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Research paper

The effects of defoliation on carbon allocation: can carbon limitation reduce growth in favour of storage?

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There is no consensus about how stresses such as low water availability and temperature limit tree growth. Sink limitation to growth and survival is often inferred if a given stress does not cause non-structural carbohydrate (NSC) concentrations or levels to decline along with growth. However, trees may actively maintain or increase NSC levels under moderate carbon stress, making the pattern of reduced growth and increased NSCs compatible with carbon limitation. To test this possibility, we used full and half defoliation to impose severe and moderate carbon limitation on 2-year-old *Quercus velutina* Lam. saplings grown in a common garden. Saplings were harvested at either 3 weeks or 4 months after treatments were applied, representing short- and longer-term effects on woody growth and NSC levels. Both defoliation treatments maintained a lower total leaf area than controls throughout the experiment with no evidence of photosynthetic up-regulation, and resulted in a similar total biomass reduction. While fully defoliated saplings had lower starch levels than controls in the short term, half defoliated saplings maintained control starch levels in both the short and longer term. In the longer term, fully defoliated saplings had the greatest starch concentration increment, allowing them to recover to near-control starch levels. Furthermore, between the two harvest dates, fully and half defoliated saplings allocated a greater proportion of new biomass to starch than did controls. The maintenance of control starch levels in half defoliated saplings indicates that these trees actively store a substantial amount of carbon before growth is carbon saturated. In addition, the allocation shift favouring storage in defoliated saplings is consistent with the hypothesis that, as an adaptation to increasing carbon stress, trees can prioritize carbon reserve formation at the expense of growth. Our results suggest that as carbon limitation increases, reduced growth is not necessarily accompanied by a decline in NSC concentrations. Therefore, a lack of NSC decline may not be evidence that reduced tree growth under cold or water stress is caused by sink limitation.

Keywords: carbon reserves, non-structural carbon, tree growth.

Introduction

Our poor understanding of what limits tree growth and survival hinders our ability to predict how forests will respond to several global change factors, including increases in CO_2, temperature, insects and pathogens. We often divide mechanisms limiting growth or survival into two categories: carbon and sink limitation. This dichotomy, while perhaps overly simplistic, is very relevant to predicting how tree growth and/or mortality will respond to these future changes. Carbon limitation occurs when growth is limited by the availability of carbon. In contrast, sink limitation occurs when growth is limited by a tree's ability to use the available carbon for growth due to such factors as insufficient nutrient supply or direct environmental constraints on the rate of biosynthesis (e.g., temperature). An increase in carbon availability should only increase tree growth if growth is carbon limited, not sink limited.

Non-structural carbohydrate (NSC) concentrations (i.e., levels) are often used to infer whether growth is carbon or sink limited. Consistently high NSC concentrations or reduced growth coupled with increased or static NSC concentrations in response to stress are used as indicators that growth and survival are sink limited (e.g., Hoch et al. 2003, Körner 2003, Palacio et al. 2008, Sala and Hoch 2009). Such conclusions have led to a decade of arguments and supposed evidence against carbon limitation. In particular, many experiments and studies examining the effects of water stress have concluded that growth is directly sink limited because NSC levels do not decline (Würth et al. 2005, Sala and Hoch 2009, Sanz-Pérez et al. 2009, Galvez et al. 2011, Piper 2011).

However, recent debate has arisen over whether NSC levels can be interpreted as evidence of sink limitation or not (McDowell and Sevanto 2010, Ryan 2011, Sala et al. 2012, Wiley and Helliker 2012). Specifically, it is not known whether increased NSCs and decreased growth are caused by the passive build-up of carbon that cannot be used because growth is directly limited by other factors (Körner 2003), or rather, represent an active stress response to avoid carbon starvation (Smith and Stitt 2007, McDowell and Sevanto 2010, McDowell 2011). In the latter case, NSC accumulation or maintenance may be an active process, occurring while growth is still carbon limited. Conditions such as drought, cold temperatures or reduced carbon uptake may all increase the chance of starvation, and a prioritization of storage over growth could enable survival over longer periods or under more severe stress.

Carbon storage dynamics, apart from their relevance to understanding how different stressors limit growth, are themselves of interest. Storage remains one of the most poorly understood and modelled carbon pools in tree growth models (Le Roux et al. 2001). Storage is also an important life history trait, often found to correlate with survival or recovery from disturbance (Webb 1981, Canham et al. 1999, Gleason and Ares 2004, Bréda et al. 2006, Myers and Kitajima 2007, Galiano et al. 2011). However, allocating carbon to reserves could reduce carbon available for immediate growth, defense or reproduction. For this reason, storage allocation is likely to be highly regulated and under strong selection. Trees' storage responses to disturbances are therefore important to understanding how individuals and whole forests cope with defoliation, drought and constantly changing environmental conditions.

When comparing growth under stressful and less stressful conditions, a sink limitation to growth is often inferred if NSC concentrations are not lower in the stressful condition. However, this inference assumes that NSCs are mainly stored passively (i.e., when growth is sink limited) and that trees will always maximize growth at the expense of maintaining carbon reserves. Non-structural carbohydrate levels and growth should both certainly decline under severe carbon limitation, as a plant must rely on stored carbon to maintain metabolism and defense for survival. But under moderate carbon limitation, if growth is more expendable than storage, storage allocation may increase relative to growth, allowing NSC levels to be maintained or even increase relative to the less stressful condition. To test this hypothesis, though, growth and storage must be monitored when carbon is limiting. This requirement can be complicated because many stressors, such as drought and cold temperature, may impose both carbon and direct physical constraints on growth, and it is unclear which is the immediate limitation.

One-time defoliation is a potential candidate to test whether NSC levels can be maintained under moderate carbon limitation. Defoliation limits carbon uptake by reducing the total leaf area, and when severe, it is known to decrease both growth and NSC levels (Ericsson et al. 1980, Vanderklein and Reich 1999, Li et al. 2002), which is expected under severe carbon limitation. In addition, the growth reduction observed following defoliation is correlated with the degree of canopy reduction (Ericsson et al. 1980, Salemaa and Jukola-Sulonen 1990, Reichenbacker et al. 1996), consistent with increasing carbon limitation with declining carbon uptake capacity. Following defoliation, the preferential allocation of resources away from below ground, as indicated by a decrease in the root to shoot ratio (Markkola et al. 2004, Snyder and Williams 2007, Eyles et al. 2009), suggests that trees are responding to a carbon, not a nutrient, limitation. Also, elevated CO_2 levels can increase the growth of defoliated trees (Kruger et al. 1998, Mattson et al. 2004, Handa et al. 2005, Huttunen et al. 2007, but also see Kruger et al. 1998, Volin et al. 2002, Handa et al. 2005), which should only be possible if growth is carbon limited.

