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Extreme defoliation reduces tree growth but not C and N storage in a winter-deciduous species

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• **Background and Aims** There is a growing concern about how forests will respond to increased herbivory associated with climate change. Carbon (C) and nitrogen (N) limitation are hypothesized to cause decreasing growth after defoliation, and eventually mortality. This study examines the effects of a natural and massive defoliation by an insect on mature trees' C and N storage, which have rarely been studied together, particularly in winter-deciduous species.
• **Methods** Survival, growth rate, carbon [C, as non-structural carbohydrate (NSC) concentration] and nitrogen (N) storage, defences (tannins and total polyphenols), and re-foliation traits were examined in naturally defoliated and non-defoliated adult trees of the winter-deciduous temperate species *Nothofagus pumilio* 1 and 2 years after a massive and complete defoliation caused by the caterpillar of *Ormiscodes amphimone* (Saturniidae) during summer 2009 in Patagonia.
• **Key Results** Defoliated trees did not die but grew significantly less than non-defoliated trees for at least 2 years after defoliation. One year after defoliation, defoliated trees had similar NSC and N concentrations in woody tissues, higher polyphenol concentrations and lower re-foliation than non-defoliated trees. In the second year, however, NSC concentrations in branches were significantly higher in defoliated trees while differences in polyphenols and re-foliation disappeared and decreased, respectively.
• **Conclusions** The significant reduction in growth following defoliation was not caused by insufficient C or N availability, as frequently assumed; instead, it was probably due to growth limitations due to factors other than C or N, or to preventative C allocation to storage. This study shows an integrative approach to evaluating plant growth limitations in response to disturbance, by examining major resources other than C (e.g. N), and other C sinks besides storage and growth (e.g. defences and re-foliation).

Key words: Climate change, plant defences, defoliation, herbivory, insect outbreaks, non-structural carbohydrates, nitrogen, *Nothofagus pumilio*, Nothofagaceae, *Ormiscodes amphimone*, Patagonia, storage.

INTRODUCTION

In temperate regions, forests are expected to experience more frequent and severe herbivory under future climate warming scenarios (Dale *et al*., 2001; Bradshaw and Holzapfel, 2010; Paritsis and Veblen, 2010). The defoliation caused by herbivory usually reduces growth of trees and accelerates die-back processes, and may ultimately cause mortality (Rose, 1958; Kulman, 1971; Kosola *et al*., 2001; Galiano *et al*., 2011; Saffell *et al*., 2014), and yet the physiological mechanisms driving these responses remain elusive. A long-standing belief is that reduced photosynthetic area by defoliation causes a carbon (C) shortage (i.e. decreases in non-structural carbohydrates, NSC) which in turn limits growth and survival (Dickson, 1989; Krause *et al*., 1993) (i.e. C limitation, Hypothesis 1, Fig. 1). Although some studies have reported this response in seedlings and juvenile trees subjected to severe defoliation (Wargo *et al*., 1972; Parker and Patton, 1975; Tschaplinski and Blake, 1994; Canham *et al*., 1999), it is largely unknown whether C limitation may explain the growth reductions observed in mature trees after defoliation. Some studies examining responses of mature trees to defoliation have found a positive relationship between C storage and subsequent crown recovery (re-foliation) in evergreen conifers, suggesting that reduced crown recovery could have been driven by limited C storage (Webb, 1981; Galiano *et al*., 2011). However, this idea is not supported by other studies on conifers. For example, Palacio *et al*. (2012) found that complete defoliation by the pine processionary moth caused long-term growth decline in *Pinus nigra* (i.e. over 6 years following defoliation) but only transient decreases in C storage. Similarly, Saffell *et al*. (2014) reported that reduced leaf area by the Swiss needle cast in Douglas fir led to stronger reductions on growth than on C storage. Evidence for broad-leaved winter-deciduous species is scarcer than for evergreen conifers. In a young plantation of poplars, for example, repeated defoliation caused long-term decreases in growth but only transient reductions in C storage (Kosola *et al*., 2001). Similarly, Anderegg and Callaway (2012) found that repeatedly defoliated

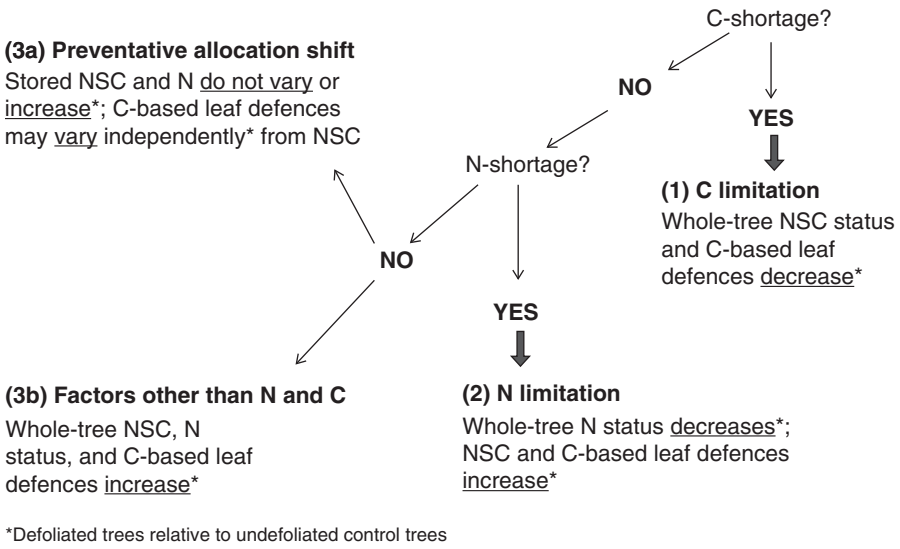

FIG. 1. Hypothetical alternative mechanisms driving tree growth reductions after complete natural defoliation. A logic tree is constructed on the basis of whether C shortage first, and then N shortage, do occur. If C shortage leads to reduced growth (C limitation), decreases in the tree's NSC status and C-based leaf defences are expected regardless of the tree's N status (1). If N shortage leads to reduced growth (N limitation), the tree's NSC status and leaf C-based defences should increase, at the same time that the tree's N status decreases (2). Under no C or N shortage two possible explanations emerge: allocation shift (3a) and growth limitation caused by other factors (3b). In the first case, no external factor limits tree growth and whole-tree NSC and N status respond concomitantly, either increasing or reaching control levels; we have no clear expectation for C-based leaf defences although up-regulation could result in independent variation with respect to the other variables. In the second case, growth is limited by factors other than N; whole-tree NSC, N status and C-based leaf defences increase concomitantly.

ramets of aspen flushed multiple canopies, enduring only moderate drawdown of NSC. To our knowledge, the effects of complete defoliation naturally caused by insects on C storage in mature trees of broadleaved winter-deciduous species have never been examined, which is notable given that this type of defoliation is most expected to result in C limitation (Körner, 2003; Palacio *et al*., 2014).

