

Infestation and Hydraulic Consequences of Induced Carbon Starvation¹

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Drought impacts on forests, including widespread die-off, are likely to increase with future climate change, although the physiological responses of trees to lethal drought are poorly understood. In particular, in situ examinations of carbon starvation and its interactions with and effects on infestation and hydraulic vulnerability are largely lacking. In this study, we conducted a controlled, in situ, repeated defoliation experiment to induce carbon stress in isolated trembling aspen (*Populus tremuloides*) ramets. We monitored leaf morphology, leaves per branch, and multitissue carbohydrate concentrations during canopy defoliation. We examined the subsequent effects of defoliation and defoliation-induced carbon stress on vulnerability to insect/fungus infestation and hydraulic vulnerability the following year. Defoliated ramets flushed multiple canopies, which coincided with moderate drawdown of nonstructural carbohydrate reserves. Infestation frequency greatly increased and hydraulic conductivity decreased 1 year after defoliation. Despite incomplete carbohydrate drawdown from defoliation and relatively rapid carbohydrate recovery, suggesting considerable carbohydrate reserves in aspen, defoliation-induced carbon stress held significant consequences for vulnerability to mortality agents and hydraulic performance. Our results indicate that multiyear consequences of drought via feedbacks are likely important for understanding forests' responses to drought and climate change over the coming decades.

Climate change is predicted to have far-reaching effects on the world's forested ecosystems, which cover about 30% of the planet's land surface and store over 45% of Earth's terrestrial ecosystem carbon (Sabine et al., 2004; Bonan, 2008). Widespread forest mortality related to drought and temperature stress has already been documented on all six plant-covered continents (Allen et al., 2010). In the western United States, massive tree mortality events have been observed recently across both deciduous and conifer tree species. Several of these events have been linked to severe drought exacerbated by high temperatures, termed "climate change-type" droughts (Logan et al., 2003; Breshears et al., 2005; Allen et al., 2010). Forest mortality events can radically transform regional landscapes and affect ecosystem function, land-atmosphere interactions, carbon sequestration, and ecosystem services provided to humans (Dale et al., 2000; Fischlin et al., 2007; Kurz et al., 2008a, 2008b).

While severe droughts are projected to increase in frequency and intensity with climate change (Fischlin et al., 2007), especially in the southwestern United States (Seager et al., 2007), we currently lack understanding of how forests are likely to respond to drought, including pathways leading to mortality (McDowell et al., 2008; Allen et al., 2010). One prominent proposal is that some forest species may be vulnerable to "carbon starvation," where trees experience negative carbon balance during drought, potentially mediated by phloem impairment, eventually leading to tissue-level starvation and mortality (McDowell et al., 2008; Sala et al., 2010). Other physiological failure pathways have been proposed, including "hydraulic failure," where drought drives a tree's transpiration rate past a critical threshold whereby excessive xylem cavitation by air interrupts plant water transport from roots to leaves. Recent syntheses highlight that carbon-related and hydraulic-related mortality pathways are fundamentally interrelated in many ways, and their interconnections are largely unexplored (McDowell et al., 2011). For instance, limitation of carbohydrate mobilization, translocation, or transport could influence carbon starvation (Sala et al., 2010; McDowell, 2011), and declines in carbon availability could affect hydraulic capacity or defense against insects or pathogens (McDowell et al., 2011). Thus, understanding the role and consequences of carbon starvation could improve our understanding of forests' vulnerability to drought.

Tests of the roles of carbon starvation have been limited to date, especially in field environments

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(McDowell et al., 2008; Hartmann, 2011). A greenhouse experiment of mature pine (*Pinus edulis*) trees subjected to severe drought found temperature-driven elevated respiration levels and earlier mortality, potentially implicating carbon starvation, although carbohydrate reserves were not measured directly (Adams et al., 2009; Leuzinger et al., 2009; Sala, 2009). Drought experiments on trembling aspen (*Populus tremuloides*) seedlings created symptoms of carbon limitation but led to increases in root stored carbohydrate reserves (Galvez et al., 2011). In one case, low carbohydrate reserves were associated with mortality in a recent study on Scots pine (*Pinus sylvestris*; Galiano et al., 2011). Yet the potential for carbon starvation in mature trees and, perhaps more importantly, the consequences of carbon limitation on other elements of physiology as a response to drought remain largely unknown.

Most evidence for carbon limitation during drought has been circumstantial, which highlights the critical need for experimental manipulation of these key physiological processes (Niinemets, 2010; McDowell, 2011). Experimental defoliation is a well-developed technique to examine carbon limitations in field settings (Li et al., 2002; Körner, 2003; Snyder and Williams, 2003; Markkola et al., 2004; Handa et al., 2005; Landhausser and Lieffers, 2012) and trade-offs in allocation to growth versus defense (Jones et al., 2004; Osier and Lindroth, 2004; Donaldson et al., 2006). We performed a repeated defoliation experiment of mature trembling aspen ramets with the goal of inducing carbon stress/starvation ("carbon stress" defined here as a combination of source limitation [removal of photosynthetic tissue] and sink enhancement [regrowth of successive canopies]; Handa et al., 2005).

