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An assessment of carbon and nutrient limitations in the formation of the southern Andes tree line

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Summary

1. Although the principal mechanism determining tree line formation appears to be carbon (C)-sink limitations due to low temperatures, few studies have assessed the complementary role of reduced soil nutrient availability with elevation. We tested the hypothesis that nutrient (especially nitrogen, N) limitations at tree line may directly (via C-source) or indirectly (via C-sink) reduce the growth of a winter deciduous tree line species.

2. If a shortage of soil N with elevation is involved in tree line formation, it should occur in two alternative ways: (i) through sink limitations because N is required for tissue formation, which would indirectly limit C investments (N decreases and C reserves increase with elevation), and (ii) through C limitations because this would lead directly to a reduction of photoassimilates (N and C reserves decrease with elevation).

3. In testing our hypothesis, we analysed tree growth rates (basal area increment), twig non-structural carbohydrate (NSC) and N concentrations, leaf N, phosphorus (P), N:P ratio concentrations, and soil nutrient levels (NO_3^-, NH_4^+, Olsen–P) in four disparate climate and soil *Nothofagus pumilio* tree lines spanning 18 degrees of latitude in the southern Andes of Chile.

4. We found a significant decrease in tree growth with elevation. Twig NSC concentrations pooled across locations also decreased significantly with elevation (starch constituted most of the NSC and was highly responsible for the negative trend), although this trend was mostly driven by the northernmost locations. Contrary to soil N availability, leaf N and P concentrations increased significantly with elevation. Twig N concentrations, soil P and leaf N:P ratios did not change with elevation.

5. *Synthesis*. The elevational decrease in NSC concentrations supports C-source limitation in *N. pumilio* trees at tree line elevation. In the light of this, we assert that the current global explanation for tree line formation (C-sink-limitation driven by low temperatures) must be revisited. Given that leaf N and P concentrations increased and twig N concentrations did not change with elevation, nutrient limitation is not likely to be involved in the C-limitations and could not therefore be an explanation for tree line formation.

Key-words: alpine tree line, carbon balance, nitrogen, non-structural carbohydrates, *Nothofagus pumilio*, Patagonia, phosphorus, plant–climate interactions, Tierra del Fuego

Introduction

Although a decline in temperature with increasing elevation has long been recognized as a major factor shaping plant community variation (von Humboldt 1849; Whittaker 1956; Körner 2003; Sundqvist, Sanders & Wardle 2013), the mechanism through which low temperatures reduce tree growth has been historically contentious. One explanation is that reduced C gain with elevation (i.e. temperature) controls tree growth and, therefore, tree line formation occurs at the elevation where C availability is insufficient to compensate for respiratory losses, i.e. the C-source limitation hypothesis (Schulze, Mooney & Dunn 1967; Stevens & Fox 1991; Wardle 1993; Sveinbjörnsson, Hofgaard & Lloyd 2002). In contrast, Körner (1998) suggested that tree line formation occurs due to low-temperature limitations on meristematic activity (i.e. carbon-sink constraints), i.e. the C-sink limitation hypothesis. A recent world-wide study in 13 alpine tree line regions confirmed the existence of this narrow isotherm for tree lines (rectified to $6.4\ °C \pm 0.7$ SD) and found supporting evidence for C-sink limitations (Hoch & Körner 2012). Rossi *et al.* (2007) also found a mean daily air temperature threshold for

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xylogenesis of 5·6–8·5 °C, along with significant correlations between the growing season air temperature, cambial activity and cell differentiation in conifers growing at the tree line elevation.

Reduced soil nutrient availability due to low temperatures could also be responsible for declining growth at tree line elevation (e.g. Sveinbjörnsson, Nordell & Kauhanen 1992; Hertel & Wesche 2008; Sullivan *et al.* 2015). Soil nutrients, especially nitrogen (N), can become less available to trees at the tree line elevation because of reduced microbial activity and low N mineralization (Sveinbjörnsson *et al.* 1995; Thébault *et al.* 2014), or due to limitations in N uptake (Weih & Karlsson 2001; Hertel & Wesche 2008; Susiluoto, Hilasvuori & Berninger 2010). Limited soil N availability may translate into reduced leaf N concentrations (Sullivan *et al.* 2015), which can limit C gain (Evans 1989; Sveinbjörnsson, Nordell & Kauhanen 1992) or C sink (i.e. tissue formation) (Herms & Mattson 1992). Consequently, N limitation may play a key role in the elevational trend of C reserves. However, we know that in some species the nutrients stored in woody tissues can buffer the asynchronies between tree nutrient demands and soil nutrient supplies (Grelet *et al.* 2001; Piper & Fajardo 2014); i.e. leaf nutrient concentrations may therefore not accurately reflect soil nutrient limitations. Although the global occurrence of tree lines at a similar isotherm, regardless of soil type, argues against the hypothesis that nutrient limitation influences tree line formation (Körner 2012), different tree line soils are occupied by different taxa that certainly have different nutrient requirements (Körner 2012; Mayor *et al.* 2016). Thus, studies using a single species at a single site may be too idiosyncratic and therefore tell little about the importance of nutrient limitation as the ultimate cause of reduced growth at the tree line. In contrast, widely distributed tree line species occurring in different climates and types of soils may represent a good model for testing the hypothesis of nutrient limitations.

Although C and N are related in net primary production and soil organic matter mineralization (Sundqvist, Sanders & Wardle 2013; Thébault *et al.* 2014), the concomitant role of C and N balances in trees is still poorly understood. Throughout all tree genera, C reserves, in the form of non-structural carbohydrates (NSC = low molecular weight sugars + starch), accumulate whenever C sinks (e.g. growth) are more limited than C sources (photosynthesis) (Chapin, Schulze & Mooney 1990). Thus, an elevational decrease in NSCs supports C-source limitations, and the opposite supports C-sink limitations. If a shortage of soil N with elevation has any involvement in tree line formation, it should occur in two alternative ways: (i) through sink limitations because N is required for tissue formation (e.g. forming structural proteins or enzymes involved in growth metabolism), which would indirectly limit C investment, and (ii) through a reduction in photoassimilates limiting C availability. Thus, if N–induced C shortage led to reduced tree growth with elevation, decreases in the tree NSC concentrations would be expected along with decreases in wood and leaf N concentrations. In contrast, if N-induced C-sink limitations caused reduced tree growth with

elevation, increases of tree NSC concentrations would be expected along with decreases of woody and leaf N concentrations. Alternatively, if branch and leaf N concentrations increased with elevation independently of NSC, this would lend support to C-sink or C-source limitations but driven by factors other than N. One of these factors could be P availability.