Another potential cause of reduced growth after severe defoliation relates to impaired nutrient status. Most herbivory usually occurs during the growing season, when stored nutrients are remobilized to the tissues under formation. Herbivory thus leads to a direct loss of nutrients from the tree, especially nitrogen (N) (Lovett *et al*., 2002). In addition, defoliation often causes root mortality, reduces root metabolism and diminishes nutrient uptake (Tuomi *et al*., 1990; Kosola *et al*., 2001). Although nutrients may limit photosynthesis, growth is even more sensitive than photosynthesis to moderate shortages in essential macronutrients (Herms and Mattson, 1992). Because of this, trees under moderate nutrient limitations are predicted to increase their C storage (Hypothesis 2, Fig. 1) (Herms and Mattson, 1992; Kosola *et al*., 2001; Palacio *et al*., 2014). Alternatively, it has been suggested that increased C storage and decreased growth in response to stressors, such as defoliation, could be driven not by a C accumulation but rather by a shift in C allocation from growth to storage, to avoid further C losses (i.e. preventative C allocation) (Wiley and Helliker, 2012). Such a C allocation shift would create an internal C limitation because, although the tree would have sufficient C to grow rapidly, changes in allocation priorities driven by defoliation would determine that the C is invested otherwise.

Whether reduced tree growth following defoliation is caused by N limitation or a preventative C allocation (as postulated by Wiley and Helliker, 2012) should be revealed by how trees store and allocate their N and C following defoliation. Because winter-deciduous species can store significant quantities of N in their woody tissues, we would expect a significant drawdown (i.e. remobilization) of woody N reserves following defoliation, if regrowth were primarily N limited (Hypothesis 2, Fig. 1) (Chapin, 1980; Millard *et al*., 2001; Millard and Grelet, 2010; Piper and Fajardo, 2014). Alternatively, if growth reduction were a consequence of preventative C allocation, as proposed by Wiley and Helliker (2012), we postulate that N storage should not decrease but rather remain invariable or increase concomitantly with C (Hypothesis 3a, Fig. 1). Our rationale is based on the facts that wild trees are often subject to soil N limitation, and that defoliation impairs nutrient root uptake. Hence, allocation of C into storage to prevent further C losses (*sensu* Wiley and Helliker, 2012) would potentially be evolutionarily unsuccessful if trees could not also acquire required N to re-allocate the C for re-foliation. In fact, high levels of both C and N storage in winter-deciduous species are thought to be an adaptation to tolerate defoliation (Grelet *et al*., 2001; Millard *et al*., 2001; Millard and Grelet, 2010; Piper and Fajardo, 2014). Additionally, if tree growth is impaired due to a preferential allocation of C to storage, N pools can be expected to remain high due to a lower demand of nutrients for growth. To date, very few studies have simultaneously examined how plants allocate both C and N into storage in responses to defoliation, leaving substantial uncertainty about how these pools interact.

Another factor that could explain suppressed growth following severe defoliation is an increase in C allocation to the production of secondary metabolites, which could divert C from growth (Herms and Mattson, 1992; Jones and Hartley, 1999; Hamilton *et al*., 2001). Secondary metabolites are expensive to synthesize, and share (i.e. compete for) common precursors and substrate with primary metabolites (e.g. structural C and NSC

FIG. 2. *Nothofagus pumilio* forest after a massive defoliation caused by an outbreak of the larvae of *Ormiscodes amphimone* (Saturniidae) during summer 2009 in the southern Andes of Chile (Aysén Region, Patagonia; left). The left-side photograph was taken in April 2009 (mid-autumn); thus, the reddish canopy at the higher elevation corresponds to the forest which was not defoliated due to the caterpillars' thermal threshold. Note the green (i.e. new) leaves produced after the defoliation event on the tree in the front of the photograph. Larvae of *O. amphimone* feeding on leaves attached to a branch of *N. pumilio* (right).

invested in growth and storage, respectively) (Herms and Mattson, 1992). Likewise, herbivory or artificial defoliation may induce changes in morphological leaf traits with defensive functions (e.g. higher leaf mass per area, LMA) (Millard *et al*., 2001; Nabeshima *et al*., 2001), and thus could increase the total cost of re-foliation. This strategy could be beneficial for species that experience low levels of competition (i.e. where fast growth is not necessary) and strong disturbance pressure caused by defoliation (e.g. herbivory outbreaks). In fact, the production of a well-defended foliage after a season of defoliation (i.e. delayed induced resistance) is a common feature in winter-deciduous species adapted to severe defoliations (e.g. *Betula* spp.) (Krause *et al*., 1993). On the other hand, in winter-deciduous species, defoliation-induced synthesis of C-based defences could be a simple consequence of an imbalance between C and N, given that defoliation is suggested to reduce N more than C (Herms and Mattson, 1992; Krause *et al*., 1993). Although it is difficult to distinguish whether increased allocation to defences along with decreased growth reflects nutrient-limited growth or up-regulation, the former would be supported if reduced growth and increased leaf C-based defences occurred with a concomitant reduction in nutrient concentration across all tissues (Hypothesis 2, Fig. 1). Alternatively, an increase of induced C-based defences under invariable or increasing whole-tree nutrient status could reflect either up-regulation of C-based defences or a C accumulation resulting from growth limitations driven by factors other than nutrients (Hypotheses 3, Fig. 1). Thus, a closer look at the C and N dynamics within a tree during recovery from severe defoliation can help to distinguish among several contrasting mechanisms proposed to explain impaired tree growth.

In this study we analysed the response of adult trees of the winter-deciduous and herbivory-tolerant broadleaved species *Nothofagus pumilio* to a complete natural defoliation caused by a moth caterpillar outbreak (*Ormiscodes amphimone*, Saturniidae) that occurred in the southern Andes during summer 2009. This particular outbreak led to the massive and complete natural defoliation of thousands of hectares of forest (Fig. 2). Herbivory in *Nothofagus pumilio* forests has been shown to be strongly controlled by temperature, and there is hence a growing concern regarding how this tree species will react to a higher herbivory, which is expected to occur under higher temperatures (Garibaldi *et al*., 2011; Mazía *et al*., 2012). For two consecutive years following the defoliation event, we monitored tree survival and measured responses of growth, re-foliation, leaf chemical and morphological defences, and C and N storage. A previous study indicated that juvenile trees of the species account for high levels of C and N storage in woody tissues which are re-mobilized to tolerate defoliation (Piper and Fajardo, 2014). Assuming that after defoliation trees effectively grow less and can eventually die, we posited multiple alternative hypotheses that can mechanistically explain such a response (Fig. 1). In brief, we hypothesized that a decrease in growth following defoliation is caused by: (1) a shortage of C (i.e. C limitation hypothesis) (Hypothesis 1, Fig. 1), (2) a shortage of N (e.g. the nutrient limitation hypothesis) (Hypothesis 2, Fig. 1) or (3) trees are not limited by either C or N, but growth is instead reduced by either preventative C allocation to storage (Hypothesis 3a, Fig. 1) or factors other than C or N (Hypothesis 3b, Fig. 1).