Trembling aspen, a clonal tree species, is the most widespread tree species in North America (Peterson and Peterson, 1992). Trembling aspens are often severely defoliated at regional scales (Landhausser and Lieffers, 2012) in boreal and temperate regions. Aspen forests also recently experienced a widespread drought-induced die-off, termed sudden aspen decline (SAD; Worrall et al., 2008, 2010). This mortality event swept across the western United States and parts of Canada after a climate change-type drought from 2000 to 2003 (Worrall et al., 2008, 2010; Michaelian et al., 2011), and mortality has continued through 2011. Drought and temperature have been implicated as the primary causes of the widespread mortality in a number of studies (Worrall et al., 2008, 2010; Rehfeldt et al., 2009; Michaelian et al., 2011; Anderegg et al., 2012). Furthermore, previous research has identified large drought-driven decreases in carbon uptake in trembling aspen in experimental settings (Galvez et al., 2011; Anderegg, 2012). Thus, aspen forests provide an exceptionally timely and relevant case to examine the multiyear consequences of carbon stress in this species, both in light of SAD and frequent defoliator outbreaks. Importantly, we did not test here the role of carbon starvation in drought-induced SAD, which has been done elsewhere (Anderegg et al., 2012) but instead

sought to understand the consequences of carbon stress, which may play a role in multiyear feedbacks and pathways to mortality.

We performed successive defoliation to induce carbon stress to ask four primary questions. (1) How do canopy patterns (e.g. leaves per branch across branch height), leaf morphology, and leaf area index change during defoliation-induced carbon stress? (2) How does carbon stress manifest in the nonstructural carbohydrate reserves in multiple tissues and over time in defoliated ramets? (3) What are the consequences of defoliation-induced carbon stress on vulnerability to insect attack? (4) What are the hydraulic effects of defoliation-induced carbon stress?

RESULTS

We used repeated defoliation on one plot in a paired-plot design in five aspen clones in the San Juan National Forest in Colorado during the summer of 2010 (see "Materials and Methods"). A nearby (less than 10 km) and similar-elevation weather station to the research sites revealed that 2010 had a relatively dry spring, 90% of average snowpack, and average snowmelt around mid-May. During the summer of 2010, the San Juan National Forest received little measurable rain (less than 0.5 cm) between snowmelt and the onset of monsoonal rains in late July, resulting in a seasonal drought. After a strong monsoonal influx of rain, water year precipitation was still slightly below average for the region (36.8 cm; 42.2 cm average). Water year precipitation for 2011 was barely below average (41.1 cm; 42.2 cm average) with monsoonal influx in early July.

Canopy Characteristics of Carbon Stress

Defoliated plots flushed three canopies over the summer (natural leaf flush [C1] plus two canopies after 100% defoliation of three ramets [C2 and C3]). Leaf area index declined substantially between the three canopies ($P = 0.0004$), although this was largely driven by different factors in the second canopy (C2) versus the third canopy (C3; Fig. 1C). Average leaf area per leaf declined overall ($P < 10^{-6}$) and sharply between C1 and C2 but much less substantially between C2 and C3 (Fig. 1A). Conversely, the number of leaves per branch declined moderately between C1 and C2 but greatly between C2 and C3 ($P < 10^{-4}$; Fig. 1B). None of these patterns differed between high and low branches in the canopy, suggesting little sun-leaf/shade-leaf differences in leaf size. We noted very few instances of whole branch die-back in defoliated ramet canopies. Average leaf net photosynthesis rates did not differ between first (natural) canopy and second (postdefoliation) canopy (A_{first} 8.4 ± 1.2 [SD] $\mu\text{mol m}^{-2} \text{s}^{-1}$; A_{second} 8.7 ± 1.4 $\mu\text{mol m}^{-2} \text{s}^{-1}$), which indicates that reflushed leaves functioned largely as well for carbon uptake as the initial leaves.