Two important considerations about the potential role of N on C-source and C-sink limitations at tree line are the timing and the plant tissue selected for sampling. First, the elevation effects on NSC concentrations can be significantly modified by the season (Hoch & Körner 2003; Gruber *et al.* 2011), thus special care should be placed on *when* the examination of NSC and N concentrations is conducted to investigate causes of reduced growth. Ideally, the collection of tissue should be done when growth is indeed occurring (e.g. spring or early summer). Secondly, if growth is limited by either a scarcity of resource availability or impediments in investing these resources into new tissue, expected changes in the concentrations of these resources should take place in tissues proximate to those where growth is occurring, e.g. twigs (Piper *et al.* 2016). This is because trees usually cover their demands by first mobilizing stored resources from the closest possible storage sites (Landhäusser & Lieffers 2003). Notably, most previous studies examining the C balance of tree line trees have assessed NSC concentrations at the end of the growing season and in major storage tissues (roots, stems or branches) (e.g. Fajardo, Piper & Cavieres 2011; Hoch & Körner 2012).

In this study, we jointly assessed the C storage, N status and tree growth in the widely distributed tree line species *Nothofagus pumilio*, with the main objective of explaining the formation of the southern Andes tree line. The southern Andes tree line is dominated by this single species (*N. pumilio*), which covers more than 20 degrees of latitude with different soil types (of both volcanic- and sedimentary-origin), thus constituting an ideal system to test tree line formation hypotheses, particularly the nutrient limitation hypothesis. Previous studies examining temperature effects on C reserves (but not nutrients) on *N. pumilio* have been inconclusive about the ultimate causes of tree line formation (Fajardo, Piper & Cavieres 2011; Hoch & Körner 2012; Fajardo & Piper 2014; Piper *et al.* 2016). The concurrent consideration of C reserves and nutrients could shed some light in this regard.

We worked in four disparate climate and edaphic tree lines spanning 18 degrees of latitude from a Mediterranean-like tree line in central Chile (36° SL) to a sub-Antarctic tree line in Tierra del Fuego (54° SL). The southern Andes tree line can be assumed to be driven solely by abiotic factors given that it has undergone no human intervention and that atmospheric N deposition driven by anthropogenic climate change is extremely low (Weathers & Likens 1997; Reay *et al.* 2008). Our specific objectives were to (i) test the nutrient limitation hypothesis for tree line formation, (ii) directly distinguish C-source and C-sink limitations and (iii) determine the relationship between N and C-source and C-sink limitations. To accomplish these objectives, we determined leaf and soil N

and P concentrations, twig N and NSC concentrations, and stem basal area increment (BAI). We finally expect that by working with several bodies of evidence (e.g. tree growth rates, C and N storages), we will be able to further decipher the potential mechanisms controlling tree line formation, and understand whether these mechanisms will limit or promote the hypothetical upslope expansion of trees following global warming.

Materials and methods

STUDY SITES AND TREE LINE SPECIES

Nothofagus pumilio (Poepp. et Endl.) Krasser (Nothofagaceae) is a deciduous broadleaf light-demanding tree species that extends through a wide latitudinal and altitudinal range in the southern Andes of Chile and Argentina. We conducted this study in four sites, covering 18 degrees of latitude, from Mediterranean-like to cold temperate climatic conditions. The first site was in Termas de Chillán (36°54′ S, 71°24′ W), where the tree line occurs at 2086 m of elevation. The annual precipitation is 1520 mm (Las Trancas weather station, Dirección General de Aguas, 2005–2014, 1250 m a.s.l.). The second site was in the Antillanca area within the Puyehue National Park (40°47′ S, 72°11′ W), where the tree line occurs at 1337 m of altitude. The annual precipitation is 3660 mm (Daniels & Veblen 2004, Aguas Calientes weather station, 1991–1997, 475 m a.s.l.). In these two sites, most precipitation occurs in the form snow from May to September, and the soils are derived from andesitic rock. The third site was in the Coyhaique National Reserve (45°31′ S, 72°02′ W), where the tree line occurs at 1214 m of altitude. The annual precipitation here is 910 mm (Coyhaique National Reserve weather station, Dirección General de Aguas, 2002–2015, 400 m a.s.l.). The soil is derived from aeolian volcanic ash deposits. The fourth site was in the Karukinka Private Reserve in Tierra del Fuego (54°06′ S, 69°21′ W), where the tree line occurs at 625 m of altitude. The annual precipitation is 565 mm (Lago Deseado weather station, Dirección General de Aguas, 2006–2013, 400 m a.s.l.) and the soil is of granitic origin.

We tried to select tree line sites that have not been noticeably affected by human activities or disturbed by landslides or avalanches. We also chose sites that represent the natural climatic tree limit of each respective region. Finding such places in the southern Andes is less difficult than, for example, in Eurasia, where traditional human land-uses near tree lines has been commonplace (Gehrig-Fasel, Guisan & Zimmermann 2007). We defined the tree line as the uppermost limit of a group of individual trees with an upright growth form of at least 3 m (Körner 2003), which also included, in some cases, krummholz (i.e. stunted individuals) that become erect after a period of horizontal growth.

FIELD SAMPLING AND INITIAL SAMPLE PROCESSING

At each site, we selected trees at four elevations, covering the last c. 300 m of the tree line ecotone. Our main objective when selecting the elevations at each site was to cover the complete tree line ecotone; from the uppermost crooked-shape (krummholz) trees at tree line elevation down to where large (DBH > 30 cm), erect trees form a close and relatively dense forest. In this way, we were certain that we were comparing individual trees at tree line elevation that are noticeably affected by low temperatures with individuals that are not affected (or much less affected) by low temperatures, such as those located at a lower elevation, which have no apparent growth constraints (high dimensions and erect stems). For clarity and brevity, we hereafter refer to elevations by their short names: E1, closed, tall, lowest elevation forest; E2, intermediate stand located between E1 and the timberline; E3, the timberline; and E4, the tree line (Table 1). Although the four tree line sites occurred at different elevations (a systematic decrease in tree line elevation with latitude), we assumed that the temperature gradient with elevation was of comparable magnitude. This assumption is based on the fact that elevational tree lines occur at analogous isotherms–tree lines world-wide share a similar mean growing season temperature of 6·4 °C, including the *N. pumilio* tree line in the Coyhaique area (Hoch & Körner 2012; Fajardo & Piper 2014). An exception is Termas de Chillán where the tree line elevation occurs at a slightly (c. 1·5 °C) warmer temperature (Körner & Paulsen 2004), possibly due to the Mediterranean climatic influence (Piper *et al.* 2016). In all the tree line ecotones, trees of comparable age may differ from up to sevenfold in terms of height between