MATERIALS AND METHODS

Species and research site

The study was carried out in the Aysén Region, Patagonia, Chile, specifically in the Reserva Nacional Cerro Castillo conservation area, where the forest comprises primarily *Nothofagus pumilio* (Nothofagaceae). Mean annual precipitation is approx. 1000 mm and is distributed regularly throughout the year (Dirección General de Aguas, Servicio Meteorológico Nacional); mean temperature for the growing season is 8·6 °C. The soil in the study area is derived from aeolian volcanic ash deposits. *Nothofagus pumilio* (Poepp. Et Endl.) Krasser (Nothofagaceae) is a broadleaved winter-deciduous tree species endemic to the southern Andes of South America, distributed from 35 to 55 °S. It is one of the most cold-resistant tree species of the region, forming high-elevation forests and being the dominant treeline species (Alberdi *et al*., 1985; Fajardo *et al*., 2013). The growing season for *N. pumilio* typically starts in

October and extends to mid-April, although this varies with latitude and elevation (Hevia *et al*., 1999). In Patagonia, leaf-out occurs in late October, maximum leaf size is reached in early December, leaf senescence (reddish colour) occurs in April and complete leaf shedding is achieved in May (A. Fajardo, unpubl. res.).

We selected four sites in the Cerro Castillo National Reserve that were defoliated during the growing season of 2009. These sites are pure second-growth forest of *N. pumilio* 50–80 years old, with diameters at breast height (dbh, 1·35 m) of 25–35 cm, heights of 8–15 m and stand densities of 1100–1800 trees ha^{-1} (A. Fajardo, unpubl. res.). At each site a frontier between trees that were fully defoliated and trees that escaped defoliation was clearly observed. Caterpillars of *Ormiscodes amphimone* have a very well-defined temperature threshold (Fig. 2), leading to an abrupt boundary between defoliated and non-defoliated trees (i.e. controls). Thus, at each site, we sampled trees 20 m below (defoliated) and 20 m above (non-defoliated) this boundary. The four sites were Estero Parada (46°05′37″S, 72°14′10″W, 805 m a.s.l.), Refugio (46°05′59″S, 72°13′58″W, 754 m a.s.l.), Cerro Castillo (46°06′28″S, 72°05′14″W, 610 m a.s.l.) and La Cuesta (46°06′18″S, 72°03′26″W, 760 m a.s.l.). In April 2009 (the end of the growing season in the austral hemisphere) we photographed these boundaries (Fig. 2) and marked defoliated (no leaves) and non-defoliated trees to simplify *a posteriori* identification of sampling sites. Non-defoliated trees at this time of the year still had leaves.

Sampling design

With the assistance of the photographs previously taken, we selected 30 defoliated (below the elevational frontier) and 30 non-defoliated *N. pumilio* adult trees (above the frontier) across four sites (seven trees at two sites, and eight trees at the other two sites). To test our hypotheses we first needed to assess whether defoliated trees survived and if so whether they effectively grew less after defoliation than non-defoliated trees. All trees were monitored for survival and signs of decay (e.g. absence of leaves, branch mortality, colonization by wood decay fungi) in March 2010, 2011 and 2014. For all trees we collected tissue samples for re-foliation measurements, NSC, N and leaf chemical defences, as well as for the determination of LMA (g m^{-2}) and other leaf traits at mid-March 2010 and 2011 (i.e. 1 and 2 years after the defoliation occurred). This time of year represents the end of the growing season in the southern hemisphere, when it is known that C and nutrient replenishment start to occur in winter-deciduous species (Barbaroux and Bréda, 2002; Hoch *et al*., 2003).

Tree growth determination

Tree growth was assessed retrospectively as an annual basal area increment. In March 2014, we measured on each individual tree dbh (1·35 m above ground), diameter at coring height (dch, approx. 0·2 m above ground) and bark thickness at dch, and extracted two increment cores to the pith at dch. Each tree was cored perpendicular to the slope using a 5·15-mm increment borer (Haglöf, Långsele, Sweden). Cores were prepared following standard dendrochronological techniques (Stokes and Smiley, 1996). For the purposes of this study, we assigned an annual ring to the calendar year in which the radial growth was completed. All samples were dated and visually cross-dated to detect the presence of either false or incomplete rings using marker rings, especially in the defoliated trees; in this case, non-defoliated trees served as chronology references. Following visual cross-dating, tree ring width was measured to the nearest 0·001 mm and assigned to calendar years using a microscope mounted on a dendrochronometer with a Velmex sliding stage (Bloomfield, NY, USA) and Accurite measuring system (St Louis, MO, USA). The annual basal area increment (BAI) was then computed for each of the last 6 years (including the 2 years after defoliation) as:

$$\mathrm{BAI} = \pi\left(R_n^2 - R_{n-1}^2\right),$$

where R is the radius of the stem without bark at dch and n is the year of the tree ring completion.

Re-foliation measurements

To evaluate the leaf area recovery after defoliation, we estimated re-foliation density as the number, mass and area of leaves per branch in March 2010 and March 2011. We also measured mean leaf area and LMA as traits associated with crown recovery and morphological defences. For each individual tree, we targeted and cut a terminal, fully expanded and sun-exposed branch at 2–3 m height using pruner scissors. Branches were labelled and placed in a cooler for transportation. Selected branches were approx. 4 years old and had a similar diameter for control and defoliated trees (6·5 ± 0·15 and 6·22 ± 0·16 mm, respectively; $P > 0{\cdot}20$, Student's t-test); however, we considered the branch diameter as a co-variable for all response variables used to quantify re-foliation (see Statistical analysis for further details). Tissue samples were collected between 1200 and 1700 h. In the laboratory, all leaves per branch were detached and counted. Some leaves were then separately laid flat on a white paper sheet and photographed with a reference square of known area using a Nikon Coolpix 5000 digital camera (Nikon, Tokyo, Japan), the total projected leaf area was then determined using SIGMAPROC image processing software (Systat Software, Richmond, CA, USA) and mean area per leaf was calculated by dividing total leaf area by the number of leaves. All leaves were then placed to dry in a forced-air stove (Memmert, Schwabach, Germany) at 70°C for 72 h and finally the photographed leaves and the remaining leaves were separately weighted on a scale at 0·0001 g precision to determine LMA and leaf mass per branch. We computed LMA as the oven-dried leaf weight divided by its total foliar surface. Finally, all leaves were ground to a fine powder using a coffee mill; they were then stored at 4°C until chemical analyses were performed.

