	0.06	0.07	0.82	0.41				
Tree age	−0.06	0.03	−1.80	0.07				
SLA	−0.09	0.009	−10.06	<0.001				
%N_l	4.79	1.27	3.78	<0.001				
Long-established × altitude	0.04	0.014	2.80	<0.05				
$\delta^{15}N_l$								
Intercept	−6.96	1.24	−5.62	<0.001	0.62	0.94	0.47	0.63
Long-established versus recent	−1.75	0.36	−4.82	<0.001				
Altitude	0.001	0.001	1.44	0.17				
DBH	0.03	0.008	4.39	<0.001				
Tree age	−0.009	0.003	−2.58	<0.05				
SLA	0.002	0.001	1.78	0.07				
%N_l	0.38	0.13	3.01	<0.05				

Both forests had a negative correlation between $\delta^{15}N_s$ and %N_s and %OM; only in long-established forests was $\delta^{15}N_s$ negatively correlated with NH_4^+ and soil PO_4^{3-} concentrations (Figure 5). In recent forests, a negative correlation for ECM fungal species richness with soil PO_4^{3-} concentrations and %N_s was found (Figure 5). For both forest types, the difference $\delta^{15}N_l$-$\delta^{15}N_s$ was significantly and positively correlated with %N_s, but it was positively correlated with NH_4^+ concentrations in long-established forests, and with NO_3^- in recent forests (Figure 5).

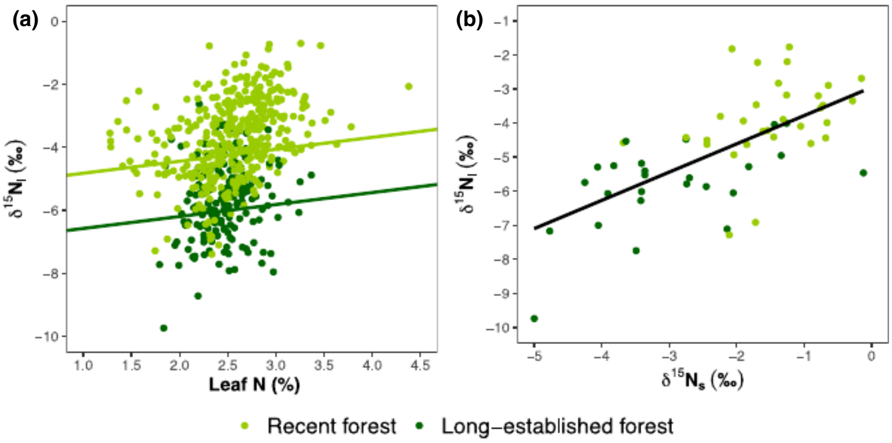

FIGURE 3 Relationships between leaf and soil nitrogen-related parameters. Relationship between leaf $\delta^{15}N$ ($\delta^{15}N_l$) and leaf %N (panel a) and soil $\delta^{15}N$ ($\delta^{15}N_s$, panel b). The regression lines in panel (a) were fitted by considering the output from the linear mixed effect model shown in Table 3, namely −6.96‰ as the global intercept, which refers to long-established forest, and −5.21‰ as intercept for recent forest, obtained as difference between the global intercept and the estimate reported in the table (−1.75), and 0.38‰ per %N for the slope. Slopes ± *SE* values for the linear regression shown in panel (b) are: 0.80 ± 0.13 ($R^2 = 0.42$, $p < 0.001$)

TABLE 4 Effects of forest type, altitude and soil parameters on soil %N, $\delta^{15}N$ and the difference between leaf and soil $\delta^{15}N$. Results of linear mixed effect (for stable nitrogen isotope composition in soil, $\delta^{15}N_s$) and linear regression (for soil %N, $\%N_s$, and the difference between leaf and soil $\delta^{15}N$, $\delta^{15}N_l$-$\delta^{15}N_s$) analyses. Forest type (long-established vs. recent), altitude and soil parameters (organic matter %, NH_4^+ and NO_3^-) were included as fixed factors, while different plots for each forest type were included as random factor in the model. The unit for NH_4^+ and NO_3^- is mg/kg dry soil sample. We also provide the proportion of variance explained by fixed factors only (marginal R^2, R^2_m) and fixed + random factors (conditional R^2, R^2_c). Simple linear regression analyses (LM) were more appropriate in the case of %N and $\delta^{15}N_l$-$\delta^{15}N_s$ models. Note that log-transformation was required in the case of $\%N_s$, to improve the quality of the model fit (particularly the normality of residuals). *SD* in the Random factor indicates standard deviation