Table 1. Mean (+1 SE) concentrations (in mg cm^{-3}) of starch and soluble sugars in twigs of *Nothofagus pumilio* tree line trees sampled at four elevations (in m above sea level) within four locations in the southern Andes of Chile covering 18 degrees of latitude (from central Chile to Tierra del Fuego). Elevations were, E1: closed forest; E2: intermediate; E3: timberline; and E4: tree line elevation

	E1	E2	E3	E4
Termas de Chillán (36° SL)				
Elevation	1544	1715	1867	2086
Starch	43·51 (7·14)	14·38 (4·05)	30·03 (4·85)	21·15 (3·28)
Soluble sugars	7·77 (0·99)	8·43 (0·72)	8·21 (0·74)	7·56 (1·46)
Antillanca (40° SL)				
Elevation	984	1088	1164	1337
Starch	34·36 (9·78)	47·78 (12·46)	17·00 (4·73)	16·59 (3·62)
Soluble sugars	12·26 (1·78)	14·49 (2·30)	10·88 (1·35)	7·75 (0·51)
Coyhaique (46° SL)				
Elevation	854	975	1094	1214
Starch	61·15 (11·20)	73·81 (13·28)	85·85 (6·91)	65·46 (6·26)
Soluble sugars	11·26 (1·37)	12·87 (0·42)	18·69 (1·83)	15·69 (1·02)
Tierra del Fuego (54° SL)				
Elevation	385	488	559	625
Starch	66·44 (4·72)	57·14 (5·17)	48·00 (7·25)	49·01 (4·18)
Soluble sugars	15·45 (2·45)	19·62 (2·37)	24·83 (1·37)	25·71 (1·22)

the tree line (c. 3 m) at E4 and the closed forest at E1 (c. 21 m) (Fajardo 2016).

Sampling was carried out in mid-summer for all tree lines, when leaf expansion and shoot growth were still occurring: in late January of 2013 for Termas de Chillán and Antillanca and in early February of 2014 for Coyhaique and Tierra del Fuego. At each of the four elevations, we selected 8–10 dominant individual trees (tree heights of 3 m at tree line, 15–20 m at the lowest elevations) for tissue collection. Each individual was separated from the next by a minimum of 30 m, and all individuals were located at the edge of a patch; i.e. no shade branches were collected, particularly at low elevations. We thus restricted the sampling to adult, unshaded trees without browsing or other damage. For each individual tree, we measured the diameter at breast height (DBH, 1·35 m) and bark thickness using a diameter tape and a bark thickness gauge, respectively. We then identified and cut one terminal, c. 1 m long, fully expanded sun-exposed branch using (when necessary) a 5·6 m telescoping pole (ARS Corporation, Sakai, Japan). From each of these branches, we collected sun-exposed leaves, and 2-year-old twig segments (with bark and phloem removed in the field with a knife). Tissue collection was conducted between 10:00 and 16:00. Annual nodes were easily identifiable by bud scars, differently coloured shoot epidermises and different shoot diameters. Finally, two to-the-pith long stem cores were taken at a height of c. 30 cm for tree growth determination. We used a 5·15 mm increment bore (Haglöf, Långsele, Sweden) for this purpose. Leaves and twigs were bagged and labelled, and stored in a cooler for transportation, while increment cores were placed in labelled straws and kept in a map tube.

At about 5 m in the downslope direction from each tree sampled, one 10 cm deep soil core was removed using a 12·4 cm diameter PVC soil–corer with a stainless steel serrated edge (volume = 1207·627 cm^3). Before collecting each soil core, we removed all recognizable litter from the soil surface (O$_i$ horizon). After collection, each core was placed in a labelled Ziploc bag that was immediately sealed and maintained in a cooler in fresh conditions (<10 °C air temperature) for c. 2 h to minimize nutrient mineralization, then they were placed in a refrigerator (−20 °C). The soil core collected from each sampling location was used for the determination of available ammonium (NH$_4^+$), nitrate (NO$_3^-$) and phosphorus (Olsen–P).

Since NSC concentrations expressed in mass are affected by tissue density, we expressed twig NSC and N concentrations in terms of volume and leaf NSC, N and P concentrations in terms of area. For this, we examined the elevational trends in wood density (g cm^{-3}) and leaf mass per area (LMA, g m^{-2}). From the same branch sampled for NSC we also collected a 4–5 cm long twig from each tree, which was bagged and labelled, and stored in a cooler for transportation. We estimated the twig wood density as the ratio between the dry mass (see below) and the volume of that mass. The fresh volume of the twig sample was determined by submerging it into a glass beaker on a scale. The difference caused by the introduction of the sample, which equals the volume of water displaced by the sample, was recorded and converted into volume based on the density of water as 1·0 g cm^{-3}. For LMA determination, 20 leaves from the original branch were separately laid flat and photographed with a reference square of known area using a Nikon Coolpix 5000 digital camera (Nikon Corporation, Tokyo, Japan). The total projected leaf area was then calculated using the image-processing software SIGMAPROC (Systat Software Inc., Richmond, CA, USA). All of the leaves (for LMA and nutrient determination) and twigs (for wood density, NSC and N determinations) were then placed to dry in a forced-air stove (Memmert GmbH, Schwabach, Germany) at 70 °C for 72 h and the 20 photographed leaves along with the twig piece were subsequently weighed with a scale calibrated to 0·0001 g of precision to determine LMA and wood density, respectively. We computed LMA as the oven-dried weight of the 20 leaves divided by their total foliar area. Finally, leaf and twig samples were ground into a fine powder and stored over silica gel at 4 °C until chemical analyses were conducted.