Leaf C-based defences

For each leaf sample, leaf extracts were created by extracting 0·2 g of ground leaves in 20 mL of 50 % methanol, which were shaken for 1 h, and separated via centrifugation (Gundale *et al*.,

2010; Sundqvist *et al*., 2012). Extracts were then analysed for total phenolics and condensed tannin concentrations using the Prussian blue technique (Stern *et al*., 1996) and acid–butanol method (Porter *et al*., 1985), using catechin (+/–) and procyanidin B2 (Sigma-Aldrich, St Louis, MO, USA) as standards, respectively. Due to potential differences in reactivity of *N. pumilio* phenolics and tannins with reagents compared with the standards, the total phenolic and tannin masses are reported on a catechin and procyanidin equivalent basis, rather than as absolute masses.

NSC and N analyses

We determined NSC (soluble sugars + starch) and N concentrations in leaves, branches and stems of all trees in mid-March 2010 and mid-March 2011 (i.e. late summer). In addition, we also determined NSC in stems and roots for five trees per treatment on 19 January 2010 (i.e. the middle of the first growing season after defoliation) to examine defoliation effects on C storage at the most active period of growth, and to examine potential differences in NSC trends between stems and roots – the two major C storage sites in winter-deciduous species (Millard *et al*., 2001), and for *N. pumilio* in particular (Piper and Fajardo, 2014). The latter helped us to ultimately decide whether an estimation of NSC in major storage sites would be sufficient by sampling only stems, given that root sampling was logistically more complicated. This allowed us to identify that NSC in roots and stems responded similarly, allowing us to exclude roots from the sampling, and focus on leaves, branches and stems to provide an integral view of C storage in the trees.

From each tree we used an increment borer to extract a 10-cm stem core at dbh, and shovels and scissors to remove a coarse piece of superficial root (approx. 0·5–1 cm in diameter, for the first sampling only) and to cut a 5-cm length of branch (approx. 0·5–1 cm in diameter), and hand-collected leaves. Bark and phloem were removed from the pieces of branch and roots in the field with a knife. Plant material was properly labelled and brought to the laboratory in a cooler with ice to reduce tissue respiration during transport (Popp *et al*., 1996). In the laboratory, all samples were divided into two pieces, one for NSC and one for N analyses. Samples for NSC analyses were heated in a microwave in three 20-s cycles at maximum power to stop enzymatic activity (Popp *et al*., 1996) and then, along with the samples for N analyses, placed in a forced-air oven at 65°C to dry until constant weight. Branch and stem samples were then ground to a fine powder using a mixer ball mill MM 200 (Retsch, Haan, Germany), and subsequently stored at 4°C until chemical analyses were performed. We determined soluble sugars and starch concentrations in approx. 15 mg of dried powder of every tissue sample. Soluble sugars were extracted with a methanol/chloroform/water solution, separated from pigments and lipids by adding water and chloroform (Rose *et al*., 1991), and then main sugars (sucrose, glucose, fructose) were determined with the phenol sulphuric method, using 2 % phenol and reading at 490 nm (Chow and Landhäusser, 2004). The residual pellet was dried overnight at 50°C in a forced-air oven and starch was then gelatinized (Rose *et al*., 1991) and hydrolysed to glucose with amyloglucosidase (Sigma-Aldrich 10115) at 45°C overnight. We determined glucose in a similar way as soluble sugars (Chow and Landhäusser, 2004). Soluble sugars and starch concentrations were expressed as mg per g dry weight. Total NSC concentrations were estimated as the sum of soluble sugars and starch. The N concentration of each tissue sample was determined from 25 mg of dry and ground powder by a combustion analyser (LECO TruSpec Micro CHN, Centro de Investigación en Ecosistemas de la Patagonia, Coyhaique, Chile). N concentrations were expressed on a dry mass basis (as % dry matter). This method quantifies total N (i.e. it does not distinguish between stored and structural N), which is widely used to examine changes in N storage (Chapin, 1980; Millard *et al*., 2001; Silla and Escudero, 2003), given that most tissue N can be potentially remobilized (Chapin *et al*., 1990; Millard and Grelet, 2010).

Statistical analyses

The influence of defoliation on BAI, re-foliation (leaf number, area and mass per branch), C and N storage, and leaf morphological and chemical properties (mean leaf area, N, tannins, phenolics and LMA) was analysed fitting linear mixed-effects models (LMMs). In the modelling, we considered defoliation condition (Control and Defoliated) as the fixed factor and sites as the random factor to account for among-site variation. When variables did not meet normality assumptions they were $\log_{10}$ transformed. In all cases, analyses were run separately for each year (2010 and 2011 for all response variables, except for growth, for which analyses were run from 2005 to 2012). For re-foliation variables, we considered branch diameter as a covariable, and tested the significance of the interaction between branch diameter and defoliation. The lack of significance for the interaction means that our re-foliation measurements were not biased by the diameter of the branches sampled in defoliated and control trees. For C and N storage, analyses were performed by tissue (leaves, branches, stems). Finally, we used Student's *t*-tests to compare NSC and N concentrations between control and defoliated trees in January 2010. We found that defoliation did not cause any mortality, and therefore no statistical analysis was performed to evaluate survival. All analyses were performed in JMP Version 8.0 (SAS Institute, Cary, NC, USA).

RESULTS

Annual BAI was not significantly different between control and defoliated trees for all years previous to defoliation ($P > 0{\cdot}05$). Defoliation occurred between January and March 2009, and thus at this time there was a non-significant growth reduction in defoliated trees when compared with control ones ($F = 0{\cdot}60$, $P = 0{\cdot}44$). However, 1 year after the defoliation (the growing season period starting in the austral spring of 2009), defoliated trees grew less than half (41 % of BAI) as much as control trees ($F = 33{\cdot}00$, $P < 0{\cdot}001$). This significant difference remained for the subsequent year 2011 ($F = 13{\cdot}39$, $P < 0{\cdot}001$, Fig. 3).