Linear mixed effect model

					Random effect (*SD*)			
Fixed effect	**Estimate**	***SE***	***t*-value**	***p*-value**	**Plots**	**Residuals**	R^2_m	R^2_c
$\delta^{15}N_s$								
Intercept	−0.28	1.16	−0.25	0.81	0.48	0.50	0.71	0.85
Long-established versus recent	−0.99	0.35	−2.83	<0.05				
Altitude	0.0005	0.001	0.39	0.70				
$\%N_s$	−1.40	0.32	−4.43	<0.001				
NH_4^+	−0.0002	0.007	−0.04	0.97				
NO_3^-	0.004	0.005	0.79	0.44				
Organic matter %	−0.01	0.008	−1.55	0.13				

Linear regression

Factor	**Estimate**	***SE***	***t*-value**	***p*-value**	R^2
$\%N_s$					
Intercept	−1.48	0.26	−5.77	<0.001	0.72
Long-established versus recent	0.12	0.09	1.44	0.16	
Altitude	0.0006	0.0003	2.61	<0.05	
NH_4^+	0.0025	0.0026	0.96	0.34	
NO_3^-	0.0005	0.0016	0.29	0.77	
Organic matter %	0.015	0.003	5.26	<0.001	
$\delta^{15}N_l$-$\delta^{15}N_s$					
Intercept	−2.03	1.08	−1.87	0.06	0.37
Long-established versus recent	−1.01	0.35	−2.91	<0.01	
Altitude	−0.002	0.001	−1.7	0.09	
$\%N_s$	1.93	0.54	3.56	<0.001	
NH_4^+	−0.008	0.011	−0.77	0.45	
NO_3^-	0.006	0.006	0.87	0.39	
Organic matter %	−0.0002	0.014	−0.015	0.99	