TREE GROWTH DETERMINATION

Cores were prepared following standard dendrochronological techniques (Stokes & Smiley 1996). Cores were dried, mounted and glued firmly on grooved wooden sticks. Cores were subsequently sanded with successively finer grades of sandpaper until optimal surface resolution allowed the annual rings to be distinguished under magnification (10×). Inside-bark bole radius and annual radial increments from the last 10 years were measured to the nearest 0·01 mm using a microscope mounted on a dendrochronometer with a Velmex sliding stage and Accurite measuring system (Bloomfield, NY, USA). Cross-dating accuracy was checked visually since rings were easily identified. We estimated a basal area increment (BAI$_{10}$) for the last 10 years, which represents a relative measure of gain in growth, as:

$$BAI_{10} = \pi(R_t^2 - R_{t-10}^2)$$

where R_t is the radius of the stem and R_{t-10} is the radius of the stem without the 10 outermost years. The radius of the stem, R_t, was computed by considering the diameter at coring height, bark depth, and the distance between pith and the last year's ring in the core.

CHEMICAL ANALYSES

Non-structural carbohydrates

Our assessment of C reserves was based on the determination of NSC concentrations, as the sum of the three most abundant low molecular-weight soluble sugars (glucose, fructose and sucrose) and starch. NSC concentrations were analysed following Wong's (1990) procedure as described in detail in Hoch, Popp & Körner (2002). About 13 mg of dried powder from twig samples were extracted with 1·6 mL of distilled water at 100 °C for 60 min. An aliquot of the extract was used to determine low molecular carbohydrates after enzymatic conversion (invertase and phosphoglucose isomerase from *Saccharomyces cerevisiae*, Sigma Aldrich I4504 and P5381, respectively, St. Louis, MO, USA) of sucrose and fructose to glucose. The concentration of free glucose was determined photometrically after the enzymatic conversion of glucose to gluconate-6-phosphate (Glucose Assay Reagent, G3293; Sigma Aldrich) on a 96-well multiplate reader. Following the degradation of starch to glucose using a purified fungal amylase ('amiloglucosydase' from *Aspegillus niger*; Sigma Aldrich 10115) at 45 °C overnight, NSC was determined in a separate analysis. The starch concentration was calculated as NSCs minus the sum of free sugars. Total soluble sugar, starch and NSC concentrations are presented on a percent of dry matter basis.

Nutrient concentrations

Leaf P was extracted from leaves by combining 20 mg of dry leaf material in 1 mL of 2% acetic acid, and digesting this mixture for 30 min before centrifuging it at 270 *g* (Fredeen, Rao & Terry 1989). Foliar P concentrations were determined following Murphy & Riley's (1962) procedure, which, briefly consists of the formation of an antimony-phosphomolybdate complex and its subsequent reduction to

phosphomolybdenum blue, a bluish-purple colour complex. The absorbance of this complex is read at 880 nm and converted into a phosphate concentration using a calibration curve of potassium dihydrogen phosphate. Foliar and twig N concentrations were determined by placing 200 mg of dry, ground tissue samples in a combustion analyser (LECO TruSpec® Micro CHN, Centro de Investigación en Ecosistemas de la Patagonia, Coyhaique, Chile). Phosphorus and N concentrations were expressed in terms of area (g m^{-2} for P_a and N_a), which is the result of P and N concentrations on a mass basis multiplied by LMA. We also computed the foliar N/P ratio as the quotient between foliar N and P concentrations. The N/P ratio (i.e. stoichiometry) has been increasingly proposed as a relevant indicator of nutrient limitation at both the species and community levels (Koerselman & Meuleman 1996; Aerts & Chapin 2000).

Soil chemical analyses

The soil samples were shipped to a soil testing laboratory (Instituto Nacional de Investigación Agropecuaria, INIA, Santiago, Chillán, Chile), where analysis of nitrate (NO_3^-, mg kg^{-1} soil d.w.), ammonium (NH_4^+, mg kg^{-1} soil d.w.) and Olsen–P (mg kg^{-1} soil d.w.) were conducted. Mineral available N (i.e. NH_4^+ and NO_3^-) was extracted using a 5:1 proportion of K_2SO_4 solution:soil, after which the extracts were used to determine NH_4^+ and NO_3^- using standard colorimetric methods (Longeri, Etchevers & Venegas 1979). Olsen–P is a widely used method appropriate to quantify P availability in soils with neutral to basic pH, although in acidic volcanic soils it shows a good correlation with other methods (e.g. Bray–P) (Rojas 1976).

DATA ANALYSIS

All of the variables were transformed to their natural logarithms before analyses to meet assumptions of normality. The influence of elevation on tree growth (BAI), twig NSC, wood density, LMA,

tissue and soil nutrient concentrations were analysed fitting linear mixed-effects models (LMM) using the *nlme* package (Pinheiro *et al.* 2009) in the R version 2.15.3 (R Development Core Team 2016). Although wood density and LMA did not significantly change with elevation (see below), tissue concentrations were expressed per unit of area and per unit of volume for leaf and wood nutrient concentrations respectively. In the modelling, we considered elevation (E1, E2, E3 and E4) as the fixed factor, with random effects for locations and the trees nested in each location; the former in order to account for the among-locations variation and the latter because tree individuals within each location were more likely to resemble one another than tree individuals from other locations. For BAI, NSC, wood density, LMA, tissue and soil nutrient concentrations, we tested the fixed effects of elevation using likelihood ratio tests (χ^2) in nested models fitted using maximum likelihood (Bolker *et al.* 2009). Finally, in cases where elevation proved to have a significant effect on a variable, we conducted post hoc multiple comparisons (Tukey's procedure) among elevations using the *multcomp* package (Hothorn, Bretz & Westfall 2008) to find out which elevations differed from others.

