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Infestation and hydraulic consequences of induced carbon starvation

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69 *Abstract*

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

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97 **Introduction**

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

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

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

137 Most evidence for carbon limitation during drought has been circumstantial, which
138 highlights the critical need for experimental manipulation of these key physiological processes
139 (Niinemets 2010; McDowell 2011). Experimental defoliation is a well-developed technique to
140 examine carbon limitations in field settings (Li et al. 2002; Körner 2003; Snyder & Williams
141 2003; Markkola et al 2004; Handa et al. 2005; Landhausser & Lieffers 2011) and trade-offs in
142 allocation to growth versus defense (Jones et al. 2004; Osier & Lindroth 2004; Donaldson et al.
143 2005). We performed a repeated defoliation experiment of mature trembling aspen (*Populus*
144 *tremuloides*) ramets with the goal of inducing carbon stress/starvation (“carbon stress” defined
145 here as a combination of source limitation (removal of photosynthetic tissue) and sink
146 enhancement (regrowth of successive canopies) (e.g. Handa et al. 2005)).

147 Trembling aspen, a clonal tree species, is the most widespread tree species in North
148 America (Peterson and Peterson 1992). Trembling aspen are often severely defoliated at regional
149 scales (e.g. Landhausser & Lieffers 2011) in boreal and temperate regions. Aspen forests also
150 recently experienced a widespread drought-induced die-off, termed sudden aspen decline (SAD)
151 (Worrall et al. 2008; Worrall et al. 2010). This mortality event swept across the western United
152 States and parts of Canada following a “climate change-type” drought from 2000-2003 (Worrall
153 et al. 2008; Worrall et al. 2010; Michaelian et al. 2011), and mortality has continued through
154 2011. Drought and temperature have been implicated as the primary cause of the widespread
155 mortality in a number of studies (Worrall et al. 2008; Worrall et al. 2010; Rehfeldt et al. 2010;
156 Michaelian et al. 2011; Anderegg et al., 2011). Furthermore, previous research has identified

157 large drought-driven decreases in carbon uptake in trembling aspen in experimental settings
158 (Galvez et al. 2011; Anderegg, 2012). Thus, aspen forests provide an exceptionally timely and
159 relevant case to examine the consequences of carbon stress in this species, both in light of SAD
160 and frequent defoliator outbreaks. Importantly, we do not test here the role of carbon starvation
161 in drought-induced SAD, which has been done elsewhere (Anderegg et al., 2011), but instead
162 seek to understand the consequences of carbon stress, which may play a role in multi-year
163 feedbacks and pathways to mortality.

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

171

172

173 **Results**

174 We used repeated defoliation on one plot in a paired-plot design in five aspen clones in
175 the San Juan National Forest, Colorado, USA during summer 2010 (see Materials and Methods).
176 A nearby (<10 km) and similar-elevation weather station to the research sites revealed that 2010
177 had a relatively dry spring, 90% of average snowpack, and average snowmelt around mid-May.
178 During the summer of 2010, the San Juan National Forest received little measurable rain
179 (<0.5cm) between snowmelt and the onset of monsoonal rains in late July, resulting in a seasonal
180 drought. Following a strong monsoonal influx of rain, water year precipitation was still slightly
181 below average for the region (36.8 cm; 42.2 cm average). Water year precipitation for 2011 was
182 barely below average (41.1 cm; 42.2 cm average) with monsoonal influx in early July.

183 *Canopy characteristics of carbon stress*

184 Defoliated plots flushed three canopies over the summer (natural leaf flush (C1) plus 2
185 canopies following 100% defoliation of 3 ramets (C2 & C3)). Leaf Area Index declined
186 substantially between the three canopies ($p=0.0004$), though this was largely driven by different
187 factors in the second canopy (C2) versus the third canopy (C3) (Figure 1C). Average leaf area

188 per leaf declined overall ($p < 10^{-6}$) and sharply between the C1 and C2, and much less
189 substantially between C2 and C3 (Figure 1A). Conversely, number of leaves per branch declined
190 moderately between C1 and C2, but greatly between C2 and C3 ($p < 10^{-4}$) (Figure 1B). None of
191 these patterns differed between high and low branches in the canopy, suggesting little sun-
192 leaf/shade-leaf differences in leaf size. We noted very few instances of whole branch die-back in
193 defoliated ramets' canopies. Average leaf net photosynthesis rates did not differ between first
194 (natural) canopy and second (post-defoliation) canopy (A_{first} : $8.4 \pm 1.2 \mu\text{mol/m}^2\text{s}$ (SD); A_{second} :
195 $8.7 \pm 1.4 \mu\text{mol/m}^2\text{s}$ (SD)), which indicates that re-flushed leaves functioned largely as well for
196 carbon uptake as the initial leaves.