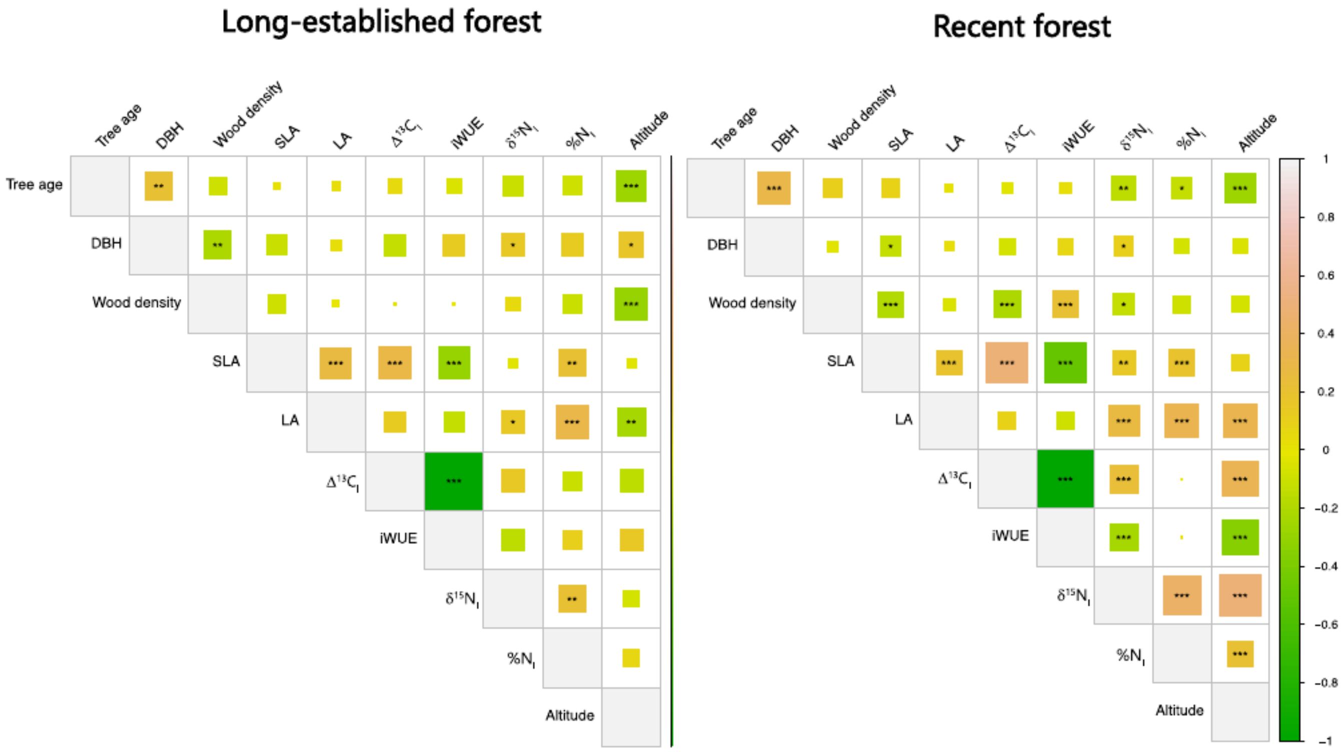

FIGURE 4 Correlation matrix including leaf, tree parameters and altitude for the two forest types. Results of a Spearman rank correlation analyses carried out for recent and long-established forests to assess differences in the strength and directionality of correlations between the leaf traits and tree parameters considered in this study. Gradient colour indicates Spearman correlation coefficients ranging from −1 to +1. Stars indicate the level of significance: (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$. Full results are reported in the Supporting Information Text S2