Results

Elevation had a significantly negative effect on tree stem growth ($\chi^2 = 22.507$, $P < 0.001$, $b = -0.279$), measured as the mean BAI in the last 10 years. On average, trees located in the lowest elevation grew 87.18% (BAI = 130.69 cm^2 year^{-1}) more than trees at the tree line elevation (BAI = 69.82 cm^2 year^{-1}; Fig. 1a). The significant decrease in stem growth with elevation is consistent with low temperature driving growth limitations at tree line elevation. Similar to BAI, when twig NSC concentrations were considered, we found a significant decrease of 20.69% with elevation ($\chi^2 = 7.333$, $P = 0.007$, $b = -0.085$), from 62.76 mg cm^{-3} at E1 to 49.78 mg cm^{-3} at E4 (Fig. 1b).
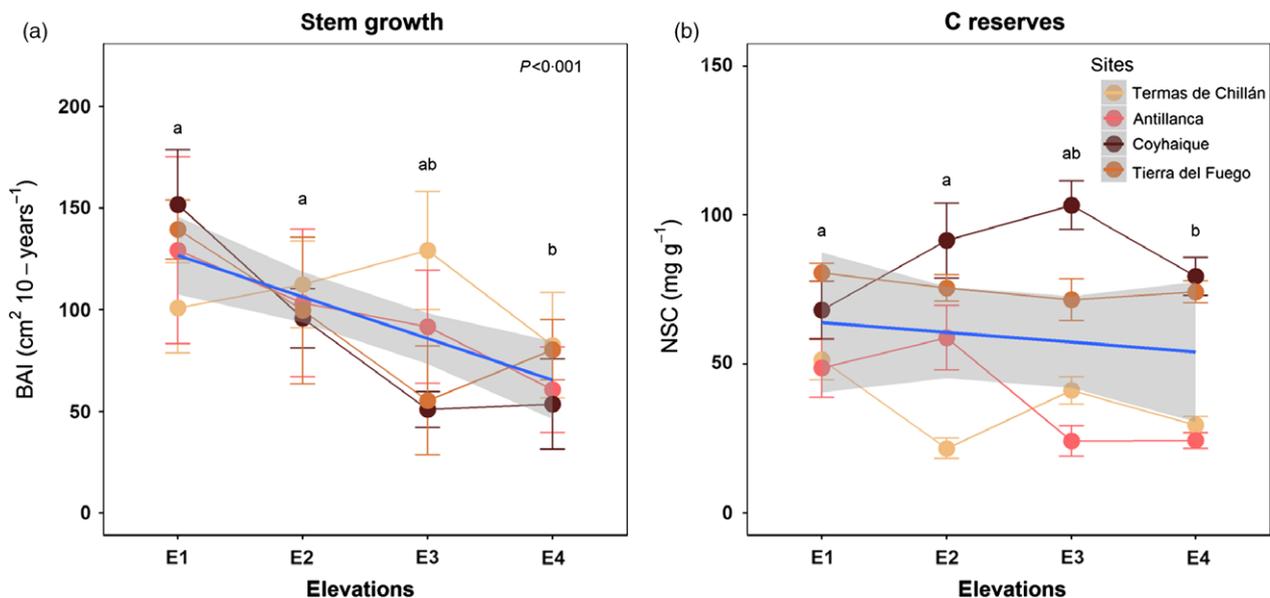

Fig. 1. Mean (a) basal area increment of the last 10 years (BAI, stem growth) and (b) twig C reserves (non-structural carbohydrates, NSC, concentrations) in tree line trees of *Nothofagus pumilio* sampled at four elevations, including tree line elevation, and at four locations covering 18 degrees of latitude in the southern Andes of Chile. Elevations were, E1: closed forest; E2: intermediate; E3: timberline; and E4: tree line elevation (see Table 1 for elevations at each location). Error bars refer to standard errors. Different letters indicate significant differences ($P < 0.05$) among elevations. [Colour figure can be viewed at wileyonlinelibrary.com]

This trend appeared to be mainly driven by the two northernmost sites, which also had much lower NSC levels than the other two sites. While starch constituted most of the NSC in twigs (Table 1) and was highly responsible for the negative trend of NSC concentrations with elevation ($\chi^2 = 6.612$, $P = 0.010$, $b = -0.120$), soluble sugars did not change significantly with elevation ($\chi^2 = 2.383$, $P = 0.123$, $b = 0.037$).

Soil available N ($NH_4^+ + NO_3^-$) concentrations decreased significantly with elevation ($\chi^2 = 8.760$, $P = 0.003$, $b = -0.167$), from 63·31 mg g^{-1} at E1 to 37·32 mg g^{-1} at E4, representing a change of 69·64% (Fig. 2a). By contrast, soil P concentrations did not change with elevation ($\chi^2 = 1.258$, $P = 0.262$, $b = -0.066$; Fig. 2b). Contrary to soil N availability, leaf N concentrations (on an area basis) increased significantly with elevation ($\chi^2 = 21.405$, $P < 0.001$, $b = 0.063$, Fig. 3a); N concentrations were particularly different ($P < 0.001$) between trees at E1 (3·0 g m^{-2} ± 1 SE = 0·23) and E4 (3·36 g m^{-2} ± 1 SE = 0·22). Leaf P concentrations also increased significantly with elevation ($\chi^2 = 6.671$, $P = 0.010$, $b = 0.062$, Fig. 3b), from 0·23 g m^{-2} (± 1 SE = 0·22) at E1 to 0·27 g m^{-2} (± 1 SE = 0·17) at E4 ($P = 0.031$). Mass-based concentrations of leaf N and P followed similar elevational trends as area-based concentrations, given that LMA did not change with elevation ($\chi^2 = 0.035$, $P = 0.851$). The leaf N:P ratio did not vary with elevation ($\chi^2 = 0.002$, $P = 0.963$, Fig. 3c). On average, the leaf N:P ratio was 15·68, indicative of a N and P balance. Finally, and in contrast to BAI and twig NSC concentrations, the N concentration of twigs showed considerable variation with a coefficient of variation of 30·4%

(mean = 2·58 mg cm^{-3}, ± 1 SE = 0·14), thus indicating that it was not affected by elevation ($\chi^2 = 0.326$, $P = 0.568$, Fig. 3d). Twig wood density ($\chi^2 = 0.102$, $P = 0.750$) did not differ with elevation either.