We used single defoliation treatments to test the following hypothesis: under severe carbon-limiting stress, both growth and NSC levels will decline, but under moderate stress, carbon-limited growth is not accompanied by a decline in NSC levels. We compared growth and starch concentrations of *Quercus velutina* Lam. saplings either 3 weeks or 4 months following full, half or no defoliation (controls). The 3-week response of half defoliated saplings and fully defoliated saplings relative to controls was used to test for growth and storage response under moderate and severe carbon limitation, respectively. Similar comparisons at 4 months allowed us to determine whether the differences observed were maintained over the longer term. We then compared the change in total biomass and starch biomass from 3 weeks to 4 months among treatments to determine whether there was evidence for an increase in storage allocation relative to growth under carbon stress. We discuss the results as well as whether our defoliation treatments actually induced a carbon limitation.

Materials and methods

Plant material and experimental design

Two-year-old *Q. velutina* saplings were purchased from New Jersey State Forest Nursery and kept dormant at 4 °C until

planting. In May 2010, saplings were planted 1 m apart in three plots on the campus of the University of Pennsylvania (Philadelphia, PA, USA; 39° 57′ N, 75° 11′ W). At planting, saplings were fertilized with a controlled release fertilizer (Osmocote® Classic 14-14-14; Scots Company LLC, Marysville, OH, USA) and mulched. All plots were weeded and well watered by either automatic sprinklers or a drip-irrigation system. Defoliation treatments were not applied until 2011 to allow a whole growing season for recovery from transplant shock.

Defoliation treatments and harvest

On 16–17 June 2011, 38 saplings (26 in plot 1, six each in plots 2 and 3) received one of the three treatments: full (12 individuals), half (14) or no defoliation (12). For full defoliation, all leaves were removed by clipping at the top of the petiole. In half defoliation, every leaf was cut in half along the mid-vein while leaving the mid-vein intact. For controls, no leaves were removed.

We took into account the substantial initial size variation when we assigned individuals to defoliation treatments. In June, we measured the initial stem diameter with a digital calliper and leaf area with a laser area meter (CI-203, CID Bio-Science, Inc, Camas, WA, USA). As ground-level diameter and total leaf area were correlated ($R^2 = 0.52$, $P < 0.01$), we took the leaf area into account when assigning individuals to treatment. The smallest sapling and largest sapling were assigned to the half defoliation treatment; the rest were divided into consecutive groups of three (first three smallest, next three smallest etc). Within each group, we randomly assigned one sapling to each of the three treatments, maintaining an even distribution of treatments among the three garden plots. After treatments were applied, we measured the total area of the leaves removed from each individual, which correlated well with our initial non-destructive estimates of the total leaf area ($R^2 = 0.74$, $P < 0.01$).

We destructively harvested six saplings in each treatment at mid-summer (9–11 July) and the remainder (eight half defoliated, six fully defoliated, six controls) at the end of the growing season (17–24 October), allowing us to examine both short and longer-term responses. By ensuring an even initial size distribution between harvest dates, harvests from plot 3 only included two treatments at each date; however, we found no evidence that this drove or obscured any results (see Supplementary Data at *Tree Physiology* Online). We dug up all saplings by hand, harvesting as much of the root as possible. Because the soil had high clay content, a portion of the root system, especially the fine root fraction, was not recovered. These fine roots would not have contributed greatly, however, to the total root mass.

Growth measurements

The defoliation treatments were expected to reduce root and stem growth, which we measured in several ways. For differences in both short- and longer-term growth, we used total biomass excluding leaves at harvest as a proxy for growth (referred hereafter as woody biomass). Because treatment groups were not different in size when treatments were applied (see below), any differences in size at the time of harvest were due to differential growth after treatment. At each harvest, above-ground woody tissue and roots were weighed after being washed, microwaved at 1000 W for 30 s to denature enzymes and oven-dried at 70 °C for 1 week. The effect of defoliation on the total woody biomass was then analysed separately for each harvest date using an ANCOVA after testing for the homogeneity of slopes. The covariate, a measure of initial size, was derived from the first principal component (explaining 83% of variation) of a principal component analysis (PCA) using the initial leaf area and initial diameters at 0 and 12 cm in height and did not differ among treatments ($P = 0.79$ and 0.92 for July and October harvests, respectively).

We also used the relative basal area increment (BAI) as a second measure of longer-term growth. The stem was marked with a permanent marker at the base of the stem (0 cm) and at 12 cm above the ground (12 cm). At each mark, the diameter was measured in four directions, 90° apart, before treatments were applied and again at the October harvest. For both 0 and 12 cm, we used the average diameter to calculate the relative BAI in the following way:

$$\frac{\text{Diameter}^2_{\text{harvest}} - \text{Diameter}^2_{\text{initial}}}{\text{Diameter}^2_{\text{initial}}}$$

Finally, because October biomass and relative BAI include growth between treatment application and the first harvest, we wanted to ensure that any significant differences seen in the longer term were not only the result of shorter-term growth differences. Therefore, we calculated the biomass increment ($\text{Biomass}_{\text{incr}}$) between July and October for each treatment as the difference in the average biomass between July and October.

Carbon storage measurements

After weighing, we sampled roots and stems—including bark, phloem and wood—for starch content at each harvest date. Roots were sampled by pooling three 1-cm-long sections, one each from the top, middle and bottom thirds of the main root axis. Stems were sampled by pooling three 2-cm-long sections, one from the base, one from the base of a branch and one from the tip of the same branch.

Starch content was then determined following the method of Baud et al. (2002). Each sample was ground to powder in a ball mill, extracted twice in 80% ethanol at 80 °C for 1 h to remove soluble sugars, vacuum dried and then stored at −20 °C. Starch content was measured via enzymatic digestion to glucose using α-amylase and amyloglucosidase (A7720,

A1602, Sigma-Aldrich, St. Louis, MO, USA). An aliquot was removed, and its glucose content determined by digesting glucose with glucose-6-phosphate dehydrogenase and hexokinase (G8529, H4502, Sigma-Aldrich) and measuring the consequent change in absorbance at 340 nm. Absorbance values were converted to moles of glucose by comparison to a standard curve derived from pure starch (S5127, Sigma-Aldrich). From these values, we calculated the starch content as total sample glucose equivalents, and starch content is reported as both total mass (g) and as starch level or concentration (i.e., percent of sample dry weight).