For the 2 years examined after defoliation, defoliated trees had significantly lower re-foliation than control trees (Fig. 4). The former had significantly fewer leaves, and lower leaf mass and leaf area per branch for the first year after the defoliation (2010) than the latter (Table 1). For the second year after

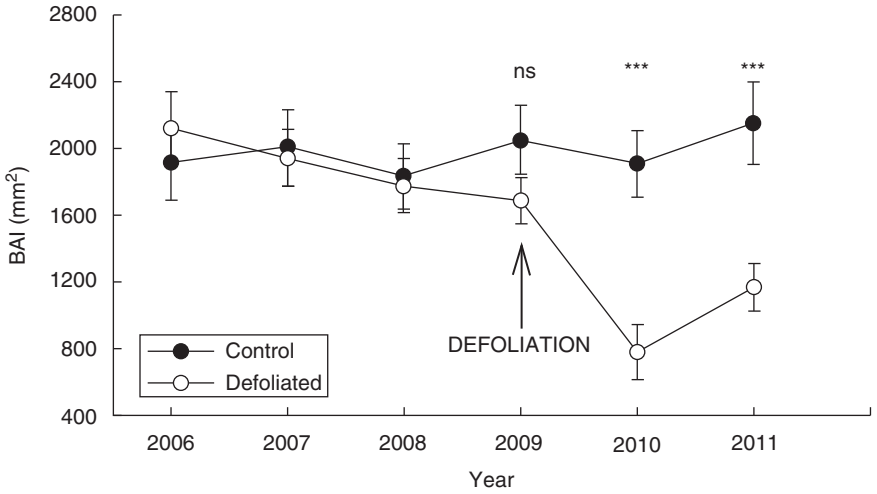

FIG. 3. Mean annual relative tree growth (basal area increment in mm^2, BAI) of non-defoliated (i.e. Control) and Defoliated trees of *Nothofagus pumilio* after a massive and complete defoliation caused by the moth caterpillar *Ormiscodes amphimone* (Saturniidae) during summer 2009 (i.e. January and February) in the southern Andes of Chile.

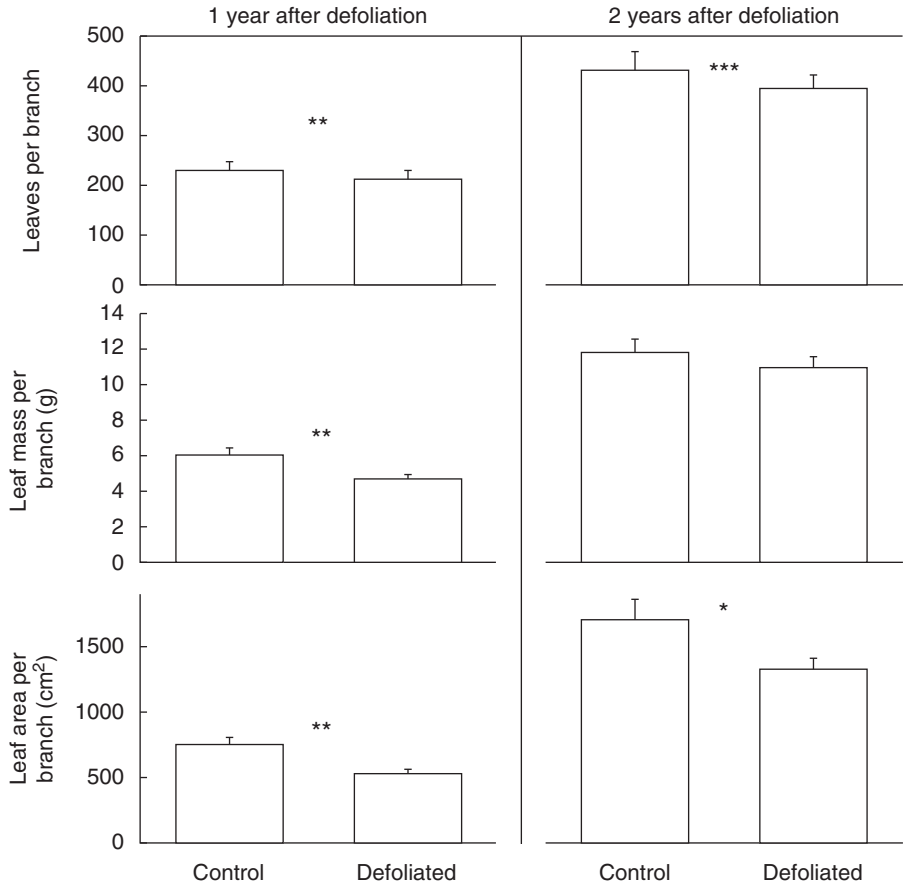

FIG. 4. Number, mass and area of leaves per branch in non-defoliated (i.e. Controls) and Defoliated trees of *Nothofagus pumilio* 1 and 2 years after a massive and complete natural defoliation caused by the moth caterpillar *Ormiscodes amphimone (*Saturniidae) during summer 2009 (i.e. January-February) in the southern Andes of Chile. Bars represent mean values, and error bars refer to standard errors ($n = 30$). Asterisks indicate significant differences between defoliated and control trees at $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ and $***P < 0{\cdot}001$.

TABLE 1. *Summary of* F-*ratios and inference* (P-*values) for the effects of defoliation (De), branch diameter (Bd), and the interaction of both on leaf number, mass and area per branch of* Nothofagus pumilio*, 1 and 2 years after a massive and complete defoliation caused by the caterpillar* Ormiscodes amphimone *(Saturniidae) during summer 2009 in the southern Andes of Chile (46 °04′S, 72 °03′W), based on linear mixed-effect models (LMM)*

Response variable	Model effects	1 year after defoliation	2 years after defoliation
Leaf number	De	0·01 (0·920)	0·25 (0·619)
	Bd	9·58 (0·004)	7·19 (0·012)
	De × Bd	0·65 (0·424)	1·15 (0·289)
Leaf mass	De	8·22 (0·006)	0·23 (0·637)
	Bd	4·68 (0·041)	17·05 (<0·001)
	De × Bd	0·27 (0·605)	2·91 (0·094)
Leaf area	De	12·45 (<0·001)	4·42 (0·040)
	Bd	1·39 (0·244)	10·64 (0·002)
	De × Bd	0·82 (0·369)	4·63 (0·036)

defoliation (2011), leaf area per branch remained significantly lower for defoliated than for control trees (Table 1, Fig. 4). Branch diameter had a significant positive effect on leaf number, area and mass per branch, but the interaction term in the analysis between branch diameter and defoliation condition was only significant for leaf area in the second year (Table 1).

We found a significant difference in leaf morphological and chemical traits between defoliated and control trees, particularly in the first year after defoliation (2010). In particular, defoliated trees displayed smaller leaves (2010: $F = 16{\cdot}53$, $P < 0{\cdot}001$; 2011: $F = 3{\cdot}77$, $P = 0{\cdot}057$), lower leaf N concentrations (Table 2) and lower tannin concentrations (2010: $F = 10{\cdot}40$, $P = 0{\cdot}002$; 2011: $F = 34{\cdot}52$, $P < 0{\cdot}001$; Fig. 5). Polyphenol leaf concentration, by contrast, was higher for defoliated than for control trees, although this difference was observed only in the first year after defoliation (2010: $F = 18{\cdot}78$, $P < 0{\cdot}001$; 2011: $F = 0{\cdot}061$, $P = 0{\cdot}805$). As an exception, LMA was similar between defoliated and control trees for both the first ($F = 0{\cdot}017$, $P = 0{\cdot}97$) and the second year after the defoliation ($F = 1{\cdot}35$, $P = 0{\cdot}250$; Fig. 5).