Discussion

CONTRASTING TRENDS IN THE C AND NUTRIENT STATUS OF *NOTHOFAGUS PUMILIO*

Across four *N. pumilio* tree line sites covering 18 degrees of latitude in the southern Andes of Chile, we found that elevation impinges disparate effects on the C and nutrient variables studied here. A significant decrease in stem growth with elevation was accompanied by a decrease in twig NSC concentrations, a decrease in soil N concentrations, an increase in both leaf N and P concentrations and no changes in twig N concentrations. According to our expectations, a significant decrease of growth and of twig NSC concentrations with elevation constitutes support for the C-source limitation hypothesis (Stevens & Fox 1991; Fajardo & Piper 2014). Given that N and P concentrations in leaves, and more importantly twig N concentrations, did not decrease with elevation, N and P are not likely to be involved in the reduction of carbohydrates in woody tissues. While C-sink limitations have been proposed to account for increasing leaf nutrient concentrations (Körner 1989; Hoch 2013), our study reveals that leaf nutrient accumulation in *N. pumilio* at the tree line elevation was not driven by C-
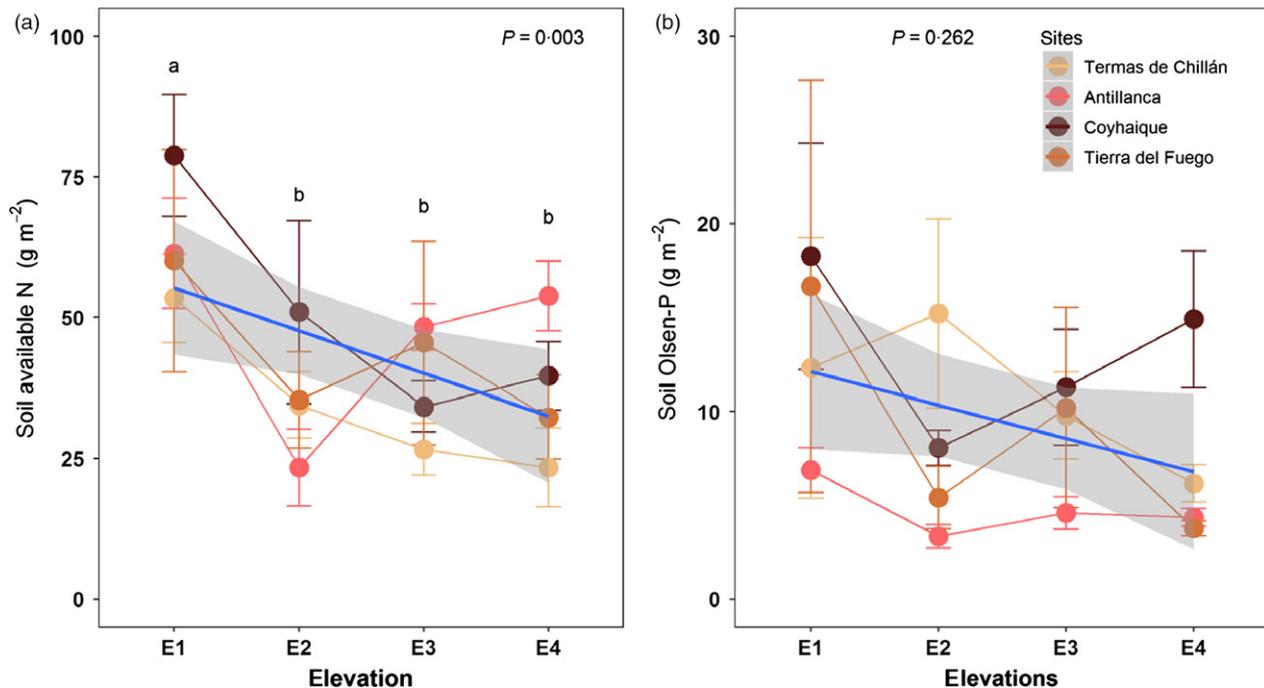

Fig. 2. Mean (a) available nitrogen (N = nitrate + ammonium) and (b) phosphorus (P) concentrations (non-structural carbohydrates) in the soil surface (0–10 cm, dry weight) sampled at four elevations, including tree line elevation, in the tree line ecotone of *Nothofagus pumilio*, and at four locations covering 18 degrees of latitude in the southern Andes of Chile. Elevations were, E1: closed forest; E2: intermediate; E3: timberline; and E4: tree line elevation (see Table 1 for elevations at each location). Error bars refer to standard errors. Different letters indicate significant differences ($P < 0.05$) among elevations. [Colour figure can be viewed at wileyonlinelibrary.com]
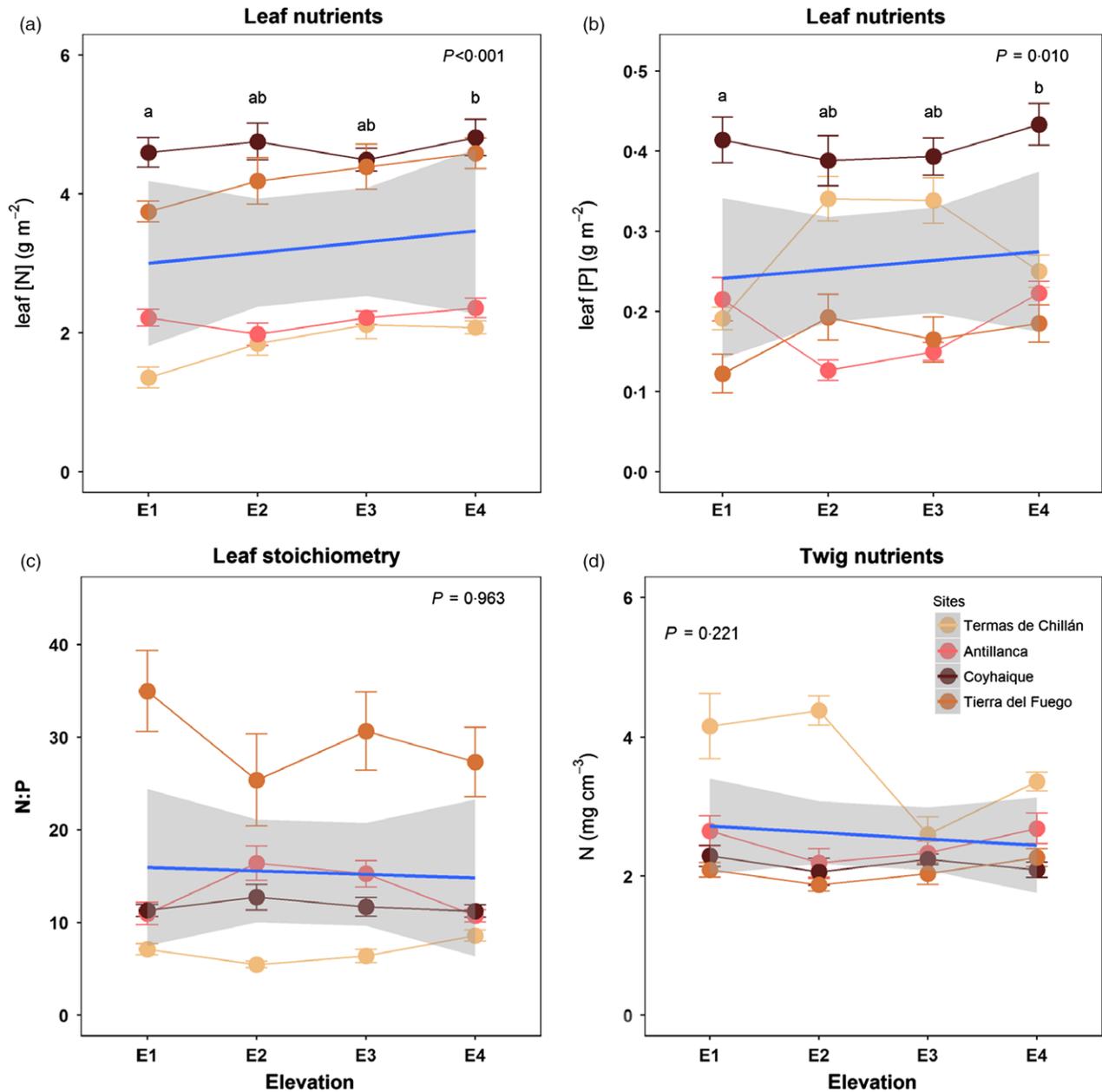
Fig. 3. Mean (a) leaf nitrogen (N), (b) phosphorus (P) concentrations, (c) leaf stoichiometry (N:P) and (d) twig N concentrations in tree line trees of *Nothofagus pumilio* sampled at four elevations, including tree line elevation, and at four locations covering 18 degrees of latitude in the southern Andes of Chile. Elevations were, E1: closed forest; E2: intermediate; E3: timberline; and E4: tree line elevation (see Table 1 for elevations at each location). Error bars refer to standard errors. Different letters indicate significant differences ($P < 0.05$) among elevations. [Colour figure can be viewed at wileyonlinelibrary.com]