We explored the effects of defoliation on starch stores in several ways. First, we used a two-way ANOVA (main effects: treatment and harvest date) to test for differences in starch levels at each harvest date. Secondly, we tested for differences in the starch concentration increment and the difference in average per cent starch content between October and July, as indicated by a significant interaction term between harvest date and treatment within the same ANOVA. A significant interaction between harvest date and treatment would indicate that starch levels increased at different rates in the different treatments. Third, we compared the total starch mass among treatments in July and October. The total starch mass is the sum of above- and below-ground starch mass, each calculated as the starch concentration multiplied by the organ dry weight. The effect of defoliation on total starch mass was analysed separately for each harvest date using an ANCOVA after testing for homogeneity of slopes. The covariate was the same as described above for biomass: the first principal component of a PCA using the initial leaf area and initial diameters at 0 and 12 cm in height.

Assessment of carbon uptake

Because we intended defoliation treatments to reduce carbon uptake throughout the experiment, and to assess whether potential carbon uptake differed between fully defoliated and half defoliated saplings following canopy refoliation, we measured the total leaf area at both harvest dates. Leaf area measurements are reported as a percentage of the initial leaf area, and the effects of defoliation and harvest date were examined using a two-way ANOVA.

We measured leaf-level photosynthesis to see whether effects of reduced canopy size on whole-plant carbon gain were negated by photosynthetic up-regulation. Leaf-level light-saturated photosynthetic rates (A_{sat}) were taken periodically from the time of treatment through September using LI-COR 6400 with the 6400-02B LED light source (LI-COR Biosciences, Lincoln, NE, USA). On each date, A_{sat} was measured between 8:00 and 13:00 h on one fully expanded leaf of two to six saplings spread among treatments, using a light level of 1200 μmol and CO_2 level of 400 μmol. Measurements were taken for 3 min every 10 s after photosynthesis had reached a plateau or after 20 min if no plateau was reached; an average A_{sat} was calculated for each measured sapling on a given day. Vapour pressure deficit (VPD) varied strongly among sampling dates and times, and visual inspection of the data showed a strong reduction of A_{sat} at VPD values >1.75. Therefore, measurements taken at VPD > 1.75 were dropped from the analysis. Data were then divided into two groups based on the date of measurement: measurements of control and half defoliated saplings taken before fully defoliated saplings completed leaf expansion (18 June–5 July) and measurements of all treatments after expansion (8 July–19 September). Then, within each of these two time periods, treatment effects were analysed using either (i) the average A_{sat} for saplings that had only one measurement during that time period or (ii) the maximum average A_{sat} for saplings that had more than one measurement during that time period.

Finally, we measured leaf starch content as another indicator of carbon availability, as the leaf starch content is the balance between carbon supply and carbon demand by the leaf and the rest of the plant. Leaf starch content was measured at three times throughout the experiment: at sunrise (AM) and sunset (PM) on 29 June and 13/14 September and at sunrise (AM) on 9 July. At each collection, a single circular 1.27-cm diameter disk was punched from two leaves per individual and stored at −80 °C until processed. Samples were ground with liquid nitrogen in 2-ml centrifuge tubes, extracted in ethanol as described above, and stored at −20 °C. Starch content was then measured as described above and reported as grams of glucose equivalents per unit leaf area. Treatment effects were analysed with separate ANOVAs for each collection date because dates differed both in the time of day when samples were collected and in treatments included (e.g., June collection did not include fully defoliated saplings, as they did not have leaves).

Leaf traits and measurements

To explore whether defoliation treatments might have imposed a sink limitation to growth, we measured several different leaf traits. We first measured leaf nitrogen content to rule out a sink limitation due to reduced nitrogen. We measured leaf $\delta^{15}N$ as it could indicate treatment differences in nitrogen dynamics, such as nitrogen uptake or nitrogen demand, caused by the loss of nitrogen and/or need to rebuild a new canopy. We also measured leaf water potential to rule out the possibility that our method of half defoliation negatively affected water balance.

Nitrogen concentration and its isotopic signature were determined for leaves collected at each harvest. Leaves removed at the time of treatment application were used to determine initial values for half and fully defoliated saplings. For most controls, a single leaf was removed at the time of treatment to determine initial values. However, for the smallest control saplings, only

half a leaf was removed to minimize leaf area reduction. Measurements were made with an elemental analyser (Costech Analytical Technologies, Valencia, CA, USA) coupled to an isotope ratio mass spectrometer (Thermo-Finnigan Delta Plus, Bremen, Germany) at the University of Pennsylvania. For each sapling, the change in per cent nitrogen content and the change in $\delta^{15}N$ ($\Delta\delta^{15}N$) were calculated as the difference between values at harvest time and initial values. The effects of defoliation and harvest date were determined for both variables using a two-way ANOVA.

Water potential was measured at pre-dawn and midday on 14 September 2011 with a pressure chamber (PMS Instruments Co., Albany, OR, USA). At both times, one leaf per sapling was removed (four saplings/treatment for pre-dawn and two or three saplings/treatment for midday), immediately inserted into a plastic bag and placed in a dark container (not longer than 45 min) until measured. Differences among treatments were determined using a two-way ANOVA with the time of day and treatment as main effects.

Data analysis

The main effects of treatment and harvest date were determined as described above for each variable. (We assumed no effect of plot, as the majority of saplings were in plot 1.) When residuals were not normally distributed or the variance was correlated with the mean, data were ln-transformed (leaf area, biomass, starch mass, specific leaf area (SLA), relative BAI) or arcsine-transformed (starch concentration). To compare treatments within each analysis, we used either the Bonferroni correction or Tukey's honestly significant difference (HSD) test to correct for multiple comparisons within the same analysis. All analyses were performed using JMP 10.0 (SAS) and graphed with Sigma Plot 12.3 (Systat). Results were considered significant for $P \leq 0.05$ and marginally significant for $0.05 < P \leq 0.10$.

Results

Carbon uptake

Both defoliation treatments reduced canopy size in the short term (until the July harvest) and longer term (July to October harvest), but total leaf area differences between defoliation levels were restricted to the short term. All fully defoliated saplings produced a new canopy. New leaf growth began around 2 weeks following defoliation, and new leaves were not fully expanded until right before the July harvest. After refoliation, both defoliation treatments maintained lower leaf area, expressed as a per cent of initial leaf area, than controls, and there was no change between harvest dates (Table 1, Figure 1). In control saplings, canopy area increased by 20% during the experiment, while canopies were reduced to 66 and 54% of the initial area in half and fully defoliated trees, respectively. Therefore, for most of the short-term period, fully defoliated saplings had smaller canopies than half defoliated saplings, but between July and October, leaf area was not significantly different between these two treatments (Figure 1).