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

202 *Carbohydrate dynamics and changes*

203 At no point did carbohydrate concentrations in control ramets differ significantly from
204 native control (un-trenched) ramets, suggesting root-trenching had little effect on carbohydrate
205 balance. Thus, we present here only carbohydrate data of trenched-control and defoliated plots.
206 No carbohydrate concentrations differed between control, defoliated, and native treatments prior
207 to the onset of the experiment. Additionally, no substantial changes were observed in tissue
208 glucose levels, thus only starch and sucrose concentrations are presented here.

209 Branch starch concentration changed significantly over time ($p < 10^{-5}$), between treatments
210 ($p < 10^{-3}$), and differently between treatments over time (time-treatment interaction; $p < 10^{-4}$)
211 (Figure 3). In contrast, bole xylem starch levels changed significantly over time ($p = 0.003$),
212 though not between treatments or time-treatment interactions ($p = 0.45$, $p = 0.66$). Bole bark starch
213 concentrations changed significantly between treatments, and these differences varied over time
214 ($p_{\text{treatment}} = 0.013$; $p_{\text{time} \times \text{treatment}} = 0.016$). Similarly, starch concentrations in
215 roots changed significantly between treatments ($p = 0.03$) and time-treatment interactions
216 ($p = 0.003$). Thus, repeated defoliation strongly influenced branch and root starch concentrations,
217 moderately influenced bark starches, and had little effect on xylem starches.

218 Branch sucrose levels exhibited significant differences between treatments, although
219 these differences varied over time ($p_{\text{treatment}}=0.002$; $p_{\text{time} \times \text{treatment interaction}}=0.01$)
220 (Figure 4). Bole xylem sucrose, however, showed significant changes only over time ($p=0.02$)
221 and bark sucrose showed no significant changes across time, treatment, or the interaction. Root
222 sucrose changed significantly only in the time-treatment interaction ($p=0.02$). This suggests that
223 repeated defoliation only decreased branch and root sucrose levels. We explore specific canopies
224 and tissue-level changes below.

225 *Canopy 1 (Natural leaf-out)*: Starch concentrations in branches plummeted following
226 initial leaf-out of ramets ($p=0.0007$) (Figure 3). This indicates that primary reserves for canopy
227 production in aspens likely come from branches. Bole xylem and bark starches declined
228 significantly as well ($p_{\text{xylem}}=0.004$; $p_{\text{bark}} < 10^{-5}$), while root starches remained steady or increasing.
229 Sucrose levels in xylem, bark, and roots all decreased slightly (Figure 4).

230 *Canopy 2*: Following experimental defoliation, newly flushed leaves did not differ in any
231 respect from previous leaves ($\text{Starch}_{\text{new}}: 11.3 \% \pm 1.1\% \text{ (SE)}$; $\text{Sucrose}_{\text{new}}: 12.8\% \pm 1.4\%$;
232 $\text{Starch}_{\text{previous}}: 11.6 \% \pm 1.2\% \text{ (SE)}$; $\text{Sucrose}_{\text{previous}}: 13.5\% \pm 1.1\%$). Branch starch
233 concentrations remained low in defoliated ramets', compared to substantial recovery in control
234 ramets ($p=0.02$), but did not decline lower (Figure 3). Bark starch concentrations exhibited the
235 same pattern as branches with control plots increasing significantly from defoliated plots
236 ($p=0.005$). Root starch levels exhibited the largest declines ($p=0.003$). This suggests that the
237 reserves for a second canopy came largely from roots. Sucrose levels declined in roots, bark, and
238 branches in defoliated ramets, but the declines were not significant after the bonferroni
239 correction (Figure 4).

240 *Canopy 3*: Following second experimental defoliation, branch starch concentrations
241 remained significantly lower in defoliated plots ($p=0.0007$) but did not decrease in C3, while root
242 and bark starches recovered to where they were no longer significantly different from control
243 plots ($p_{\text{root}}=0.07$, $p_{\text{bark}}=0.052$). Branch and root sucrose continued to decline, remaining
244 significantly lower in defoliated plots ($p=0.0005$), while xylem and bark sucrose largely
245 recovered to control levels.