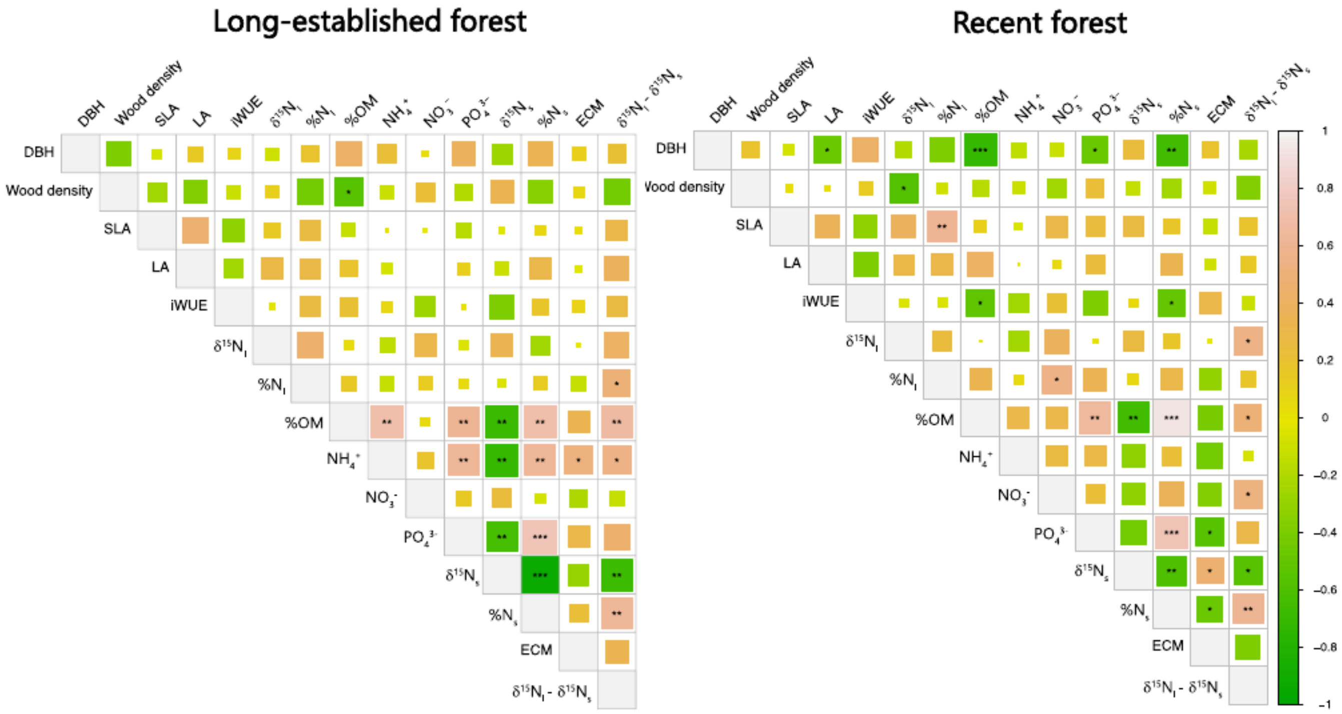

FIGURE 5 Correlation matrix including leaf, tree and soil parameters for the two forest types. Results of Spearman rank correlation analyses carried out for recent and long-established forests to assess differences in the strength and directionality of correlations between the tree and soil parameters obtained in this study and a related study performed at the same forest sites (i.e. ECM fungi richness, from Correia et al. 2021). Gradient colour indicates Spearman correlation coefficients ranging from −1 to +1. Stars indicate the level of significance: (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$. Full results are reported in the Supporting Information Text S2

4 | DISCUSSION

4.1 | Do recent and long-established forests differ in terms of leaf traits?

As expected, trees in recent forests had higher iWUE than those in long-established forests. The physiological mechanism underlying this difference seems to be associated more with a (i.e. thicker and/or denser leaves) than with a higher %N, suggesting a more conservative strategy occurred in trees in recent versus long-established forests to minimize transpirative water losses. An explanation for the observed difference in SLA could be that trees from recent forests experienced high levels of solar radiation during their establishment on former pastureland and so developed a leaf structure that was able to cope with higher evaporative demand. Thus, the lower SLA—and higher iWUE—in recent forests could be seen as an adaptation to water limitation that trees may have undergone during their establishment and hence represents an 'ecological memory' (Johnstone et al., 2016; Mausolf et al., 2018) of the shift between land uses (pastureland towards 'new' forest). Previous studies reported a reduction in SLA and enhancement of iWUE (Poorter et al., 2009; Rosas et al., 2019) as well as a negative relationship between iWUE and leaf mass per area (LMA)—the inverse of SLA (Medrano et al., 2009; Prieto et al., 2018) with increasing aridity. Moreover, lower SLA has been associated also with lower mesophyll and hydraulic conductances—the former controlling the CO_2 diffusion from the leaf intercellular space to the chloroplast, and the latter controlling the water flow from xylem through leaf—both contributing to reducing *A* and, to greater extent, transpiration (e.g. Flexas et al., 2013; Théroux-Rancourt et al., 2014).

Differences in SLA could also arise from the tree establishment process itself in the two forest types and its effects on the size and architecture of root systems. Whereas all extant trees in the recent forests naturally grew from seeds (as these patches have never been exploited), some trees in long-established forests may have resprouted after past logging events carried out as part of the traditional forest management (coppicing) practiced in beech forests in southern Europe (Cullotta et al., 2016). Such trees may have higher SLA and lower iWUE due to the benefits of a deep, well-developed root system (Alfaro-Sánchez et al., 2020; Castell et al., 1994), which would set them apart from recently established forests (Mausolf et al., 2018).

4.2 | Leaf nitrogen isotopic composition is a better proxy than %N for detecting changes in N dynamics

Forest regrowth on abandoned pastureland has a profound effect on soil properties, nutrients and C and N stocks, even though any changes may only become apparent decades after forest establishment (Li et al., 2012; Pellis et al., 2019). Our test to ascertain whether there was any legacy of previous land use in ecosystem N availability revealed no differences between the two forest types on soil NH_4^+ and NO_3^- concentrations (Table 2) and no effects of forest type or on $\%N_s$ (Table 4). This result could be explained by the spatial heterogeneity in nutrient availability and the dynamics of nutrient cycling, which can be difficult to detect when looking at average values (Fraterrigo et al., 2005).