There was no evidence that photosynthetic up-regulation compensated for differences in canopy size. In the short term, in fact, half defoliation reduced A_{sat} from control levels ($F_{1,22} = 4.65$, $P = 0.04$; Table 2). The significantly lower leaf starch levels of half defoliated leaves in late June (across AM and PM: $F_{1,33} = 4.93$, $P = 0.03$; Figure 2) also suggest reduced carbon uptake. In the longer term, after leaf-out was completed, A_{sat} was lowest in fully defoliated saplings, but differences among treatments were not significant ($F_{2,19} = 1.01$,

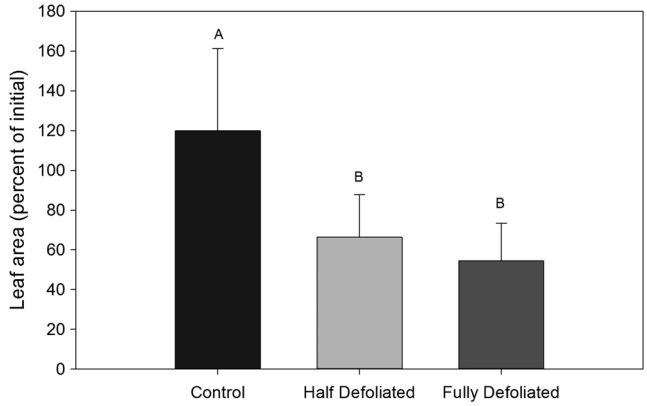

Figure 1. Leaf area at harvest as a per cent of initial leaf area. Values represent back-transformed treatment averages pooled across harvest dates (treatment × harvest date, not significant). Error bars represent 95% confidence limits, and different letters represent significantly different values according to Tukey's HSD.

Table 1. Two-way ANOVA results for analyses with harvest date and treatment as main effects.

Response variable	Defoliation			Harvest date			Defoliation × harvest date		
	F	df	*P*	*F*	df	*P*	*F*	df	*P*
Leaf area	7.96	2,32	**0.002**	0.001	1,32	0.975	0.49	2,32	0.615
Δ Leaf percent nitrogen	3.24	2,32	*0.052*	1.81	1,32	0.189	1.03	2,32	0.369
$\Delta\delta^{15}N$	4.53	2,32	0.212	0.59	1,32	0.447	6.43	2,32	**0.005**
Above-ground starch concentration	8.38	2,32	**0.001**	56.76	1,32	**<0.001**	4.48	2,32	**0.019**
Below-ground starch concentration	13.77	2,32	**<0.001**	31.46	1,32	**<0.001**	3.02	2,32	*0.063*

Significant results are bold; marginally significant results are italicized.

Table 2. Treatment averages for A_{sat}, change in leaf percent nitrogen, $\Delta\delta^{15}N$, leaf water potential and relative BAI

	Control	Half defoliated	Fully defoliated
A_{sat} (μmol CO_2 m^{-2} s^{-1})			
6/18–7/5	11.9 (0.9)[a]	9.4 (0.7)[b]	—
7/8–9/19	13.8 (1.4)[a]	14.4 (1.3)[a]	11.7 (1.4)[a]
Δ percent nitrogen			
July	0.10 (0.07)[a]	−0.36 (0.23)[a]	−0.05 (0.08)[a]
October	−0.37 (0.14)[a]	−0.55 (0.25)[a]	0.01 (0.13)[a]
$\Delta\delta^{15}N$			
July	0.18 (0.16)[a]	−0.43 (0.56)[a]	0.35 (0.47)[a]
October	−0.02 (0.30)[a*]	1.71 (0.59)[b*]	−0.70 (0.41)[a]
$\Psi_{predawn}$ (MPa)	−0.5 (0.1)[a]	−0.2 (0.1)[b]	−0.1 (0.1)[b]
Ψ_{midday} (MPa)	−2.1 (0.2)[a]	−1.9 (0.1)[a]	−1.6 (0.1)[a]
Rel. BAI_{0cm} (mm^2/mm^2)	1.07 (.61–1.67)[a*]	0.32 (0.06–0.64)[b]	0.43 (0.11–0.85)[b*]
Rel. BAI_{12cm} (mm^2/mm^2)	0.88 (0.20–2.21)[a*]	0.28 (−0.07–0.89)[ab]	0.17 (−0.16 to 0.82)[b*]

Average A_{sat} is given for two time periods: before and after new leaf expansion of fully defoliated saplings. Changes in leaf percent nitrogen and in leaf $\delta^{15}N$ are given for both harvest dates relative to pretreatment values. Leaf water potential (Ψ) measurements were taken at both pre-dawn and midday on 14 September. Relative BAI were measured at both 0 and 12 cm above the ground. Standard errors or 95% confidence intervals are shown in parentheses following averages. Different letters represent significant differences among treatments, and different letters each with asterisk represent marginally significant differences. Significant differences were determined by Tukey's HSD except for $\Delta\delta^{15}N$ and change in leaf percent nitrogen, which were determined using the Bonferroni correction (no. of comparisons = 6).

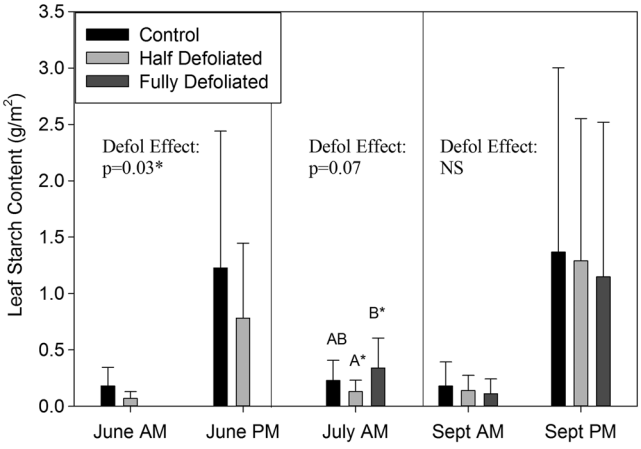

Figure 2. Leaf starch content at three separate dates. Error bars are 95% confidence intervals. Defoliation effects are from the separate ANOVAs performed for each date. Marginally significant differences according to Tukey's HSD are indicated by different letters with asterisks (only in July). In June, across AM/PM, half defoliated trees had significantly lower starch levels, while there were no differences in September.

$P = 0.38$). Similarly, early July leaf starch levels did not differ from controls for either defoliation treatment, although half defoliated saplings had marginally less leaf starch than fully defoliated saplings ($F_{2,15} = 3.20$, $P = 0.07$; Figure 2). In September, there were no differences in leaf starch among treatments ($F_{2,34} = 0.36$, $P = 0.70$; Figure 2).

Leaf traits and sink limitation

The trends in leaf nitrogen dynamics were not consistent with nitrogen limitation increasing with defoliation severity. Defoliation had an inconsistent but marginally significant effect on the change in leaf percent nitrogen when harvest dates were combined (Table 1), with half defoliated saplings having the greatest reduction and fully defoliated trees having the least. However, within each harvest date, none of the treatments differed significantly (Table 2). Across both harvest dates, defoliation treatment did not affect the $\Delta\delta^{15}N$ (Table 1), but differences were apparent in $\Delta\delta^{15}N$ in October when half defoliated saplings underwent an enrichment in ^{15}N that was significantly greater than fully defoliated saplings and marginally significantly greater than controls (Table 2).