246 *Next Year (NY)*: By July of 2011, branch starch concentrations remained significantly
247 lower in defoliated plots than control plots ($p=0.002$), but had recovered in roots to where

248 defoliated root starch concentrations exceeded those of control plots ($p=0.47$; Figure 3). Similar
249 recovery and higher levels was observed in branch and root sucrose levels.

250 *Infestation vulnerability*

251 A year following defoliation, rates of infestation increased substantially in defoliated
252 ramets (Figure 5). Frequency of infestation by *Cytospora* canker and black canker increased
253 significantly ($p=0.004$; $p=0.04$ respectively). Though lower levels of infestation occurred with
254 poplar borer and aspen bark beetle, control ramets experienced no infestation by these agents at
255 all.

256 *Hydraulic vulnerability*

257 Hydraulic conductivity did not differ prior to defoliation in 2010, yet measurements in
258 2011 indicate large shifts in hydraulic capability. By July 2011, defoliated ramets had
259 significantly lower levels of refilled basal area-specific hydraulic conductivity ($p=0.007$) (Figure
260 6). This held true for native (un-refilled conductivity) as well ($p=0.003$), and thus the treatments
261 had no significant difference in PLC ($p=0.15$). Vulnerability curves indicated a slight increase in
262 vulnerability (higher levels of PLC at less negative water potentials), but this was not
263 significantly different for any water potential ($p>0.17$) (Figure 6).

264

265 **Discussion**

266 We characterize here the patterns of induced carbon stress and evidence for subsequent
267 year consequences of carbon stress in trembling aspen. Defoliation-induced carbon stress led to
268 distinct leaf morphology and distribution in subsequent canopies and the following year. This
269 pattern is characterized by smaller leaf area per leaf with few instances of whole-branch dieback,
270 especially in early stages of carbohydrate drawdown. This pattern has been observed in moderate
271 frost-driven defoliation in aspen as well, though severe frost damage led to patchy second
272 canopies (St. Clair et al. 2009). Later stages reveal large decreases in the number of leaves per
273 branch, and most leaves that were flushed tended to be at the distal ends of branches. This
274 pattern contrasts with the observed branch dieback from SAD, which coincides with areas of the
275 canopy more likely to be water stressed due to decreasing xylem water potentials with height and
276 increasing radiation load. Branch die-back tended to occur abruptly across a whole branch.
277 Aspen has highly “sectored” xylem, and SAD crown dieback patterns align with expected
278 patterns of hydraulic failure from aspen xylem structure (Lu et al. 2010).

279 Likely due to both decreased photosynthesis (source decrease) and the carbon cost of
280 canopy construction (sink increase), defoliation led to carbohydrate decreases in ramets. The
281 source of carbon reserves for the first, natural canopy (C1) and the subsequent post-defoliation
282 canopies (C2,C3) appear to have varied. Branch carbohydrates appeared to have been drawn
283 down for C1, while root reserves declined for C2. A combination of sources may have been used
284 for C3. This aligns with previous research that has suggested the primary storage reserve of
285 aspen carbohydrates is in the clonal root network (Shepherd & Smith 1993; Landhausser &
286 Lieffers 2011). Root carbohydrate drawdown has been observed in natural defoliation events in
287 aspen clones as well (Landhausser & Lieffers 2011). It also suggests that the seasonal
288 carbohydrate pattern in these forests may be similar to those of the Northern Rocky Mountains,
289 as carbon to construct a normal canopy was drawn from the branches in Alberta (Landhausser &
290 Lieffers 2003). We scaled root and branch starch concentrations by a tissue's biomass, drawn
291 from our measured root biomass and a previously published branch biomass allometric equation
292 calibrated on the same diameter of trees in Utah (Johnston & Bartos 1977), to generate a rough
293 estimate of total tissue starch reserves. Plot-level branch biomass averaged 11.4 kg/plot, while
294 root biomass averaged 6.8 kg/plot. Thus, the branch starch drawdown associated with
295 constructing canopy C1 was approximately 720 g/plot, while the root drawdown associated with
296 C2 was approximately 250 g/plot. This aligns well with the respective biomass and LAI
297 differences between C1 and C2 (Figure 1).