sink limitations (i.e. NSC concentrations decreased with elevation).

NO SUPPORT FOR THE NUTRIENT LIMITATION HYPOTHESIS FOR TREE LINE FORMATION

Our results do not support the hypothesis of N limitation as an explanation for tree line formation. Support for this hypothesis has come from some studies conducted in Alaska, that showed elevational reductions in both leaf and soil nutrient concentrations (Sveinbjörnsson *et al.* 1995; Loomis 2006; McNown & Sullivan 2013; Sullivan *et al.* 2015). In

contrast, we found a significant increase in leaf nutrient (N and P) concentrations with elevation–in spite of reduced soil N concentrations–when considering four different climate and edaphic study tree line sites in the southern Andes that are dominated by a single species. Similarly, Hertel, Therburg & Villalba (2008) and Hertel & Wesche (2008) found an elevational increase of leaf N concentrations in a *N. pumilio* and a *Polylepis tarapacana* tree line ecotones in Argentina and in eastern Bolivia, respectively. Likewise, at a global scale, Mayor *et al.* (2016) working in seven temperate tree line ecotones (with five sites each)–including the Austrian/Swiss Alps, the New Zealand Southern Alps, the southern Andes

(Patagonia, Chile), the Canadian and US Rocky mountains, the Snowy Mountains (Australia), and the Taisetsu Mountain (Japan)–found that, on average, tree leaf N increased by 12% with elevation and that tree leaf P did not vary with elevation. Thus, our large-scale results point to an absence of nutrient limitation at tree line elevation (see also Körner 1989, 2012; Birmann & Körner 2009).

How do tree line trees increase their leaf nutrient concentrations with elevation? Higher leaf N and P concentrations at the tree line elevation may help to increase hardiness (e.g. increasing concentrations of N-rich proteins and P-rich RNA) against colder temperatures (Reich & Oleksyn 2004; Elser *et al.* 2010), something that could still be compatible with N limited growth (Sveinbjörnsson, Nordell & Kauhanen 1992). However, if this were the case with *N. pumilio*, the increase in leaf N concentrations should have been coupled by a decrease in twig N concentrations (i.e. a current C sink), as the new leaves and developing shoots (i.e. C sinks) rely on the closest storage sites (Landhäusser & Lieffers 2003). Similar to other winter deciduous species (Grelet *et al.* 2001), *N. pumilio* mobilizes its stored N whenever growth is being N limited (Piper & Fajardo 2014). By sampling ≤2 year-old twigs during the mid-growing season, we were certain to capture any potential N depletion or accumulation driven by growth. An alternative explanation for elevational increases in leaf nutrient concentrations would be that life in harsh environments may select for inherently slow growth–ecotypes–(Güsewell 2004), as was also shown in provenance trials in common gardens (Oleksyn *et al.* 1998), and this retarded growth may be associated with luxuriant nutrient consumption (Chapin 1980; Körner & Larcher 1988; Körner 1989). However, evidence from plantations with adult trees from a single common source has shown that the growth decrease and C accumulation with elevation are environmentally driven processes (Fajardo *et al.* 2012), thus rejecting the presence of ecotypes as the cause of, at least, C differences.

In general, the use of N:P ratios is claimed to be more instructive than just looking at nutrient concentrations, given that some element concentrations may vary more than others, i.e. relative nutrient limitations (Güsewell 2004; Körner 2012). We found that the leaf stoichiometry of *N. pumilio* did not change with elevation and averaged an N:P ratio of 15·68, a value that, according to Koerselman & Meuleman (1996) and Aerts & Chapin (2000), provides evidence for a N and P balance. In contrast to our results, Reich & Oleksyn (2004), working across large latitudinal gradients, showed that tree N:P ratios increased with temperature. It is possible that we did not detect this trend because we worked at a much narrower range of temperatures.