Finally, there was no evidence that either defoliation treatment caused water stress that might have led to a sustained sink limitation. Saplings in both defoliation treatments had significantly higher water potential than controls across both predawn and midday measurements ($F_{2,14} = 10.48$, $P < 0.01$; time × treatment = not significant; Table 2).

Growth

Both defoliation treatments reduced woody growth in the short term ($F_{2,14} = 11.08$, $P < 0.01$; Figure 3). In July, total dry weight of controls was significantly greater than half and fully defoliated saplings, indicating that they had grown more since treatments were applied.

In the longer term (October), multiple growth measurements indicate that control saplings maintained higher growth rates than saplings in both defoliation treatments. First, controls still had significantly greater mass than fully defoliated saplings and marginally significantly greater than half defoliated saplings ($F_{2,16} = 4.73$, $P = 0.02$; Figure 3). Secondly, relative BAI at both 0 and 12 cm height was also greater for control saplings than

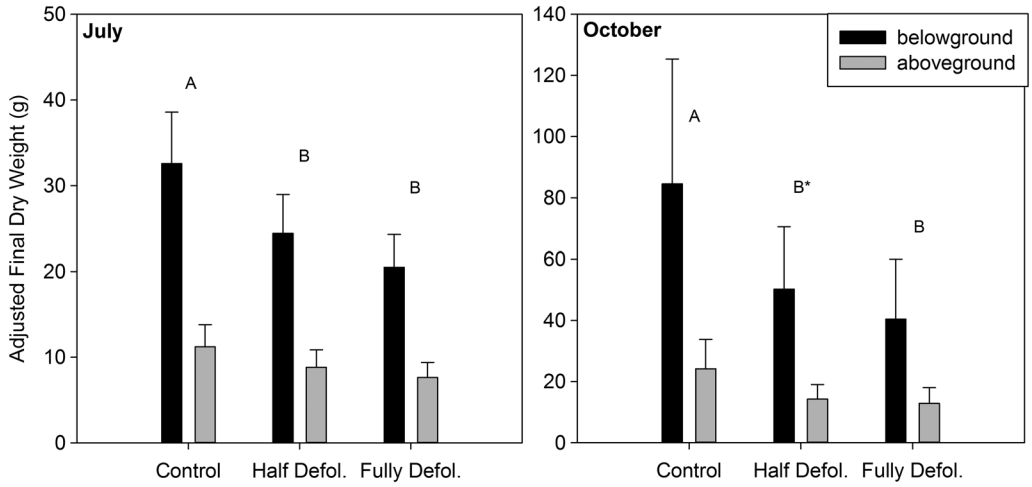

Figure 3. Adjusted averages for final dry weight of above- and below-ground woody tissue. Values are adjusted averages at harvest from the ANCOVA (principal component representing initial size used as covariate). Differences among treatments reflect differential growth following defoliation, as sapling size did not differ before treatment application. Error bars represent 95% confidence intervals. Different letters represent Tukey's HSD between treatments for total (above + below-ground) dry weight; different letters each with asterisk represent marginally significant differences.

for defoliated saplings ($F_{2,17} = 4.60$, $P = 0.03$ and $F_{2,17} = 3.03$, $P = 0.07$, respectively; Table 2). Relative BAI_{0cm} was greatest in controls, which differed significantly from that of half defoliated saplings and marginally from that of fully defoliated saplings. Relative BAI_{12cm} was also highest for controls, but this difference was only marginally significant relative to fully defoliated saplings. Thirdly, the biomass increment ($Biomass_{incr}$) between harvest dates for controls was approximately twice that of either defoliation treatment (Figure 5), indicating that defoliation had in fact reduced growth in the longer term, as well as in the short term. Growth did not significantly differ between half and fully defoliated saplings by any of these measures, although biomass and biomass increment tended to be greater in half defoliated saplings.

Storage

We predicted that only severe carbon limitation would reduce starch levels. The short-term response of storage levels was consistent with this prediction, but the response was slightly altered in the longer term. In July, while fully defoliated saplings had significantly lower above- and below-ground starch levels than both control and half defoliated saplings, half defoliated saplings did not differ significantly from controls (Table 1, Figure 4). In October, there were still no significant differences in above- or below-ground starch levels between control and half defoliation treatments (Table 1, Figure 4). Unexpectedly, while fully defoliated saplings tended to have lower below-ground starch levels than the other treatments, differences among treatments were not significant. In fact, fully defoliated saplings had above-ground starch levels nearly identical to controls (Figure 4).

Total (above- and below-ground) starch mass, adjusted for initial sapling size, declined with increasing defoliation severity at both harvest dates (July: $F_{2,14} = 11.99$, $P < 0.01$; October: $F_{2,16} = 4.72$, $P = 0.03$; see Figure S1 available as Supplementary Data at *Tree Physiology* Online). In July, fully defoliated saplings had significantly lower total starch mass than saplings of the other two treatments. In October, fully defoliated saplings still had significantly lower starch mass than controls. Half defoliated saplings had starch mass intermediate between fully defoliated and control saplings, but they did not differ significantly from either group.

As an estimate of storage allocation over the longer term, starch concentration increment (change in percent starch between harvests) increased significantly with defoliation severity (Figure 4), suggestive of greater starch allocation. This is indicated by the significant and marginally significant interactions between treatment and harvest date for above- and below-ground, respectively (Table 1). Linear contrasts revealed that the interaction was primarily due to the greater increase in starch levels by fully defoliated trees. Fully defoliated trees had a significantly greater increase in starch content relative to controls above-ground (linear contrast sum of squares (SS) = 98% of interaction SS; $P < 0.01$) and trended in the same way below-ground (SS = 91% of interaction SS; $P = 0.03$), although not significantly after Bonferroni correction. Fully defoliated saplings had a greater, but not significant, increase in starch content than half defoliated trees ($P = 0.07$ and 0.08 for above- and below-ground, respectively). Half defoliated saplings and controls did not differ significantly in starch concentration increment ($P > 0.10$).