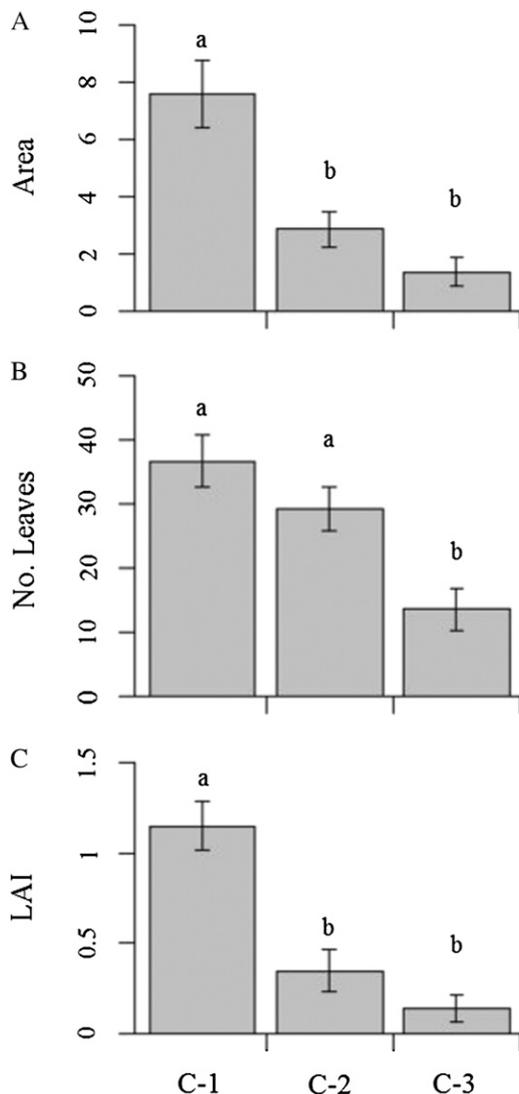


Figure 1. Canopy characteristics (mean \pm SE) of defoliated ramets after first canopy flush (C1), second canopy flush (C2), and third canopy flush (C3). A, Average area per leaf (cm²). B, Average number of leaves per branch. C, Leaf area index (LAI; m² m⁻²).

In contrast, ramets undergoing SAD exhibited strongly directional patterns in canopy die-back. These ramets had much higher rates of mortality in the top and south sides of the canopy (Fig. 2). Our observations of ramets at different stages of canopy dieback suggest that canopy dieback during aspen decline generally starts on high and south-facing branches and proceeds downward and northward.

Carbohydrate Dynamics and Changes

At no point did carbohydrate concentrations in control ramets differ significantly from native control (untrenched) ramets, suggesting that root trenching had little effect on carbohydrate balance. Thus, we

present here only carbohydrate data of trenched-control and defoliated plots. No carbohydrate concentrations differed between control, defoliated, and native treatments prior to the onset of the experiment. Additionally, no substantial changes were observed in tissue Glc levels; thus, only starch and Suc concentrations are presented here.

Branch starch concentration changed significantly over time ($P < 10^{-5}$), between treatments ($P < 10^{-3}$), and differently between treatments over time (time-treatment interaction; $P < 10^{-4}$; Fig. 3). In contrast, bole xylem starch levels changed significantly over time ($P = 0.003$), although not between treatments or time-treatment interactions ($P = 0.45$, $P = 0.66$). Bole bark starch concentrations changed significantly between treatments, and these differences varied over time ($P_{\text{treatment}} = 0.013$, $P_{\text{time-treatment interaction}} = 0.016$). Similarly, starch concentrations in roots changed significantly between treatments ($P = 0.03$) and time-treatment interactions ($P = 0.003$). Thus, repeated defoliation strongly influenced branch and root starch concentrations, moderately influenced bark starches, and had little effect on xylem starches.

Branch Suc levels exhibited significant differences between treatments, although these differences varied over time ($P_{\text{treatment}} = 0.002$, $P_{\text{time-treatment interaction}} = 0.01$; Fig. 4). Bole xylem Suc, however, showed significant changes only over time ($P = 0.02$), and bark Suc showed no significant changes across time, treatment, or their interaction. Root Suc changed significantly only in the time-treatment interaction ($P = 0.02$). This suggests that repeated defoliation only decreased branch and root Suc levels. We explore specific canopies and tissue-level changes below.

Canopy 1 (Natural Leaf-Out)

Starch concentrations in branches plummeted after initial leaf-out of ramets ($P = 0.0007$; Fig. 3). This indicates that primary reserves for canopy production in aspens likely come from branches. Bole xylem and bark starches declined significantly as well ($P_{\text{xylem}} = 0.004$, $P_{\text{bark}} < 10^{-5}$), while root starches remained steady or increased. Suc levels in xylem, bark, and roots all decreased slightly (Fig. 4).

Canopy 2

After experimental defoliation, newly flushed leaves did not differ in any respect from previous leaves (starch_{new}, 11.3% \pm 1.1%; Suc_{new}, 12.8% \pm 1.4%; starch_{previous}, 11.6% \pm 1.2%; Suc_{previous}, 13.5% \pm 1.1%). Branch starch concentrations remained low in defoliated ramets compared with substantial recovery in control ramets ($P = 0.02$), but they did not decline lower (Fig. 3). Bark starch concentrations exhibited the same pattern as branches with control plots, increasing significantly from defoliated plots ($P = 0.005$). Root starch levels exhibited the largest declines ($P = 0.003$). This suggests that the reserves for a second canopy came