Lastly, and contrary to what Frangi *et al.* (2005) found in *N. pumilio* tree lines in Tierra del Fuego, we found a significant decrease in mineral available N ($NH_4^+ + NO_3^-$) with elevation. Soil P concentrations did not change with elevation. The significant increase in foliar nutrient concentrations and decrease in soil nutrients demonstrates a decoupling between soil nutrient availability and the plant nutrient status. Although the elevational decrease in soil nutrient availability

might be interpreted as evidence of nutrient limitation (McNown & Sullivan 2013; Thébault *et al.* 2014; Sullivan *et al.* 2015), Körner (2012) argues that what truly matters is the nutrient release rate and the way in which these nutrients ultimately get to the plant, with soil microbes and mycorrhizae playing a vital role. In this respect, Thébault *et al.* (2014) suggested that the *N. pumilio* tree line in Tierra del Fuego would be limited by the competition between trees and soil microbial communities for decreasing soil inorganic N with elevation. However, this competition for capturing soil nutrients should not lead to a higher leaf nutrient concentration at the tree line elevation when compared to lower elevations, as we found. It is very likely that *N. pumilio* displays traits that compensate for reduced soil nutrient concentrations with elevation. For instance, Hertel & Wesche (2008) found elevational increases in both leaf N concentrations and fine root biomass in *P. tarapacana*, along with elevational decreases in soil N concentrations, and suggested that the root biomass increase is a response to limited soil nutrient availability. Finally, Mayor *et al.* (2016), at a global scale, found that soil microbial community composition (phospholipid fatty acids, PLFA, biomarkers) was not associated with elevation-related temperature, but with soil properties linked to plant community transitions (i.e. from tree line to alpine vegetation).

IS C LIMITATION A GENERAL FEATURE OF DECIDUOUS TREES?

Hoch & Körner (2012), working with 13 tree line sites located around the world and representing different floras (conifer and angiosperms), found that NSC concentrations increased with elevation, with one exception: leaves of *N. pumilio* in Patagonia, Chile. Using an experimental approximation, Fajardo & Piper (2014) also found support for the C-source limitation when seedlings of *N. pumilio* were exposed to lower temperatures (e.g. above tree line elevation, 2 m above-ground to simulate air-temperature coupling) and decreased their NSC concentrations. Overall, support for either the C-source or C-sink limitation hypotheses to explain tree line formation seems inconclusive in *N. pumilio* (e.g. Fajardo, Piper & Cavieres 2011; Fajardo, Piper & Hoch 2013) and it is very dependent on which tissue is sampled and whether factors other than temperature (e.g. drought) are involved (e.g. Mediterranean tree lines) (Piper *et al.* 2016). Also, the timing of sampling seems to be crucial in the evaluation of C-source vs. C-sink limitations (Piper *et al.* 2016). Our results also suggest that the general pattern supporting the C limitation was mostly driven by the two northernmost locations (i.e. Termas de Chillán and Antillanca), and in a minor degree by the southernmost locations (Tierra del Fuego). These location disparities are consistent with the hypothesis that other environmental factors might be influencing the C balance of *N. pumilio* at high elevations (e.g. Piper *et al.* 2016). In addition, we argue that the deciduous character of *N. pumilio* may have some influence on the C balance of trees at tree line elevation. Studies comparing growth

responses to atmospheric CO_2 enrichment in deciduous and evergreen species suggest that the former may undergo C limitation when exposed to low temperatures or drought. For example, *Larix decidua* (a deciduous tree line species), but not *Pinus mugo* spp. *uncinata* (an evergreen tree line species), increased its shoot growth and ring width over time when exposed to higher CO_2 concentrations in a tree line in the Swiss Alps (Hättenschwiler *et al.* 2002; Handa, Körner & Hättenschwiler 2005; Dawes *et al.* 2011). This indicates that *L. decidua* benefited from extra C, i.e. C-limited.

Winter deciduous species produce leaves with higher photosynthetic rates per unit mass than evergreens (Chabot & Hicks 1982; Givnish 2002); they therefore have higher nutrient (N and P) concentrations (Aerts 1996; Reich *et al.* 1998b; Piper & Fajardo 2014). The higher leaf nutrient concentrations of deciduous species may be associated with a different physiological response to low temperatures when compared to evergreens. The maintenance of high levels of leaf N is costly and implies high respiration rates (Reich *et al.* 1998a); dark respiration rates are indeed higher in deciduous than in evergreen species (Villar, Held & Merino 1995). Increasing leaf nutrient concentrations with elevation to maintain tissue integrity (e.g. freezing resistance) under harsh climatic conditions, as proposed by Sveinbjörnsson, Nordell & Kauhanen (1992), could mean additional respiratory costs. Also, compensatory root growth under reduced nutrient availability or uptake (Hertel & Wesche 2008) may occur at some C cost. If so, the NSC pool available for storage would most likely be lower at higher than at lower elevations, which could ultimately explain why our study showed that *N. pumilio* reduced its twig NSC concentrations with elevation. It is perhaps not a coincidence that the strongest elevational decrease (i.e. between E1 and E4) was found in the two northernmost locations, which also had the lowest soil N (Termas de Chillán) and soil P (Antillanca) availability at tree line elevation (Fig. 2). On the other hand, the pronounced negative NSC–elevation trend in these two locations could reflect C sink limitations at the lowest elevations driven by summer drought (which in Chile may occur as far south as 40° SL, Armesto, León-Lobos & Arroyo 1995) increasing NSC (Piper *et al.* 2016), given that growth is more sensitive to drought than photosynthesis (Muller *et al.* 2011). In this respect, it is uncertain whether drought effects on NSC concentrations differ between deciduous and evergreen species. Beyond the exact mechanism involved, our results show that while NSC concentrations can be used to distinguish between C-source and C-sink limitations (C balance) in evergreen conifers at tree line elevation they do not give straightforward results in deciduous species.

Conclusions

Temperature is a major limiting factor for high elevation (Körner 2003; Sundqvist, Sanders & Wardle 2013; Mayor *et al.* 2016) and high latitude plant communities, which are expected to be particularly responsive to global warming (Innes 1991; IPCC 2007). Our understanding on how temperature effects on physiological processes in plants are modulated by other more local and stochastic factors, including soil nutrition,

still need to be refined (Körner 2012). In particular, tree C and N storages have rarely been studied together across elevational gradients. Although in this study we demonstrated that nutrient limitation is not likely to be responsible for global tree line formation, the most common temperature-driven physiological mechanism (sink-limitation) must be reconsidered as a global explanation for tree line formation given that it does not apply to our widespread deciduous tree line species, *N pumilio* (see also the case of *L. decidua* under CO_2 enrichment experiments at the Swiss Alps tree line). It may be possible that the physiological mechanism behind tree line formation be modulated by species features such as foliar habit, implying different C and nutrient requirements and allocation paths.

Authors' contributions

A.F. and F.I.P. generated hypotheses and designed the research. A.F. collected the field data. Both authors analysed the data and wrote the manuscript.

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Data accessibility

Data available from the Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.jh65f (Fajardo & Piper 2016)..

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