Discussion

Defoliation likely caused a carbon limitation to growth

We used defoliation to determine how NSC levels and storage allocation change as growth becomes increasingly carbon

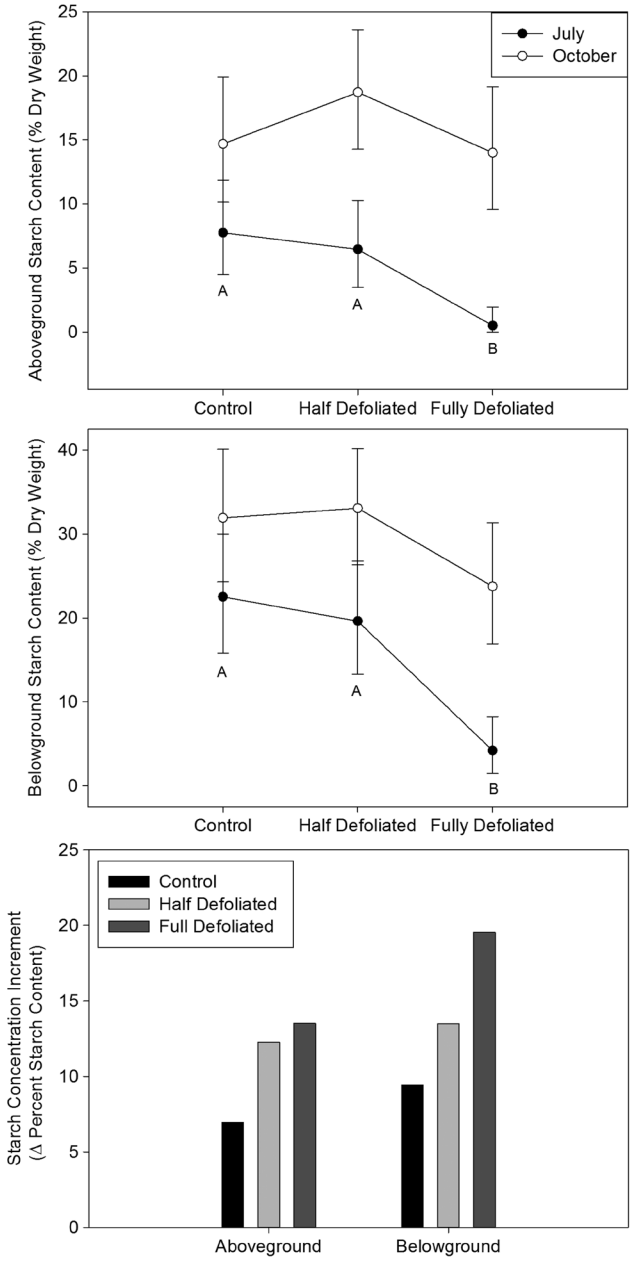

Figure 4. Starch content of above- and below-ground woody tissue at July and October harvests. Error bars represent 95% confidence intervals. Different letters represent significant differences using Bonferroni correction (no. of comparisons = 9) for July averages; there were no differences among treatments for October. Starch concentration increment, a measure of allocation to storage, is the difference between back-transformed October and July average starch levels. The effect of defoliation on starch increment was determined by the interaction of treatment and harvest date. The significant (above-ground) and marginally significant (below-ground) interaction between treatment and harvest date is due primarily to the difference between controls and fully defoliated saplings. Fully defoliated saplings had the largest starch increments, increasing from significantly lower starch levels in July to statistically indistinguishable levels in October.

limited. As expected, defoliation reduced woody growth by several measures in both the short and longer term. To demonstrate that this growth reduction was due to carbon limitation,

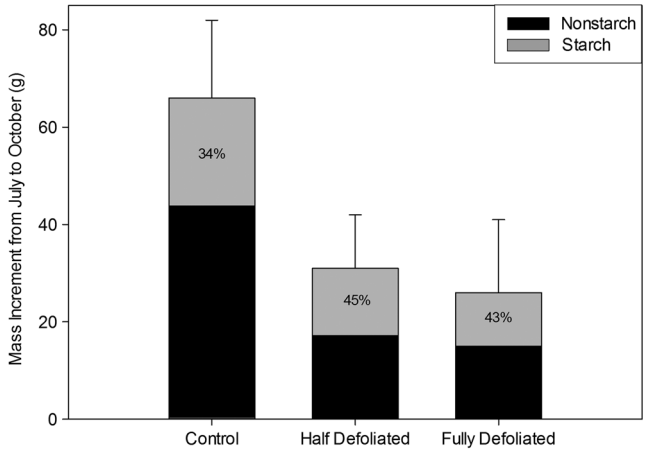

Figure 5. Total woody mass increment ($Biomass_{incr}$) between July and October harvests, divided into starch and non-starch components. The average total mass and starch mass increments were calculated as the difference between the adjusted averages from July and October (adjusted by covariate of size as described in Materials and methods). Error bars for $Biomass_{incr}$ are jack-knife estimates of standard error (Ephron 1981). Non-starch mass increment is the difference between total and starch mass increment. Numbers represent the proportion of total mass increment due to starch, or $Conc_{incr}$. While defoliation tends to decrease $Biomass_{incr}$, it tends to increase the proportion of increment due to starch.

the following must be true: (i) carbon uptake was reduced; and (ii) growth reduction was due to reduced carbon availability, not enhanced sink limitation. Defoliation reduced whole-plant carbon uptake over both the short and longer term. In the short term, carbon uptake declined with increasing defoliation severity, as fully defoliated trees had no leaves for at least 2 weeks of this period. In the longer term, however, both defoliation treatments had similar total leaf area. Leaf starch and A_{sat} measurements throughout the experiment indicate that there was no leaf-level photosynthetic up-regulation, as is often observed (Hoogesteger and Karlsson 1992, Vanderklein and Reich 1999, Pinkard et al. 2007, Eyles et al. 2011), to cancel out the effects of reduced canopy area. On the contrary, A_{sat} and leaf starch in half defoliated saplings were initially even lower than in controls, which suggests an even greater reduction in total carbon uptake. This reduction may be an effect of removing a portion of leaves instead of whole leaves, which we did to mimic herbivory more realistically. Effects of herbivory and/or mechanical damage on photosynthesis may expand, often transitively, beyond the area immediately affected (Zangerl et al. 2002, Delaney et al. 2008).

We found no evidence that defoliation reduced woody growth due to nitrogen loss or water stress. While repeated defoliation has been argued to impose nitrogen limitation (Tuomi et al. 1990), we do not think that this is likely in our experiment. First, saplings were well fertilized and only experienced a single defoliation. Secondly, at each harvest date, the change in leaf nitrogen did not differ significantly

among treatments. Furthermore, the change in nitrogen content with increasing defoliation severity was not consistent with increasing nitrogen limitation, as fully defoliated trees had the smallest reduction in leaf nitrogen overall. A difference in leaf age was apparently not obscuring a nitrogen reduction in fully defoliated saplings as fully defoliated leaves in October (~3.5 months old) did not have significantly lower leaf nitrogen than control leaves in July (~2.5–3 months old). In addition, complete defoliation increased SLA (data not shown), which is usually observed to increase with fertilization and decrease with nutrient stress (VanArendonk et al. 1997, Knops and Reinhart 2000, Calfapietra et al. 2005). Defoliated plants likewise did not suffer greater water stress, as leaf water potential was actually higher in defoliated saplings than in controls.