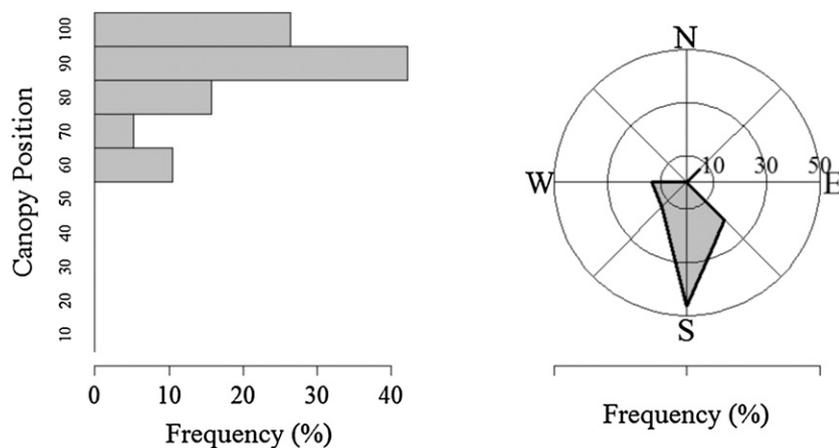


Figure 2. Distribution of average canopy mortality of SAD-affected ramets. Average height of mortality within the canopy (left) and direction of mortality (right) are shown.

largely from roots. Suc levels declined in roots, bark, and branches in defoliated ramets, but the declines were not significant after the Bonferroni correction (Fig. 4).

Canopy 3

After the second experimental defoliation, branch starch concentrations remained significantly lower in defoliated plots ($P = 0.0007$) but did not decrease in C3, while root and bark starches recovered to where they were no longer significantly different from control plots ($P_{\text{root}} = 0.07$, $P_{\text{bark}} = 0.052$). Branch and root Suc continued to decline, remaining significantly lower in defoliated plots ($P = 0.0005$), while xylem and bark Suc largely recovered to control levels.

Next Year

By July 2011, branch starch concentrations remained significantly lower in defoliated plots than in control plots ($P = 0.002$), but they had recovered in roots to where defoliated root starch concentrations exceeded those of control plots ($P = 0.47$; Fig. 3). Similar recovery and higher levels was observed in branch and root Suc levels.

Infestation Vulnerability

One year after defoliation, rates of infestation increased substantially in defoliated ramets (Fig. 5). Frequency of infestation by *Cytospora* canker and black canker increased significantly ($P = 0.004$ and $P =$

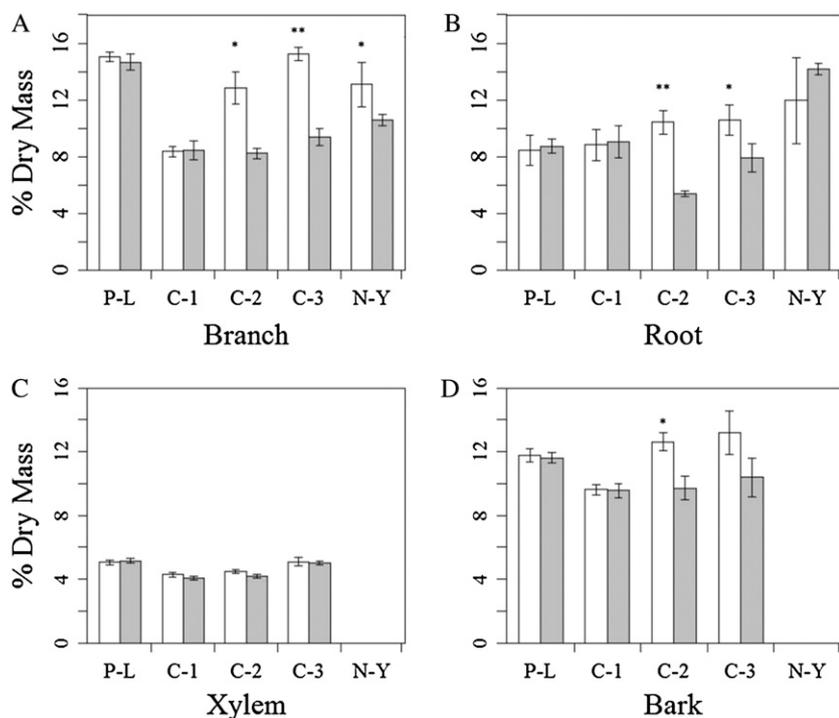
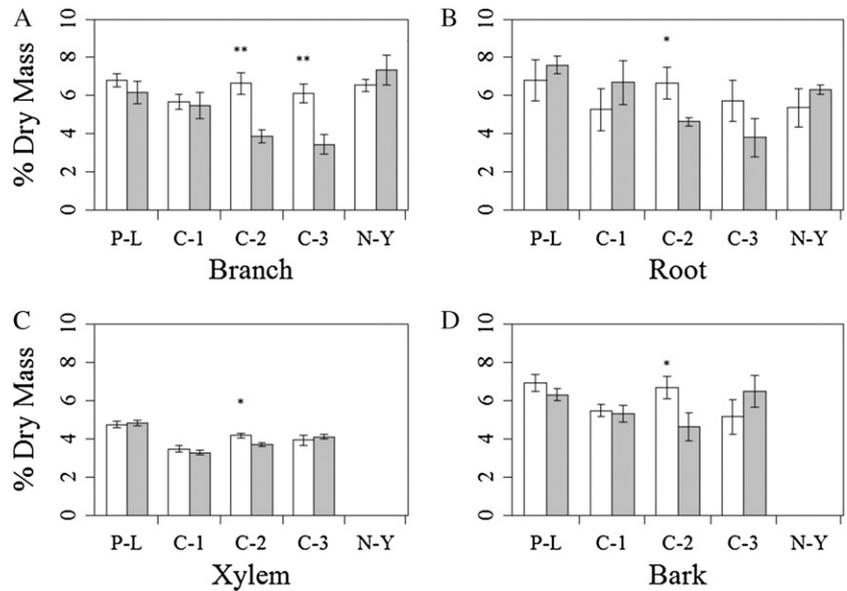