298 The generally modest declines in carbohydrate reserves, capability for multiple leaf
299 flushes, and relatively rapid recovery of root starch concentrations all suggest substantial
300 carbohydrate reserves in aspen clones. Several other tree species have been found to store
301 enough carbohydrate reserves for multiple canopies as well, which suggests this could be
302 relatively common in mature trees (Niinemets 2010). Notably, despite substantial remaining
303 carbohydrate reserves, subsequent aspen canopies exhibited smaller leaves, fewer leaves per
304 branch, and lower leaf area. This could imply that either: (1) these carbohydrates were not
305 accessible, (2) another nutrient such as nitrogen was limiting for canopy growth, (3) leaf growth
306 in subsequent canopies was slower, or (4) these ramets maintained carbohydrate reserves over
307 canopy growth. Such carbon allocation could allow deciduous trees such as aspens to maintain
308 ample reserves over winter months to flush a healthy canopy in subsequent years. This allocation
309 may also favor increased defense compound synthesis to protect against subsequent defoliations,

310 which has been documented in defoliated aspen seedlings (Donaldson et al. 2005). Maintaining
311 substantial root NSC reserves may be especially evolutionarily advantageous for aspens because
312 it could allow maintenance of clonal root networks in the soil for longer amounts of time. For
313 instance, aspen root biomass in stands transitioning to conifer forest via succession have been
314 observed to remain relatively constant, despite large losses in aboveground cover (Shepperd et
315 al. 2001).

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

325 The multi-year consequences and feedbacks from carbon stress may particularly relevant
326 for drought-induced forest mortality (Landhausser & Lieffers 2011). Defoliated ramets
327 experienced much higher levels of fungus and insect attacks one year following defoliation,
328 which was not documented one year following drought-induced hydraulic failure in a co-
329 occurring experiment (see Anderegg et al. 2011). This is a correlational relationship; increased
330 infestation could have also been influenced by physical or chemical stress responses from the
331 defoliation itself. This caveat is worth bearing in mind regarding application to drought stress,
332 but would certainly occur with natural insect defoliation events. Yet field observations revealed
333 that the spread and severity of the documented insect attacks, even when they did occur in
334 control ramets, appears to have been more severe in defoliated ramets. Combined with the
335 significantly lower levels of branch starch concentrations in 2011, this suggests that carbon stress
336 may have played an important role in vulnerability to infestation. Many of these mortality agents,
337 notably *Saperda calcarata*, can also damage sapwood xylem and therefore could impair
338 hydraulic function as well.

339 Defoliation-induced carbon stress appears to have had strong effects on hydraulic
340 performance as well. Defoliated ramets had significantly lower branch conductivities one year

341 following experimental treatment, likely due to declines in growth and new xylem formation.
342 This also highlights the extent to which trembling aspen relies on recent 1-3 years growth for
343 conducting capability, which has been noted in other studies as well (Sperry et al. 1994). Thus,
344 we hypothesize that carbon stress-induced declines in growth during drought may feedback to
345 increased hydraulic vulnerability to drought in subsequent years. Given the multi-year nature of
346 the “climate change-type” drought from 2000-2003, this could have played a role in SAD and
347 die-off events in other species triggered by the same drought (e.g. Breshears et al. 2005).

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

356

357 **Materials and Methods**

358 *Field sites*

359 We performed our experiments on aspen clones in the San Juan National Forest (SJNF),
360 located in southwestern Colorado, USA (Lowry et al. 2007). The San Juan Mountains experience
361 a summer rainy season that usually begins in July due to an influx of monsoonal air from the
362 Gulf of Mexico and the Gulf of California. Previous studies suggested a mean annual
363 temperature of 3.2 °C and an average annual precipitation of 428 mm at high elevation weather
364 stations (2660-2710 m), though this varies considerably across elevation (Elliot & Baker, 2004).
365 Aspen forests are found from around 2350-3250 m elevation in this region, co-occurring with
366 ponderosa pine (*Pinus ponderosa*) forests at the lower end and Engelmann spruce (*Picea*
367 *engelmannii*)/subalpine fir (*Abies lasiocarpa*) forests at the upper end (Worrall et al., 2008) .

368 *Defoliation-induced carbon stress experiment*

369 We performed successive 100% canopy defoliation on isolated parts of mature aspen
370 clones to artificially induce carbon stress. Prior to forest leaf-out in May 2010, we located five

371 aspen clones at low elevation (<2700 m) and largely southern-facing aspects. These attributes are
372 associated with patterns of aspen mortality in previous studies (Worrall et al. 2008) and are thus
373 likely to be vulnerable stands. In these clones, we isolated from the rest of the clone two sets of
374 three healthy ramets in plots of constant area (10 m²) via mechanical trenching down to bedrock.
375 In these trenches, we measured the diameter of all lateral roots that crossed the root-trench plane
376 along a 2 m-long stretch to calculate root biomass (as in Worrall et al. 2008). All ramets were 3-5
377 m in height with diameters between 10-14 cm, and contained few to no known aspen pathogens.
378 The two plots were less than 5 m apart in all clones. These five clones represent the replication
379 unit for all analysis of the study, as one of each paired plot was defoliated and the other plot was
380 a control (see below). In addition, we included a “native stems” control treatment of 3 stems per
381 clone >20 m from the paired plots which received no manipulation of any kind.