Studies along environmental gradients report that increases in N availability are likely to be reflected in more positive $\delta^{15}N_l$ values (Craine et al., 2009, 2015). Our results also confirmed this pattern, as positive relationships between $\delta^{15}N_l$ and $\%N_l$ and $\delta^{15}N_s$ were observed in the studied beech forests. Specifically, our data suggest that $\delta^{15}N_l$ and $\delta^{15}N_s$ were better indicators than $\%N_l$ and $\%N_s$ of the legacy of previous land use on ecosystem N dynamics, since soil and leaves in recent forests were more enriched in ^{15}N than long-established forests. This result suggests that in recent forests there is a greater contribution from external N input, putatively from livestock excreta, more enriched in heavier isotopes (Choi et al., 2017) and/or that they experienced an increase in N loss pathways, which would confirm our first hypothesis. Indeed, under high N availability, losses of N through NO_3^- leaching, denitrification and, particularly, ammonia volatilization from urea (the main N-compound in excreta) can lead to significant enrichment in ^{15}N in the residual soil N pools, which is then reflected in an increase in $\delta^{15}N_l$ (Craine et al., 2015; Högberg, 1997).

4.3 | Greater coupling between leaf physiology, tree productivity and N dynamics in recent forests

As hypothesized, we found greater coordination (i.e. correlation strength, ref. e.g. Rosas et al., 2019; Wright et al., 2004) between traits associated with physiology, productivity and N availability in recent forests than in long-established forests. The putative water conservative strategy of trees recently established on former pasturelands is again confirmed by the negative correlation between SLA and wood density, and by the positive correlation between iWUE and wood density. Greater wood density implies greater C investment in building a safer water transport system, which reduces the risk of cavitation (e.g. Chave et al., 2009; Hacke et al., 2001) and so enhances iWUE (via a reduction in the transpirative water loss).

The strong correlations between iWUE and leaf %N across the two forest types, and between iWUE or BAI and $\delta^{15}N_l$ (only in recent forests) suggest a positive effect of higher N availability on photosynthesis (Wright et al., 2004), which will ultimately lead to greater productivity. The latter points to $\delta^{15}N_l$ being a more reliable proxy of N availability and its link to tree productivity than either $\%N_s$ or $\delta^{15}N_s$, and suggests that the results reported for tree and shrub species in dry landscapes (Ruiz-Navarro et al., 2016) are relevant too to beech forests at high altitudes. However, the negative correlation—or no correlation—between iWUE and BAI in recent and long-established forests, respectively, suggests a decoupling between C gain (through photosynthesis) and C allocation to secondary growth probably due to stronger control of moisture limitations on g_s and hence iWUE (e.g. Guerrieri et al., 2019; Olano et al., 2014; Peñuelas et al., 2008) and/or shifts in C allocation to storage versus growth (Hartmann & Trumbore, 2016).

Our results also underscore important differences between these two forest types in terms of N availability and uptake by trees, which are not obvious if we only analyse the soil %N. Nutrient uptake in beech trees is known to be closely controlled by ECM associations, particularly under N limitation (e.g. Pena et al., 2010). This was confirmed by our study given, firstly, the overall more negative $\delta^{15}N$ values in leaf versus soil (e.g. Craine et al., 2015) and, secondly, the observed negative correlation between ECM species richness and both soil PO_4^{3-} concentrations and $\%N_s$. Mycorrhizal fungi transfer preferably ^{15}N-depleted N to trees (Amundson et al., 2003; Hobbie & Ouimette, 2009), which is then reflected in more negative $\delta^{15}N$ values for trees with ECM associations than for non-mycorrhizal trees (Craine et al., 2009). Nevertheless, the ^{15}N enrichment in leaf and soil samples in recent compared to long-established forests is most likely associated with greater N availability and increase in N loss pathways. Indeed, high nitrification and removal of ^{15}N-depleted NO_3^- though leaching both contribute to enriching in ^{15}N the remaining NO_3^- in soil, which is then reflected in more positive leaf $\delta^{15}N$ (Pardo et al., 2013). The positive correlation between $\delta^{15}N_l$-$\delta^{15}N_s$ and NO_3^- in recent forests is thus indicative of greater nitrification and NO_3^- leaching. By contrast, the difference $\delta^{15}N_l$-$\delta^{15}N_s$ in long-established forests was positively associated with both NH_4^+ and ECM richness. A number of studies have reported preferential uptake of NH_4^+ compared to NO_3^- by ECM fungi, which is then transferred to plants (Leberecht et al., 2016). Together, these findings suggest more N retention within the system in long-established forests than in recent forests that suffer greater N losses and have less immobilization by ECM fungi. This difference in soil N dynamics helps explain the greater ECM fungal species richness in long-established versus recent forests (Correia et al., 2021), which is most likely associated with higher N availability in the latter than the former (e.g. review by Lilleskov et al., 2019 of a N deposition gradient).