Figure 3. Starch levels (mean \pm SE) of branch, xylem, bark, and root tissues in control ramets (white bars) and defoliated ramets (gray bars) over the course of the experiment. Sampling events were preleaf flush (P-L), first canopy flush (C1), second canopy flush of defoliated ramets (C2), third canopy flush of defoliated ramets (C3), and the next year (N-Y) after defoliation. Note that next-year samples were not taken from xylem/bark tissues.

Figure 4. Suc levels (mean \pm SE) of branch, xylem, bark, and root tissues in control ramets (white bars) and defoliated ramets (gray bars) over the course of the experiment. Sampling events were preleaf flush (P-L), first canopy flush (C1), second canopy flush of defoliated ramets (C2), third canopy flush of defoliated ramets (C3), and the next year (N-Y) after defoliation. Note that next-year samples were not taken from xylem/bark tissues.



0.04, respectively). Although lower levels of infestation occurred with poplar borer and aspen bark beetle, control ramets experienced no infestation by these agents at all.

Hydraulic Vulnerability

Hydraulic conductivity did not differ prior to defoliation in 2010, yet measurements in 2011 indicated large shifts in hydraulic capability. By July 2011, defoliated ramets had significantly lower levels of refilled basal area-specific hydraulic conductivity ($P = 0.007$; Fig. 6). This held true for native (unrefilled conductivity) as well ($P = 0.003$); thus, the treatments had no significant difference in percent loss of conductivity ($P = 0.15$). Vulnerability curves indicated a slight increase in vulnerability (higher levels of percent loss of conductivity at less negative water potentials), but this was not significantly different for any water potential ($P > 0.17$; Fig. 6).

DISCUSSION

We characterize here the patterns of induced carbon stress and evidence for subsequent-year consequences of carbon stress in trembling aspen. Defoliation-induced carbon stress led to distinct leaf morphology and distribution in subsequent canopies and the following year. This pattern is characterized by smaller leaf area per leaf with few instances of whole-branch dieback, especially in early stages of carbohydrate drawdown. This pattern has been observed in moderate frost-driven defoliation in aspen as well, although severe frost damage led to patchy second canopies (St. Clair et al., 2009). Later stages revealed large decreases in the number of leaves per branch, and most leaves that

were flushed tended to be at the distal ends of branches. This pattern contrasts with the observed branch die-back from SAD, which coincides with areas of the canopy more likely to be water stressed due to decreasing xylem water potentials with height and increasing radiation load. Branch die-back tended to occur abruptly across a whole branch. Aspen has highly “sectored” xylem, and SAD crown dieback patterns align with expected patterns of hydraulic failure from aspen xylem structure (Orians et al., 2004).

Likely due to both decreased photosynthesis (source decrease) and the carbon cost of canopy construction (sink increase), defoliation led to carbohydrate decreases in ramets. The sources of carbon reserves for the first, natural canopy (C1) and the subsequent postdefoliation canopies (C2 and C3) appear to have varied. Branch carbohydrates appeared to have been

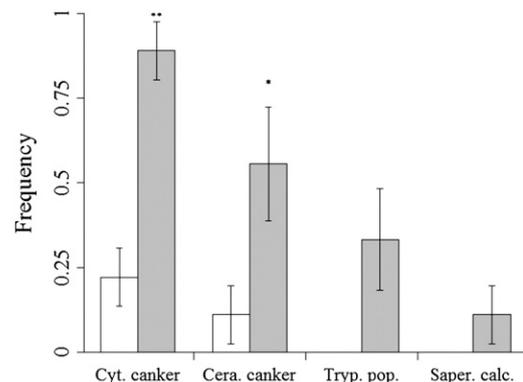


Figure 5. Frequency (mean \pm SE) of fungus or insect attacks in control (white bars) and defoliated (gray bars) ramets 1 year after defoliation for *Cytospora* canker, black canker, poplar borer, and aspen bark beetle.