By January 2010, NSC concentrations in roots and stems were similar between control and defoliated trees ($F_{1,9} = 1{\cdot}86$, $P = 0{\cdot}21$ for roots; $F_{1,9} = 2{\cdot}91$, $P = 0{\cdot}13$ for stems; Fig. 6 inset). By March 2010, NSC concentrations were also similar between defoliated and control trees for leaves ($F = 0{\cdot}52$, $P = 0{\cdot}470$), branches ($F = 0{\cdot}12$, $P = 0{\cdot}730$) and stems ($F = 0{\cdot}15$, $P = 0{\cdot}700$; Fig. 6). Likewise, after 2 years (March 2011), defoliation had no significant effect on the NSC concentrations of leaves ($F = 0{\cdot}73$, $P = 0{\cdot}390$) or stems ($F = 1{\cdot}63$, $P = 0{\cdot}210$; Fig. 6). However, and in contrast to the first year's results, we found a significant increase in branch NSC concentration of defoliated trees ($F = 5{\cdot}43$, $P = 0{\cdot}020$; Fig. 6). In more detail, soluble sugars represented the main NSC component of leaves, whilst starch dominated in branches and stems (Table 2). Soluble sugars in leaves and branches experienced a significant reduction after 1 year of defoliation. This effect, however, disappeared in the second year, when soluble sugar concentrations in stems were lower for the defoliated trees (Table 2). In contrast, foliar starch was higher in defoliated than in control trees, for both the first and the second year after defoliation, while branch starch concentration was higher in defoliated trees than in control trees for the second year only (Table 2). Finally, N storage in woody tissues did not vary in response to defoliation. N concentrations in both branches and stems were similar between defoliated and control trees for both of the two years they were measured (Table 2). The same pattern was found for the January 2010 measurements, when stem N concentrations were $0{\cdot}38 \pm 0{\cdot}03$ and $0{\cdot}37 \pm 0{\cdot}02$ % for defoliated and control trees, respectively ($F_{1,9} = 0{\cdot}02$, $P = 0{\cdot}900$), and root N concentrations were $0{\cdot}35 \pm 0{\cdot}03$ and $0{\cdot}35 \pm 0{\cdot}02$ % for defoliated and control trees, respectively ($F_{1,9} = 0{\cdot}01$, $P = 0{\cdot}990$).

DISCUSSION

Two years after the widespread complete defoliation event occurred, none of the defoliated trees died or showed evidence of die-back. This highlights the tolerance of *Nothofagus pumilio* to herbivory, a winter-deciduous species, which is supported by a previous study in which juvenile trees of the same species survived complete and chronic artificial defoliation for 3 years (Piper and Fajardo, 2014). Nonetheless, complete defoliation reduced stem growth and re-foliation for the two subsequent years following the event. Over this period, NSC concentrations in defoliated and in control (non-defoliated) trees were similar or even higher for the former, providing no support that growth reduction of *N. pumilio* 2 years after complete defoliation was due to C limitation. It has been suggested that C limitation may occur even when high concentrations of NSC are present in storage tissues, if trees were unable to remobilize this C (i.e. C sequestration) (Millard *et al*., 2007). However, this is unlikely for *N. pumilio*; juvenile trees of *N. pumilio* were able to rely strongly (i.e. remobilize) on their woody NSC and N storages to re-foliate after complete artificial defoliation (Piper and Fajardo, 2014). Further evidence that C limitation did not explain the reduced growth is seen in the response of C-based defences. Under C limitation, total polyphenols are expected to decrease because they are C costly (Herms and Mattson, 1992) (Hypothesis 1, Fig. 1). Contrary to this expectation, we found a significant increase in total polyphenol leaf concentrations by the end of the first growing season following defoliation, which is more suggestive of a C surplus than a deficit (Herms and Mattson, 1992).

We think that the lack of C limitation in *N. pumilio* after defoliation is probably due to its winter-deciduous leaf habit. It has been proposed that evergreen species are more prone than winter-deciduous species to become C limited after defoliation, given that the former store more C in leaves than the latter (Herms and Mattson, 1992; Krause *et al*., 1993). In fact, significant decreases of C storage and growth, indicative of C limitation, have been found in adult evergreen conifers 1 year after leaf loss caused by disease or defoliation (Li *et al*., 2002; Galiano *et al*., 2011; Palacio *et al*., 2012). In contrast, winter-deciduous trees seem to be less prone to C limitation after defoliation, probably because woody tissues serve as their main location for C storage, which are generally protected from herbivory (Millard *et al*., 2001). In deciduous species, neither single-season severe defoliations nor moderate chronic defoliation appear to provoke reductions in C storage (Reichenbacker

TABLE 2. *Soluble sugars, starch (on a dry mass basis, mg g^{-1}) and nitrogen (%) concentrations (mean ± 1 s.e.) and statistical inference, for different tissues in adult trees of* Nothofagus pumilio *growing naturally in Patagonia, Chile, which were or were not affected by a massive defoliation, after 1 and 2 years of an outbreak of* Ormiscodes amphimone *(Saturniidae); data were analysed using linear mixed-effects models*

	1 year after defoliation			2 years after defoliation		
	Control	Defoliated	*F*-ratios (*P*-values)	Control	Defoliated	*F*-ratios (*P*-values)
Soluble sugars						
Leaf	148·7 (3·31)	139·2 (4·05)	6·36 (0·015)	122·8 (7·29)	116·8 (6·56)	0·68 (0·41)
Branch	25·9 (1·16)	22·0 (1·29)	4·88 (0·031)	27·9 (1·54)	27·2 (1·89)	0·13 (0·72)
Stem sapwood	15·6 (0·87)	16·0 (1·19)	0·10 (0·75)	16·2 (1·08)	13·4 (0·95)	6·64 (0·013)
Starch						
Leaf	41·0 (1·83)	54·0 (1·81)	26·3(<0·001)	35·9 (1·40)	48·5 (1·77)	37·12 (<0·001)
Branch	58·4 (2·55)	63·9 (3·74)	1·66 (0·202)	51·8 (2·01)	62·6 (3·05)	10·71 (0·002)
Stem sapwood	28·2 (2·80)	26·0 (2·41)	0·35 (0·553)	21·55 (1·04)	21·1 (1·59)	0·06 (0·807)
Nitrogen						
Leaf	1·84 (0·05)	1·60 (0·05)	13·85 (<0·001)	1·82 (0·04)	1·60 (0·04)	14·30 (<0·001)
Branch	0·46 (0·01)	0·48 (0·02)	0·95 (0·330)	0·44 (0·01)	0·45 (0·02)	0·06 (0·800)
Stem sapwood	0·29 (0·01)	0·29 (0·01)	0·60 (0·440)	0·30 (0·01)	0·29 (0·01)	0·52 (0·470)