382 For baseline non-structural carbohydrate (NSC) analysis, we sampled a small amount of
383 root, branch (twig), bole sapwood xylem, and bole bark tissues (2-3 cm tissue; N=3 per tissue per
384 plot) within plots and the same tissues (N=3 per tissue per clone) of nearby native ramets for
385 carbohydrate analysis. We sampled coarse roots (diameter 3-6 mm) via careful excavation with a
386 shovel.

387 Following leaf-out (termed Canopy 1 or C1 henceforth), we sampled the same tissues in
388 plot ramets and native ramets for carbohydrate analysis. Additionally, we marked two branches
389 per ramet (diameter: 3-6 mm), one near the apical leader and one near the lower edge of the
390 canopy, in defoliated ramets. We recorded number of leaves per branch and, post-defoliation,
391 average leaf area per leaf by calculating area of defoliated leaves via ImageJ (ImageJ). We
392 measured the photosynthetic rates of ramet leaves via gas exchange (LI-COR 6400) at ambient
393 humidity and CO₂ with photosynthetically active radiation (PAR) provided by an LED light at
394 1500 (μmol/m²s). We then randomly assigned one of the two plots to be experimentally
395 defoliated to impose carbon stress. We manually defoliated 100% of the three ramets’ canopies
396 from ladders (June 12-19), taking exceptional care not to damage any branch tissue and to
397 remove leaves at the petiole. We saved and dried leaves for dry-mass leaf area index
398 calculations.

399 We noted the dates of leaf-flush and defoliation in each plot, standardizing the length of
400 flush + leaf growth period between plots. Within 10-15 days, defoliated ramets re-flushed leaves
401 (Canopy 2; C2). We allowed the same growth period in each plot as in the first canopy flush. We

402 sampled all major tissues (leaf, branch, root, bole xylem, and bole bark) for carbohydrate
403 analysis from all three treatments (defoliated, trenched-control, and native-control). We
404 measured leaves per branch and average leaf area per leaf in all ramets. Additionally, we
405 measured photosynthetic performance of the new leaves via gas exchange. Immediately
406 following these measurements, we then defoliated the ramets once more with the same methods
407 (July 15-25). Within 12-17 days, defoliated ramets re-flushed leaves again (Canopy 3; C3). We
408 waited the same amount of time as before and then made final tissue collections and branch
409 measurements, identical to previous sampling methods. We calculated Leaf Area Index per plot
410 at each sampling event by developing an average ratio of leaf area to dry mass (specific leaf
411 area) and then multiplied this ratio by the total leaf dry-mass from each ramet.

412 *Carbohydrate analysis*

413 After excising from the ramet, we kept samples on ice (typically 4-8 hours) for transport
414 to a lab. We then oven-dried all samples at 50 °C and ground samples in a wiley-mill (30 mesh)
415 and ball-mill. In the case of roots, we combined root samples of each plot (three roots per plot
416 into the same sample) at the ball-mill stage by adding equal weights of each root to the ball-mill,
417 thereby averaging the individual roots. This has been done successfully in previous carbohydrate
418 analysis of aspens (Landhausser & Lieffers 2002; Anderegg et al., 2011) and we ran several
419 analyses with roots separate and combined, which yielded no significant differences (students t-
420 test, $t=0.51$, $p=0.86$). We extracted glucose, sucrose, and starch from all collected tissues. We
421 followed the carbohydrate extraction procedure described in Raab (1995), which involves
422 chloroform extraction of free sugars, enzymatic digestion of sucrose via invertase (Sigma-
423 Aldrich: I9274), and enzymatic digestion of starch via beta-amylase (Sigma-Aldrich: A7130) and
424 amyloglucosidase (Sigma-Aldrich: 10115-1G-F). After sucrose and starch digestion, levels of
425 carbohydrates were measured colormetrically via MBTH dye (3-Methyl-2-benzothiazolinone
426 hydrazone hydrochloride; Sigma-Aldrich: 149022-15-1) at 595 nm.