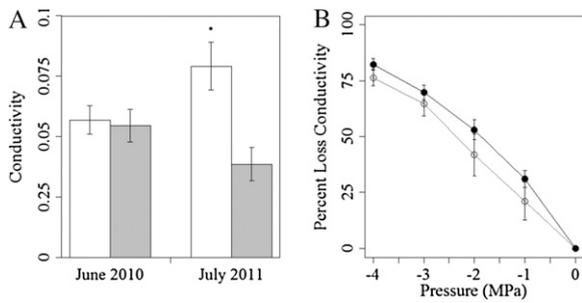


Figure 6. A, Refilled basal area-specific hydraulic conductivity (mean \pm SE; $\text{g mm}^{-1} \text{kPa}^{-1} \text{s}^{-1}$) in control (white bars) and defoliated (gray bars) ramets in 2010 prior to defoliation and in 2011. B, Percent loss of conductivity of control (white circles) and defoliated (black circles) ramets as a function of branch water potential.

drawn down for C1, while root reserves declined for C2. A combination of sources may have been used for C3. This aligns with previous research that has suggested that the primary storage reserve of aspen carbohydrates is in the clonal root network (Shepperd and Smith, 1993; Landhausser and Loeffers, 2012). Root carbohydrate drawdown has been observed in natural defoliation events in aspen clones as well (Landhausser and Loeffers, 2012). It also suggests that the seasonal carbohydrate pattern in these forests may be similar to those of the northern Rocky Mountains, as carbon to construct a normal canopy was drawn from the branches in Alberta, Canada (Landhausser and Loeffers, 2002). We scaled root and branch starch concentrations by a tissue's biomass, drawn from our measured root biomass and a previously published branch biomass allometric equation calibrated on the same diameter of trees in Utah (Johnston and Bartos, 1977), to generate a rough estimate of total tissue starch reserves. Plot-level branch biomass averaged $11.4 \text{ kg plot}^{-1}$, while root biomass averaged 6.8 kg plot^{-1} . Thus, the branch starch drawdown associated with constructing canopy C1 was approximately 720 g plot^{-1} , while the root drawdown associated with C2 was approximately 250 g plot^{-1} . This aligns well with the respective biomass and leaf area index differences between C1 and C2 (Fig. 1).

The generally modest declines in carbohydrate reserves, capability for multiple leaf flushes, and relatively rapid recovery of root starch concentrations all suggest substantial carbohydrate reserves in aspen clones. Several other tree species have been found to store enough carbohydrate reserves for multiple canopies as well, which suggests that this could be relatively common in mature trees (Niinemets, 2010). Notably, despite substantial remaining carbohydrate reserves, subsequent aspen canopies exhibited smaller leaves, fewer leaves per branch, and lower leaf area. This could imply that (1) these carbohydrates were not accessible, (2) another nutrient such as nitrogen was limiting for canopy growth, (3) leaf growth in subsequent canopies was slower, or (4) these ramets

maintained carbohydrate reserves at the expense of canopy growth. Such carbon allocation could allow deciduous trees such as aspens to maintain ample reserves over winter months to flush a healthy canopy in subsequent years. This allocation may also favor increased defense compound synthesis to protect against subsequent defoliations, which has been documented in defoliated aspen seedlings (Donaldson et al., 2006). Maintaining substantial root nonstructural carbohydrate reserves may be especially evolutionarily advantageous for aspens, because it could allow the maintenance of clonal root networks in the soil for longer amounts of time. For instance, aspen root biomass in stands transitioning to conifer forest via succession have been observed to remain relatively constant, despite large losses in aboveground cover (Shepperd et al., 2001).

Moderate drought did occur during the early part of the 2010 growing season, which is a caveat to our results. While it may have slightly influenced the observed canopy characteristics and regrowth, it seems unlikely to have influenced carbohydrate concentrations, as carbohydrate reserves in native aspen clones did not change notably due to the drought (Anderegg, 2012). For the infestation and hydraulic analyses, both control and defoliated plots experienced the same drought conditions in both 2010 and 2011. In fact, moderate drought would be most likely to conservatively bias (e.g. decrease signal) infestation and hydraulic consequences, because defoliated plots experienced less water stress due to sharp decreases in transpiration from lower leaf area and canopy duration (Hart et al., 2000).

The multiyear consequences and feedbacks from carbon stress may be particularly relevant for drought-induced forest mortality. Defoliated ramets experienced much higher levels of fungus and insect attacks 1 year after defoliation. This is a correlational relationship; increased infestation could have also been influenced by physical or chemical stress responses from the defoliation itself. This caveat is worth bearing in mind regarding application to drought stress, but it would certainly occur with natural insect defoliation events. Yet, field observations revealed that the spread and severity of the documented insect attacks, even when they did occur in control ramets, appear to have been more severe in defoliated ramets. Combined with the significantly lower levels of branch starch concentrations in 2011, this suggests that carbon stress may have played an important role in vulnerability to infestation. Many of these mortality agents, notably *Saperda calcarata*, can also damage sapwood xylem and therefore could impair hydraulic function as well.