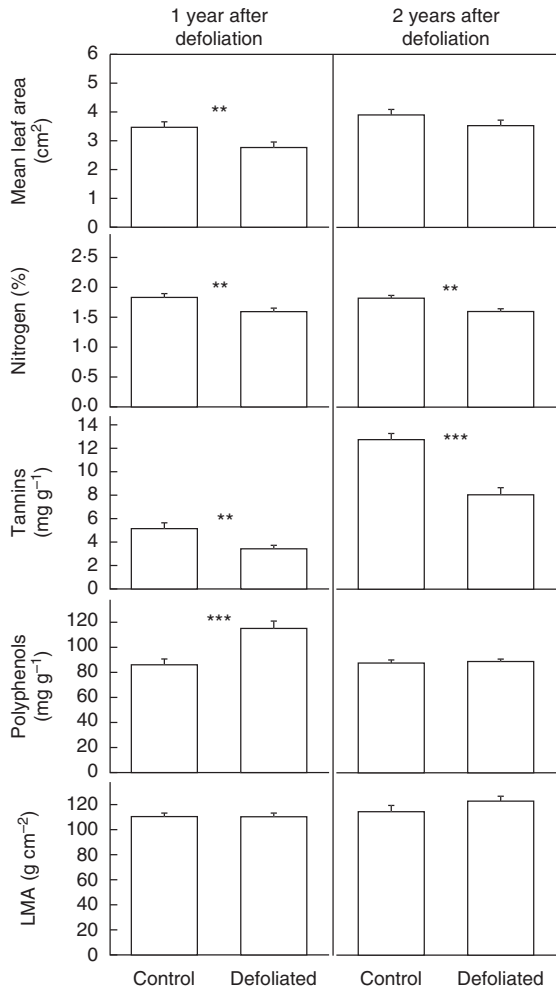

FIG. 5. Morphological and chemical characteristics of leaves in non-defoliated (i.e. Controls) and Defoliated trees of *Nothofagus pumilio* 1 and 2 years after a massive and complete forest natural defoliation caused by the moth caterpillar *O. amphimone* (Saturniidae) in the southern Andes of Chile during summer 2009 (i.e. January and February 2009). Bars represents mean values, and error bars indicate standard errors ($n = 30$). Asterisks indicate significant differences between defoliated and control trees at $^{**}P < 0{\cdot}01$ and $^{***}P < 0{\cdot}001$.

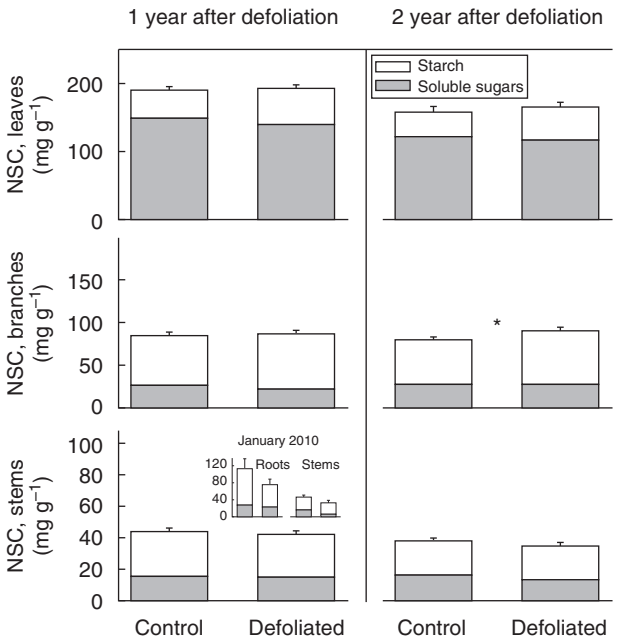

FIG. 6. Non-structural carbohydrate (NSC) concentrations per unit of dry mass in leaves, branches and stem sapwood in non-defoliated (i.e. Control) and Defoliated trees of *Nothofagus pumilio* after 1 and 2 years of a massive outbreak defoliation by the moth caterpillar *Ormiscodes amphimone* (Saturniidae) during summer 2009 in the southern Andes of Chile. Bars represent mean total NSC values; grey and white sections represent soluble sugars and starch, respectively; error bars refer to standard errors ($n = 30$). Asterisk indicates a significant difference between control and defoliated trees, at $^{*}P < 0{\cdot}05$, according to linear mixed-effect models. Inset: NSC concentrations for roots and stems measured in a subset of control and defoliated trees ($n = 5$) earlier in the season (January 2010) of the first year after the defoliation.

et al., 1996; Kosola *et al*., 2001; Palacio *et al*., 2008). Rather, the evidence gathered so far illustrates that only when defoliation is both complete and chronic is C storage reduced in these species (Wargo *et al*., 1972; Piper and Fajardo, 2014).

In addition to the C limitation hypothesis, our results also do not support our second hypothesis that N limitation could

impede growth recovery. Complete defoliations in *N. pumilio* reduced N concentrations in leaves but not in woody tissues. Woody tissues (i.e. root and trunk sapwood) are known to represent the main pool of N storage in winter-deciduous species (Millard, 1994; Grelet *et al*., 2001; Silla and Escudero, 2003; Millard and Grelet, 2010). This storage is thought to be an adaptation of deciduous species to severe defoliation, as it can be re-mobilized to meet demands for re-foliation and growth when root nutrient uptake fails (Millard *et al*., 2001; Millard and Grelet, 2010). Although our approach to estimate N storage (i.e. total N concentration) may not be as precise as others (e.g. isotopes), a previous study showed that juvenile trees of *N. pumilio* subjected to complete simulated defoliation over three consecutive years exhausted their N storage in woody tissues at the time that they increased their leaf N concentrations (Piper and Fajardo, 2014). In contrast, the mature trees that were naturally defoliated in this study did not show any changes in their N storage (i.e. their levels were comparable to control trees) and did not use their N reserves to re-foliate as predicted by our second hypothesis (Hypothesis 2, Fig. 1). The discrepancy between these studies is probably due to the defoliation regime: in both cases trees were completely defoliated but in the first case it was more frequent and therefore more likely to induce N limitations. Other factors could be also involved. For example, artificial defoliation may elicit different physiological responses from natural herbivory (Quentin *et al*., 2010; Musser *et al*., 2012), and the ability to remobilize NSC (and probably also N) and compensate for leaf damage may be lower in mature trees than in saplings (Boege, 2005). Also, whereas in Piper and Fajardo's (2014) experiment leaf nutrients were exported out of the system, in the natural defoliation examined here the N contained in the insect's frass was perhaps easily available for immediate uptake (Frost and Hunter, 2008). It may be possible that the access to frass N prevented defoliated trees reducing their leaf N concentrations below the threshold required to induce N mobilization from storage in woody tissues. The lack of support for the N limitation hypothesis as an explanation for growth reduction in defoliated trees of *N. pumilio* is further supported by the mismatch between trends in leaf C-based defences and trends in NSC concentrations after defoliation. If growth had been reduced as a result of N limitation, we expected that a concomitant increase in NSC and C-based defence concentrations would have occurred, as growth is more sensitive than photosynthesis to nutrient shortages and this in turn determines a C surplus (Tuomi *et al*., 1990; Herms and Mattson, 1992; Palacio *et al*., 2014) (Hypothesis 2, Fig. 1). However, we did not observe this trend in our study, suggesting that the increased concentration of total polyphenols for the first year was a result of up-regulation. Likewise, the similar concentration between defoliated and control trees for the second year, when NSC accumulated in the branches of defoliated trees, seems to reflect down-regulation of these defences.