4.4 | Variations in iWUE and N dynamics with altitude and tree size in beech forests

Previous studies have shown, variously, that $\Delta^{13}C_l$ (and hence iWUE) did not change (Lins et al., 2016; Wang et al., 2010), decreased (in humid climates, e.g. Hultine & Marshall, 2000; Körner et al., 1991; Marshall & Zhang, 1994) or increased (in arid or semi-arid regions, e.g. Lajtha & Getz, 1993; Van de Water et al., 2002) with altitude. Our results point to greater $\Delta^{13}C_l$ (Figure S2) and lower iWUE with increasing altitude, confirming that in more arid regions (such as the Mediterranean) more favourable moisture conditions at higher altitude may lead to lower iWUE (Peñuelas et al., 2008) and enhance tree growth (Lendzion & Leuschner, 2008). Beech trees find their optimum in mesic conditions, thus limitations on soil and atmospheric moisture negatively affect ecophysiological processes (e.g. Aranda et al., 2005; Gessler et al., 2007; Lendzion & Leuschner, 2008). Our results also suggested differences in the effect of altitude between recent and long-established forests (Table 3; Figures S1 and S3). However, it should be noted that we had fewer long-established forest plots at higher altitude where the abandonment of previous land uses (i.e. pasturelands) occurs more frequently, and secondary forests are now predominant. Further studies are needed to fully confirm whether recent forests are truly more sensitive than long-established forests to environmental changes with increasing altitude.

Unlike iWUE, though, variation in $\delta^{15}N_l$ was not correlated with altitude (and associated changes in environmental conditions), but instead to tree age or size. An increase in $\delta^{15}N_l$ with tree age has often been reported (e.g. Pardo et al., 2013) as a consequence of root development and better access to deeper soil layers, where inorganic N is more enriched in heavier isotopes than in the more superficial soil layers (Craine et al., 2015). We did not observe this effect in our study and instead noted an increase in $\delta^{15}N_l$ with tree size but a reduction with tree age. These apparently contrasting results may reflect a combination of mechanisms including changes in root development and shift in fine root distributions along the soil profile with age, which affect the source of N (and relative isotopic signature) taken up by trees, variations in root colonization by ECM fungi with tree age, as well as isotope fractionations associated with N uptake and/or N derived from ECM fungi association (e.g. Compton et al., 2007; Hobbie & Högberg, 2012 and references therein).

5 | CONCLUSIONS

Legacies of former land uses ('ecological memory' sensu Johnstone et al., 2016; Mausolf et al., 2018) significantly contribute to explaining differences between recent and long-established beech forests in terms of ecophysiological processes (iWUE, SLA) and N dynamics (as detected by leaf and soil $\delta^{15}N$). Our results point to a higher sensitivity in recently established beech forests to environmental changes that are associated with increased altitude (and particularly to improvements in moisture conditions). Knowing whether these results hold when looking at long-term changes in iWUE in relation to tree growth will provide important information on the trade-off between C uptake and allocation and water loss through transpiration under climate change. They also reveal important differences between the two forest types in terms of their N dynamics, with recent forests having, on the one hand, a greater coupling between traits associated with physiology (iWUE), productivity (BAI, wood density) and N availability ($\delta^{15}N_l$), and on the other hand, a 'leaky' N cycling, with less N retention by trees and associated ECM fungi, and greater N losses.

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AUTHORS' CONTRIBUTIONS

R.G., J.P. and J.M.E. conceived and designed the study; M.C., R.A.-S. and J.M.E. carried out sampling, field data analyses and sample preparation for leaf and soil parameter measurements; I.M.-F. provided assistance with the preparation of samples for stable isotope analyses; R.G. elaborated stable isotope data and carried out statistical analyses; R.G. and J.M.E. led the paper writing, with contribution from all the co-authors.

DATA AVAILABILITY STATEMENT

Data deposited in the Dryad Digital Repository https://doi.org/10.5061/dryad.j0zpc86db, (Guerrieri et al., 2021).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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