427 *Observational measures of canopy mortality*

428 As a reference for comparison, we determined the canopy characteristics of dieback from
429 SAD. We estimated canopy mortality in seven clones near the defoliated plots that have a <100
430 m-long mortality gradient within the same clone from a largely healthy area to a largely dead
431 area (see Anderegg et al. 2011 for full methods). Briefly, clones did not differ significantly in
432 elevation, aspect, hydrology, or stem diameter across the mortality gradient. To ensure we were

433 working within the same clone (genetically identical ramets) we distinguished adjacent clones by
434 observing leaf-flush and leaf coloration phenology, which occur largely simultaneously across a
435 given clone. We assessed canopy mortality visually in ten ramets per clone as in Worrall et al.
436 (2010). Additionally, we estimated average height of dead branches, and average direction of
437 canopy mortality by using dead branches as references of canopy mortality. To validate these
438 canopy mortality estimates and improve standardization of methods, we compared estimated
439 mortality to Leaf Area Index assessed via fish-eye photography. Our method agreed well
440 ($r=0.93$) with photographic estimates of leaf area and canopy cover.

441 *Follow up, insect vulnerability, and hydraulic analysis*

442 To determine the multiyear effects of carbon stress, we returned to the defoliated plots in
443 July 2011. We examined changes in tissue carbohydrate concentration, vulnerability to
444 infestation, and hydraulic vulnerability. We collected tissue samples of branches and roots for
445 carbohydrate analysis with the same methods. We noted the presence or absence of pathogen or
446 insect attack of defoliated ramets compared to control ramets for four of the main infestation
447 agents involved with SAD. While no primary mortality agents (pathogens or insects that
448 typically kill healthy ramets) are involved with SAD, 4-6 secondary agents are associated with
449 the decline (Worrall et al. 2010). We measured the frequency of attack of *Cytospora* canker
450 (*Cytospora chrysosperma*), black canker (*Ceratocystis populicola*), poplar borer (*Saperda*
451 *calcarata*), and aspen bark beetle (*Trypophloeus populi*) within our plots. We also collected two
452 branches per treatment in each plots for hydraulic analysis. All branches were cut, immediately
453 immersed in water, placed in plastic bags for rapid transport to the lab, and then re-cut under
454 water with a razor blade prior to measurement. We conducted measurements of native
455 conductivity, refilled conductivity, and percent loss of conductivity (PLC) per the pressure-flow
456 method of Sperry et al. (1988). In addition, we quantified a hydraulic ‘vulnerability curve,’ a
457 standard relationship between branch water potential and PLC, via the air injection technique
458 (Sperry and Saliendra 1994).

459 *Statistical Analyses*

460 We examined the effect of defoliation on carbohydrate reserves in each tissue via
461 repeated-measures ANOVA (within samples: plot over time; treatment: control versus
462 defoliation), testing the assumption of sphericity using Mauchly’s Sphericity Test. We further
463 tested differences in average leaf area per leaf, leaves per branch, and leaf area index over time

464 in the defoliated plots by using a one-way ANOVA (factor: time period). We performed pairwise
465 t-tests on selected means from ANOVAs for post-hoc tests, provided assumptions of sphericity
466 and homogeneity of variances held, correcting for multiple hypothesis testing with a bonferroni
467 correction. All analyses were conducted in R (R-project.org Version 2.12.1).

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Figure Legends

Figure One: Canopy characteristics (Mean +/- 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; and (C) Leaf Area Index (m²/m²).

Figure Two: Distribution of average canopy mortality of Sudden Aspen Decline-affected ramets. Average height of mortality within the canopy (left) and direction of mortality (right).

Figure Three: Starch levels (Mean +/- SE) of branch, xylem, bark, and root tissues in control ramets (white) and defoliated ramets (gray) over the course of the experiment. Sampling events were Pre-Leaf flush (PL), first canopy flush (C1), second canopy flush of defoliated ramets (C2) and third canopy flush of defoliated ramets (C3), and the next year (NY) following defoliation. Note: NY samples were not taken from Xylem/Bark tissues.

Figure Four: Sucrose levels (Mean +/- SE) of branch, xylem, bark, and root tissues in control ramets (white) and defoliated ramets (gray) over the course of the experiment. Sampling events were Pre-Leaf flush (PL), first canopy flush (C1), second canopy flush of defoliated ramets (C2) and third canopy flush of defoliated ramets (C3), and the next year (NY) following defoliation. Note: NY samples were not taken from Xylem/Bark tissues.