Is defoliation a sink limitation?

We did not find evidence that defoliation imposed a direct sink limitation on growth by nitrogen limitation or water stress (due to tissue damage), but sink limitation could also be imposed internally (e.g., plant architecture or phenology). Architectural constraints such as determinate growth patterns or the number of preformed buds may constrain leaf or stem growth following browsing or defoliation (Millard et al. 2001). Such architectural constraints may have limited the final canopy size of fully defoliated saplings by limiting the number of refoliated leaves. However, leaf mass was not considered in our growth measurements, and any constraint on canopy regrowth would further enhance the carbon stress of defoliation, likely exacerbating carbon limitation to non-leaf growth.

Because defoliation can delay processes such as flowering time (Freeman et al. 2003), it may also delay certain aspects of plant growth, causing a phenological sink limitation. In oaks, root growth often declines or halts during canopy expansion (Reich et al. 1980, Willaume and Pagès 2006), and a delay in growth due to canopy refoliation may shorten the total time available for root or radial growth. There are several reasons why this is not likely to explain our results. First, while fully defoliated saplings grew more new leaf area than controls, half defoliated saplings did not. Therefore, any delay in woody growth due to leaf expansion that occurred in half defoliated saplings should also have occurred in controls. Secondly, if defoliation caused a sink limitation to growth by limiting the time for this process, it is unclear why such a delay would not also apply to storage and reduce NSC levels. Finally, it is not clear that a phenological growth limitation must be a sink limitation. The arrest of root growth during leaf expansion is believed to be caused by competition for carbohydrates (Willaume and Pagès 2006, 2011), and is therefore a carbon limitation. Similarly, in *Arabidopsis*, defoliation delays the phase change from juvenile to adult leaf production (Yang et al. 2011). However, the delay is diminished when plants are treated with exogenous glucose, indicating that the plant's carbon status controls the phase shift as well as the delay (Yang et al. 2013). Finally, defoliation may alter hormonal levels or other growth regulators that could potentially impose sink limitations on growth by truncating the period of growth or by simply reducing maximum potential growth rates. However, hormonally induced growth reductions are not necessarily sink limitations either. For example, auxin levels and translocation from leaves affect root growth (Reed et al. 1998, Fu and Harberd 2003) and are likely reduced under defoliation (Willaume and Pagès 2006), but they are in turn affected by a plant's carbon status (Lilley et al. 2012). Growth regulator levels may ultimately determine how much a plant grows, but if these levels are dependent on carbon availability, then growth is still carbon limited.

Storage levels following defoliation

Under moderate carbon limitation, we predicted that NSC levels would not decrease as growth declined, but as limitation became severe, NSC levels would also decline. The latter is expected when trees must rely on stored carbon for maintenance, canopy regrowth and other processes necessary for survival. In the short term, our results were consistent with this hypothesis. In the longer term, both defoliation treatments matched our predictions for moderate carbon limitation. Control starch levels were still maintained in half defoliated trees, while growth continued to be reduced. Fully defoliated saplings, whose severe carbon stress was partially relieved by canopy regrowth, also maintained lower growth rates but recovered to near control starch levels by the second harvest. Other species have also displayed a recovery of NSC levels before growth rate following defoliation or browsing (Reichenbacker et al. 1996, Palacio et al. 2008, 2012, Susiluoto et al. 2010).

The maintenance of and recovery to control starch levels under carbon-limiting conditions is not consistent with passive storage that accumulates only when growth is carbon saturated. Rather, our findings suggest that storage can be an active sink competing with growth for carbon (Chapin et al. 1990, Lacointe et al. 2004, Silpi et al. 2007). Assuming there was no sink limitation, defoliated saplings maintained control storage levels at the expense of growing larger. Such a pattern of reduced growth while maintaining similar NSC levels has been observed under other forms of reduced carbon availability including reductions in light levels (Canham et al. 1999), shading in tree branches (Lacointe et al. 2004) and shortened day length in *Arabidopsis* (Gibon et al. 2009).

The maintenance of similar starch levels across treatments suggests that a given NSC concentration may be a general requirement before growth can proceed, leading to simultaneous and proportional allocation to both storage and growth as seen in seedlings (Imaji and Seiwa 2010) and other plants (e.g., sugar beet, Watson et al. 1972). While we should always be careful when extrapolating from saplings to adults, it seems

logical that mature trees of at least some species may have a similar allocation strategy. As these trees acquire more carbon, they would only grow as much as would allow them to maintain some baseline level of provisioning, which could vary seasonally or with environmental conditions. Therefore, trees that grow more must also increase total storage more; otherwise storage compound concentrations would decline. Such an allocation scheme seems reasonable as a bigger plant would likely require more total stored carbon during periods when photosynthesis cannot meet the immediate carbon demands for survival. By ensuring that new growth does not dilute storage levels, increasing size would not necessarily increase the risk of starvation, should some disturbance occur. Of course, this storage requirement would be revoked under extreme cases (e.g., severe defoliation) when growth may increase in priority above storage because it becomes necessary for survival.

Most of our results are consistent with previous defoliation and browsing experiments, though our interpretations differ. Heavy defoliation or browsing often leads to a reduction in both growth and NSC levels (Ericsson et al. 1980, Langstrom et al. 1990, Vanderklein and Reich 1999, Li et al. 2002), but NSC levels recover before growth (Reichenbacker et al. 1996, Palacio et al. 2008, Susiluoto et al. 2010). Repeated defoliation and browsing can also cause reduced growth and similar or even increased NSC levels (Van der Heyden and Stock 1995, Palacio et al. 2008, 2012). In many of these cases, the initial NSC decline is seen as evidence of carbon limitation, but the recovery of NSC levels is often interpreted as evidence of sink limitation to growth, with potential causes ranging from nutrient limitation to removal of apical meristems. However, all of these cases are also consistent with carbon-limited growth as the result of reduced sink priority relative to storage.

Storage allocation following defoliation

Our findings are consistent with the idea that storage can be prioritized over growth, especially under carbon-limiting conditions. In the longer term, defoliated saplings maintained control starch levels, but this does not necessarily imply that they allocated relatively more incoming carbon to storage than control saplings. Even the greater starch concentration increment exhibited by fully defoliated saplings may not be due to differential allocation. To determine whether defoliated saplings did allocate more carbon to storage than growth between harvest dates, we considered how final starch concentration is related to initial starch concentrations and to additional biomass allocation. October and July starch concentrations can be related in the following way:

$$\mathrm{Conc_{Oct}} = \mathrm{Conc_{July}} \times \left(\frac{\mathrm{Biomass_{July}}}{\mathrm{Biomass_{Oct}}}\right) + \mathrm{Conc_{incr}} \times \left(\frac{\mathrm{Biomass_{incr}}}{\mathrm{Biomass_{Oct}}}\right)$$

where $\mathrm{biomass_{Oct}} = \mathrm{biomass_{July}} + \mathrm{biomass_{incr}}$.