Defoliation-induced carbon stress appears to have had strong effects on hydraulic performance as well. Defoliated ramets had significantly lower branch conductivities 1 year after experimental treatment, likely due to declines in growth and new xylem formation. This also highlights the extent to which trembling aspen relies on recent, 1 to 3 years of

growth for conducting capability, which has been noted in other studies as well (Sperry et al., 1994). Thus, we hypothesize that carbon stress-induced declines in growth during drought may feed back to increased hydraulic vulnerability to drought in subsequent years. Given the multiyear nature of the climate change-type drought from 2000 to 2003, this could have played a role in SAD and die-off events in other species triggered by the same drought (Breshears et al., 2005).

The mechanisms through which forests respond to severe drought, especially over multiple years, are poorly understood but important for projecting and managing the response of forests to future changes in climate. Studies of the physiological and dynamic processes during stress hold great promise for improving our understanding of tree physiology during drought and informing predictive models. We present a multitissue, temporal description of defoliation-induced carbon stress and highlight previously little-considered feedbacks of carbon stress, which could influence forests' vulnerability to drought and climate change in the coming decades.

MATERIALS AND METHODS

Field Sites

We performed our experiments on trembling aspen (*Populus tremuloides*) clones in the San Juan National Forest, located in southwestern Colorado (Lowry et al., 2007). The San Juan Mountains experience a summer rainy season that usually begins in July due to an influx of monsoonal air from the Gulf of Mexico and the Gulf of California (Keen, 1996). Previous studies suggested a mean annual temperature of 3.2°C and an average annual precipitation of 428 mm at high-elevation weather stations (2,660–2,710 m), although this varies considerably across elevation (Elliot and Baker, 2004). Aspen forests are found from around 2,350 to 3,250 m elevation in this region, co-occurring with ponderosa pine (*Pinus ponderosa*) forests at the lower end and Engelmann spruce (*Picea engelmannii*)/subalpine fir (*Abies lasiocarpa*) forests at the upper end (Worrall et al., 2008).

Defoliation-Induced Carbon Stress Experiment

We performed successive 100% canopy defoliation on isolated parts of mature aspen clones to artificially induce carbon stress. Prior to forest leaf-out in May 2010, we located five aspen clones at low elevation (less than 2,700 m) and largely southern-facing aspects. These attributes are associated with patterns of aspen mortality in previous studies (Worrall et al., 2008) and are thus likely to be vulnerable stands. In these clones, we isolated from the rest of the clone two sets of three healthy ramets in plots of constant area (10 m²) via mechanical trenching down to bedrock. In these trenches, we measured the diameter of all lateral roots that crossed the root-trench plane along a 2-m-long stretch to calculate root biomass (Worrall et al., 2008). All ramets were 3 to 5 m in height, with diameters between 10 and 14 cm, and contained few to no known aspen pathogens. The two plots were less than 5 m apart in all clones. These five clones represent the replication unit for all analysis of the study, as one of each paired plot was defoliated and the other plot was a control (see below). In addition, we included a "native stems" control treatment of three stems per clone more than 20 m from the paired plots, which received no manipulation of any kind.

For baseline nonstructural carbohydrate analysis, we sampled a small amount of root, branch (twig), bole sapwood xylem, and bole bark tissues (2–3 cm of tissue; $n = 3$ per tissue per plot) within plots and the same tissues ($n = 3$ per tissue per clone) of nearby native ramets for carbohydrate analysis. We sampled coarse roots (diameter of 3–6 mm) via careful excavation with a shovel.

After leaf-out (termed C1), we sampled the same tissues in plot ramets and native ramets for carbohydrate analysis. Additionally, we marked two branches per ramet (diameter of 3–6 mm), one near the apical leader and one near the lower edge of the canopy, in defoliated ramets. We recorded the number of leaves per branch and, postdefoliation, the average leaf area per leaf by calculating the area of defoliated leaves via ImageJ (<http://rsbweb.nih.gov/ij/index.html>). We measured the photosynthetic rates of ramet leaves via gas exchange (LI-COR 6400) at ambient humidity and CO₂ with photosynthetically active radiation provided by a light-emitting diode at 1,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. We then randomly assigned one of the two plots to be experimentally defoliated to impose carbon stress. We manually defoliated 100% of the three ramets' canopies from ladders (June 12–19), taking exceptional care not to damage any branch tissue and to remove leaves at the petiole. We saved and dried the leaves for dry-mass leaf area index calculations.