Figure Five: Frequency (Mean +/- SE) of fungus or insect attacks in control (white) and defoliated (gray) ramets one year following defoliation for Cytospora canker (*Cytospora chrysosperma*), black canker (*Ceratocystis populicola*), poplar borer (*Saperda calcarata*), and aspen bark beetle (*Trypophloeus populi*).

622 Figure Six: (A) Refilled basal area-specific hydraulic conductivity (Mean +/- SE; g/mm*kPa*s)
623 in control (white) and defoliated (gray) ramets in 2010 prior to defoliation and in 2011. (B)
624 Percent loss conductivity of control (white) and defoliated (black) ramets as a function of branch
625 water potential.

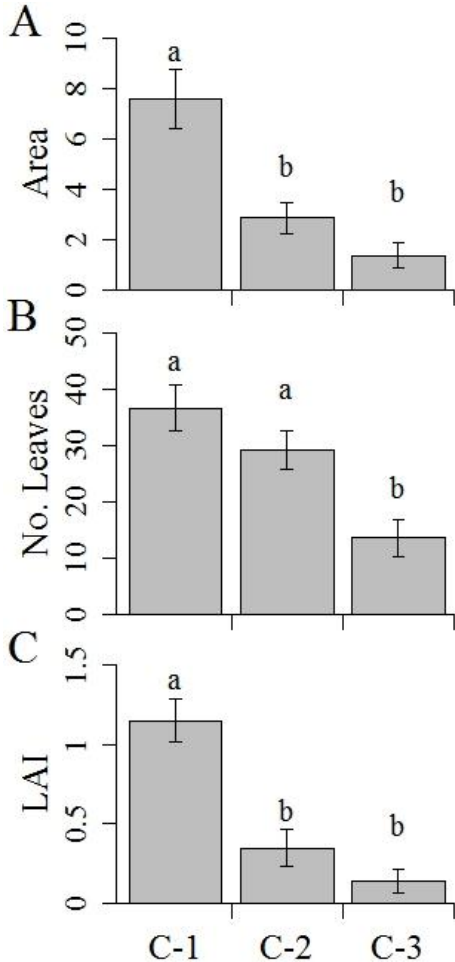


Figure One: Canopy characteristics (Mean \pm SE) of defoliated ramets after first canopy flush (C1), second canopy flush (C2) and third canopy flush (C3). (A) Average area per leaf (cm²); (B) Average number of leaves per branch; and (C) Leaf Area Index (m²/m²).

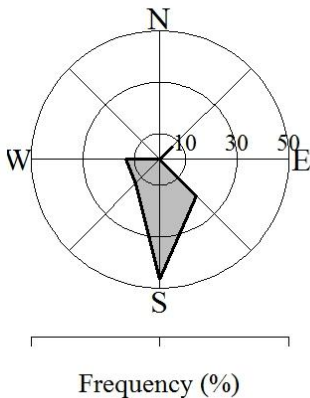
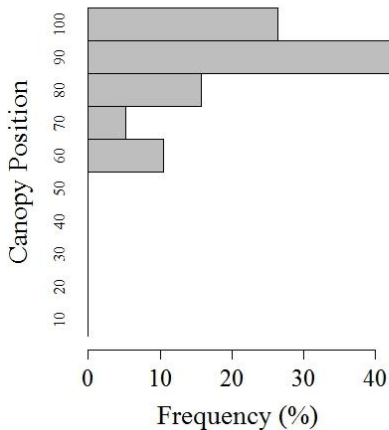