In this formulation, October starch concentration ($\mathrm{Conc_{Oct}}$) is the weighted average of the starch concentration in July ($\mathrm{Conc_{July}}$) plus the starch concentration of the added biomass, or biomass increment, between July and October ($\mathrm{Conc_{incr}}$). Each concentration is weighted by the relative contribution of total July or total increment biomass to the October total biomass ($\mathrm{Biomass_{July}}/\mathrm{Biomass_{Oct}}$ and $\mathrm{Biomass_{incr}}/\mathrm{Biomass_{Oct}}$). $\mathrm{Biomass_{incr}}$ and $\mathrm{Conc_{incr}}$ represent what is added and, therefore, reflect the net allocation of carbon to storage versus structural growth pools. Compared with controls, if fully defoliated saplings went from significantly lower to statistically indistinguishable starch levels (i.e., $\mathrm{Conc_{Oct}}$ remained the same while $\mathrm{Conc_{July}}$ declined), then fully defoliated saplings must have either (i) allocated proportionally more to storage than controls (higher $\mathrm{Conc_{incr}}$), indicating a shift in allocation between growth and storage, or (ii) have added relatively more new biomass than controls with the same starch concentration (greater $\mathrm{Biomass_{incr}}/\mathrm{Biomass_{Oct}}$ and conversely lower $\mathrm{Biomass_{July}}/\mathrm{Biomass_{Oct}}$), representing a difference in the relative growth rate only.

Our calculations support scenario (i) because defoliation increased the starch proportion of total biomass increment from July to October (higher $\mathrm{Conc_{incr}}$; Figure 5). Since $\mathrm{Biomass_{incr}}/\mathrm{Biomass_{Oct}}$ was actually lower for defoliated trees (see Figure S2 available as Supplementary Data at *Tree Physiology* Online), scenario (ii) is unlikely. Even though control saplings accumulated more total starch mass between harvest dates, starch concentration increment increased more for fully defoliated saplings because the *relative* amount of biomass allocated to storage versus growth was greater in this treatment.

If the increased storage allocation is active, it may be a response to defoliation-induced carbon limitation or carbon limitation in general. Following defoliation, the chance of starvation may be increased by reduced carbon uptake, depleted carbon stores and/or increased risk of further defoliation (if defoliation is indicative of high defoliator densities or increased likelihood of damage). It has been argued that plants should store more when the risk of carbon starvation increases, and this may only be possible by foregoing potential growth (Chapin et al. 1990, Gibon et al. 2009, Wiley and Helliker 2012). Storage—particularly below-ground—may always be a high-priority carbon sink for species like black oak that can resprout from the root collar. Maintaining high NSC concentrations at the expense of growth may ensure that these trees can survive by resprouting or refoliating after drought, fire or gypsy moth outbreaks, even if above-ground mortality is unrelated to carbon status.

Half versus full defoliation

By the October harvest, half defoliated saplings had not grown or stored significantly more than fully defoliated saplings, which was expected if full defoliation imposed a greater carbon

limitation. The reason we did not detect a difference could be due to (i) small sample sizes or (ii) no actual difference in either growth or storage. If there was no difference in growth or October starch levels, then fully defoliated saplings—which started with significantly lower starch levels in July—must have decreased carbon allocation to some other pool (i.e., root exudates or respiration) and/or increased carbon gain relative to half defoliated saplings.

Fully defoliated saplings may have had an opportunity to increase carbon gain relative to half defoliated saplings right before the October harvest, due to differences in the timing of canopy senescence. While we did not measure leaf senescence, we did observe some at the time of the October harvest, particularly in half defoliated saplings. Differences in canopy senescence between half and fully defoliated saplings are supported by the October leaf $\delta^{15}N$ data. ^{15}N enrichment has been observed in senescing leaves (Nasholm 1994, Keskitalo et al. 2005), and half defoliated leaves underwent a significantly greater increase in $\delta^{15}N$ than fully defoliated leaves in October. Delayed senescence may be due to the younger age of refoliated leaves (Palacio et al. 2013). However, we found that the change in $\delta^{15}N$ between treatment application and the October harvest ($\Delta\delta^{15}N$) correlated significantly with October starch concentration (weighted average of above + below-ground) when all treatments were combined ($R^2 = 0.27$; $P = 0.02$). One explanation for this correlation is that the timing of senescence might be modified by current carbon reserve status, allowing poorly provisioned trees (e.g., fully defoliated) to slightly delay senescence to make up for lost carbon.

Limitations of our approach

We used defoliation to impose a carbon limitation on woody growth. However, the effects of defoliation may be very complex, and we cannot rule out the possibility that growth was sink limited. Though we have argued against a nitrogen limitation to growth, leaf nitrogen levels may not always be a reliable indicator of limitation. Nitrogen and carbon limitations—both source limitations—may often be difficult to distinguish in practice and perhaps in theory as well. The acquisition and allocation of carbon and nitrogen within the plant are closely linked, and shortages of carbon will affect a tree's ability to acquire nitrogen and vice versa. But if growth was strongly limited by nitrogen, it is surprising that saplings did not exude more of the stored carbon below-ground to facilitate nutrient acquisition, unless starch was still actively stored.

Sink limitation could also be plant mediated, with defoliation initiating a hormonal response that down-regulates growth to a degree that is completely independent of carbon availability. Such a growth reduction is an active response, though, and the concurrent increase in relative carbon storage might still be regarded as the prioritization of storage over growth and part of a tree's recovery strategy.

Non-structural carbohydrate concentrations and growth limitation

We have argued that defoliation caused a carbon limitation to woody growth. If true, our results demonstrate that a lack of NSC decline is consistent with carbon limitation. Carbon limitation has been assumed to reduce NSC levels, due to the expectation that carbon-limited trees will either use already stored carbon for growth and/or will not shunt any incoming carbon to storage. However, for this to be true, storage must mostly be passive and always have a lower allocation priority than growth. Our results suggest that neither is the case under moderate carbon limitation imposed by defoliation. In fact, instead of allocating relatively *less* carbon to storage when carbon was limited, saplings actually allocated *more*. The mechanisms underlying the observed allocation shift are not known, but it has been suggested that low carbon availability per se may trigger enhanced expression of negative growth regulators, leaving more carbon available for storage (Smith and Stitt 2007, McDowell 2011). In any case, we should not assume that the absence of NSC concentration decline is indicative of carbon-saturated growth.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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