Our scheme of *a priori* hypotheses leads us to two remaining mechanisms driving growth decrease in defoliated trees of *N. pumilio* (Fig. 1). First, the remarkable growth reduction in defoliated trees of *N. pumilio* may relate to a defoliation-driven preventative shift in C allocation from growth to storage (Wiley and Helliker, 2012), and possibly to defences as well (Hamilton *et al*., 2001) (Hypothesis 3a, Fig. 1). Preventative C allocation has been interpreted as a form of C limitation, i.e. although the tree has enough C to grow, it does not use this C in growth because it would prioritize other physiological functions. Thus, the process of growth is internally C limited. Compatible with this hypothesis, we found similar (or even higher) NSC concentrations in woody tissues of defoliated trees relative to control trees at the end of the first and second growing seasons following defoliation. Our results are also partially consistent with a defoliation-induced shift in C allocation from growth (and perhaps from storage) to defences (Hamilton *et al*., 2001); leaf polyphenol concentration increased for the first year but not for the second year despite C surplus in branches. Interestingly, condensed tannins actually declined after defoliation for the first year after defoliation, when total polyphenols increased (Fig. 5). This pattern may have been the result of an up-regulation of specific classes of polyphenols other than tannins at deterring the specific herbivore responsible for the outbreak in our study system. Altogether, our results are consistent with the view that tree responses of storage and defences to defoliation are highly regulated and not a mere result of C and/or N imbalances (Chapin *et al*., 1990; Anderegg and Callaway, 2012; Wiley and Helliker, 2012).

A second possible explanation for the remarkable growth reduction in defoliated trees of *N. pumilio* is that growth could be directly limited by factors other than N and C (Hypothesis 3b, Fig. 1). For example, limited bud availability has been suggested to constrain re-foliation in another winter-deciduous species, *Betula pendula*, subjected to browsing (Palacio *et al*., 2008). Although we are certain that after the defoliation event of 2009 no other pathogen or herbivore fed on trees used in this study, the possibility that the outbreak caterpillars consumed the buds that were forming at the time of defoliation cannot be discarded. Species with an indeterminate growth pattern and with buds capable of neoformed growth are expected to have a greater potential capacity for compensatory growth than those with a fixed growth pattern driven by tissue preformation (Millard *et al*., 2001). Shoot and foliage expansion in *N. pumilio* are driven mostly by preformation during the previous season, while neoformation accounts only for a low proportion of leaves and does not occur in all branches (Souza *et al*., 2000; Guédon *et al*., 2006). Thus, potential bud herbivory during the outbreak would have reduced shoot growth and re-foliation in the next growing season (i.e. 2010). Furthermore, organogenesis in *N. pumilio* could depend more on current photoassimilation than on storage, and hence poor re-foliation in 2010 could have limited preformation of tissues expected to expand in the next season (i.e. 2011, second year after defoliation). On the other hand, defoliation may also cause hormonal imbalances that in turn can impede or limit growth (Kulman, 1971; Boege, 2005). Leaves exert a strong hormonal control on budburst, so leaf removal may stimulate renewed growth of buds (i.e. flushing) that otherwise would break in the following season (Collin *et al*., 2000). This would limit the bud availability in the following season. Indeed, we observed premature budburst in defoliated trees of *N. pumilio* (e.g. new, green leaves were observed in autumn, Fig. 2). It has also been indicated that the utilization of photosynthates for stem growth is regulated by hormones produced in the foliage (Kulman, 1971). Consistent with this, Palacio *et al*. (2012) suggested that defoliation in *Pinus nigra* reduced the levels of indole-3-acetic-acid near the cambial region, leading to decreased import of photoassimilates and

eventually to reduced radial growth. Under such conditions of growth limitation, however, defoliated trees should have concomitantly increased their NSC and N storage and C-based defences (Hypothesis 3a, Fig. 1), which was not the case here: among the tissues examined, a slight increase in NSC was found only for branches in the second year, while total polyphenols increased in the first year (i.e. both increases were not concomitant).

CONCLUSIONS

Currently, a growing debate centres on whether extreme disturbances associated with climate change (e.g. defoliation, drought) provoke C or growth limitations in trees (Sala *et al*., 2010; McDowell *et al*., 2011; Wiley and Helliker, 2012). The classical approach to distinguish between these two mechanisms has been to assess plant NSC, which has been recently recognized as imperfect and incomplete (Millard and Grelet, 2010; Sala *et al*., 2012; Wiley and Helliker, 2012; Palacio *et al*., 2014). Our study provides a more integrative approach to evaluating plant growth limitations in response to disturbance, by examining major resources other than C (e.g. N), and other C sinks besides storage and growth (e.g. defences and re-foliation). In doing so, we show that the significant reduction in growth of *N. pumilio* in response to herbivory was not caused by insufficient C or N availability, as suggested by several studies. By doing this, we were not only able to discard C limitation (as traditionally defined, i.e. insufficient C availability for growth), but also N limitation, which have been proposed as a major cause of growth limitation in trees affected by defoliation. We propose that the growth reduction in defoliated trees of a deciduous species, such as *N. pumilio*, may relate to other factors that can limit growth (e.g. hormonal disruption), or, alternatively, to a highly regulated C and nutrient conservation strategy (i.e. preventative allocation) driven by a defoliation-induced shift in allocation priorities that is compatible with C and N limitation in spite of non-reduced levels of C and N storage. We finally suggest that these allocation shifts reduce leaf palatability (lower leaf N concentration, higher polyphenol concentrations) over the seasons following the defoliation to repel potential new defoliators and allow trees to more quickly replenish NSC and nutrient stores. Large-scale severe defoliation events, such as the one we describe in our study, are increasingly reported in ecosystems around the world, with many of them associated with global warming or other environmental change factors (van Mantgem *et al*., 2009). Our study highlights that a more integrative plant physiological approach is needed to understand how tree growth is regulated in response to disturbance or environmental change.

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