We noted the dates of leaf flush and defoliation in each plot, standardizing the length of the flush + leaf growth period between plots. Within 10 to 15 d, defoliated ramets reflushed leaves (C2). We allowed the same growth period in each plot as in the first canopy flush. We sampled all major tissues (leaf, branch, root, bole xylem, and bole bark) for carbohydrate analysis from all three treatments (defoliated, trenched control, and native control). We measured leaves per branch and average leaf area per leaf in all ramets. Additionally, we measured photosynthetic performance of the new leaves via gas exchange. Immediately following these measurements, we then defoliated the ramets once more with the same methods (July 15–25). Within 12 to 17 d, defoliated ramets reflushed leaves again (C3). We waited the same amount of time as before and then made final tissue collections and branch measurements, identical to previous sampling methods. We calculated leaf area index per plot at each sampling event by developing an average ratio of leaf area to dry mass (specific leaf area) and then multiplied this ratio by the total leaf dry mass from each ramet.

Carbohydrate Analysis

After excising from the ramet, we kept samples on ice (typically 4–8 h) for transport to a laboratory. We then oven dried all samples at 50°C and ground samples in a Wiley mill (30 mesh) and a ball mill. In the case of roots, we combined root samples of each plot (three roots per plot into the same sample) at the ball mill stage by adding equal weights of each root to the ball mill, thereby averaging the individual roots. This has been done successfully in previous carbohydrate analyses of aspens (Landhausser and Loeffers, 2002; Anderegg et al., 2012), and we ran several analyses with roots separate and combined, which yielded no significant differences (Student's t test: $t = 0.51$, $P = 0.86$). We extracted Glc, Suc, and starch from all collected tissues. We followed the carbohydrate extraction procedure described by Raab and Terry (1995), which involves chloroform extraction of free sugars, enzymatic digestion of Suc via invertase (Sigma-Aldrich; I9274), and enzymatic digestion of starch via β -amylase (Sigma-Aldrich; A7130) and amyloglucosidase (Sigma-Aldrich; 10115-1G-F). After Suc and starch digestion, levels of carbohydrates were measured colorimetrically via 3-methyl-2-benzothiazolone hydrazone hydrochloride dye (Sigma-Aldrich; 149022-15-1) at 595 nm.

Observational Measures of Canopy Mortality

As a reference for comparison, we determined the canopy characteristics of dieback from SAD. We estimated canopy mortality in seven clones near the defoliated plots that had a more than 100-m-long mortality gradient within the same clone from a largely healthy area to a largely dead area (for full methods, see Anderegg et al., 2012). Briefly, clones did not differ significantly in elevation, aspect, hydrology, or stem diameter across the mortality gradient. To ensure that we were working within the same clone (genetically identical ramets), we distinguished adjacent clones by observing leaf flush and leaf coloration phenology, which occur largely simultaneously across a given clone. We assessed canopy mortality visually in 10 ramets per clone, as described by Worrall et al. (2010). Additionally, we estimated the average height of dead branches and the average direction of canopy mortality by using dead branches as references of canopy mortality. To validate these canopy mortality estimates and improve standardization of methods, we compared estimated mortality to leaf area index assessed via fish-eye photography. Our method agreed well ($r = 0.93$) with photographic estimates of leaf area and canopy cover.

Follow-Up, Insect Vulnerability, and Hydraulic Analysis

To determine the multiyear effects of carbon stress, we returned to the defoliated plots in July 2011. We examined changes in tissue carbohydrate concentration, vulnerability to infestation, and hydraulic vulnerability. We collected tissue samples of branches and roots for carbohydrate analysis with the same methods. We noted the presence or absence of pathogen or insect attack of defoliated ramets compared with control ramets for four of the main infestation agents involved with SAD. While no primary mortality agents (pathogens or insects that typically kill healthy ramets) are involved with SAD, four to six secondary agents are associated with the decline (Worrall et al., 2010). We measured the frequency of attack of Cytospora canker (*Cytospora chrysosperma*), black canker (*Ceratocystis populicola*), poplar borer (*Saperda calcarata*), and aspen bark beetle (*Trypophloeus populi*) within our plots. We also collected two branches per treatment in each plot for hydraulic analysis. All branches were cut, immediately immersed in water, placed in plastic bags for rapid transport to the laboratory, and then recut under water with a razor blade prior to measurement. We conducted measurements of native conductivity, refilled conductivity, and percent loss of conductivity per the pressure-flow method of Sperry et al. (1988). In addition, we quantified a hydraulic “vulnerability curve,” a standard relationship between branch water potential and percent loss of conductivity, via the air-injection technique (Sperry and Saliendra, 1994).

Statistical Analyses

We examined the effect of defoliation on carbohydrate reserves in each tissue via repeated-measures ANOVA (within samples, plot over time; treatment, control versus defoliation), testing the assumption of sphericity using Mauchly's sphericity test. We further tested differences in average leaf area per leaf, leaves per branch, and leaf area index over time in the defoliated plots by using a one-way ANOVA (factor, time period). We performed pairwise *t* tests on selected means from ANOVAs for posthoc tests, provided assumptions of sphericity and homogeneity of variances held, correcting for multiple hypothesis testing with a Bonferroni correction. All analyses were conducted in R (R-project.org version 2.12.1).

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