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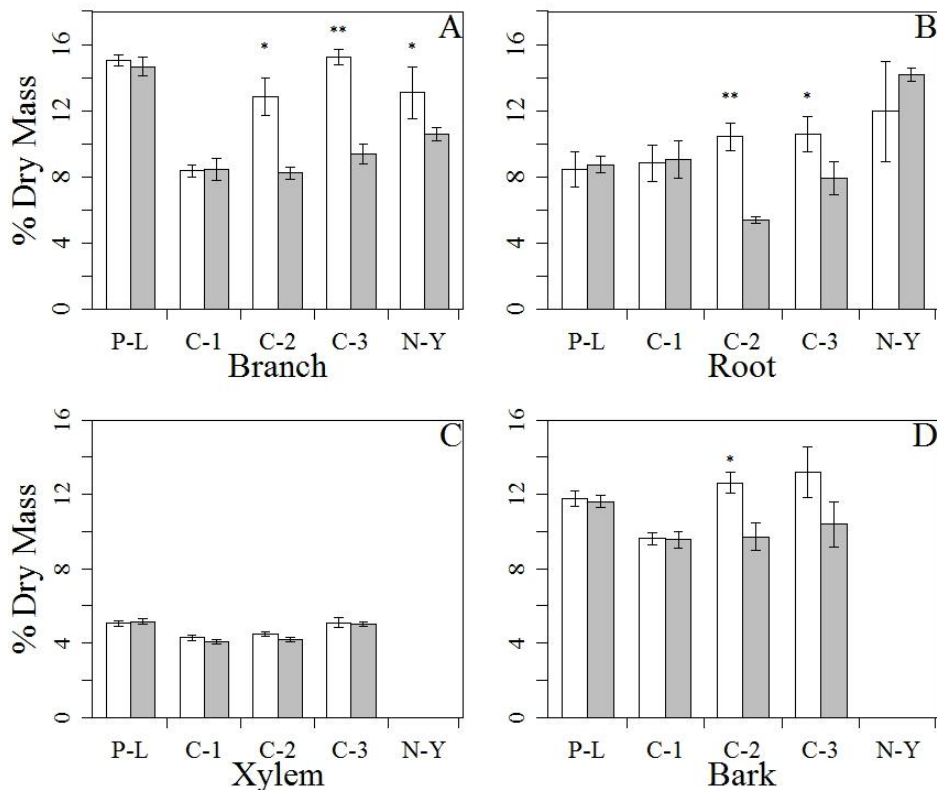


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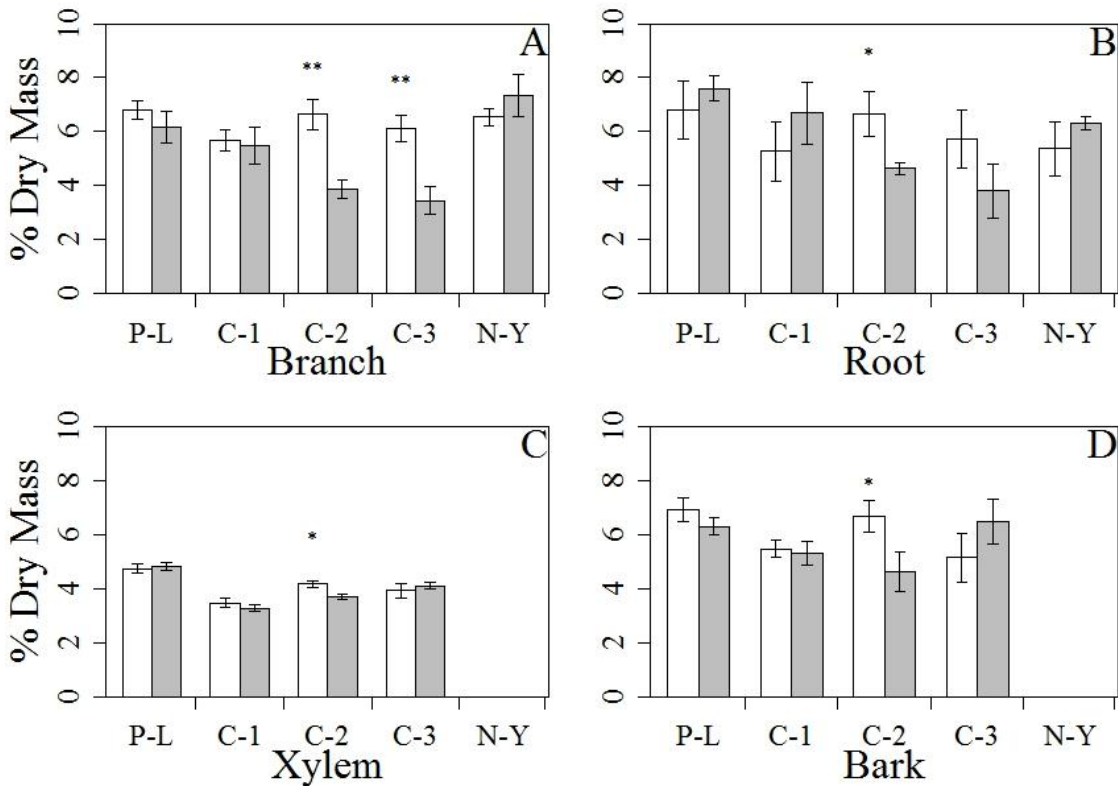


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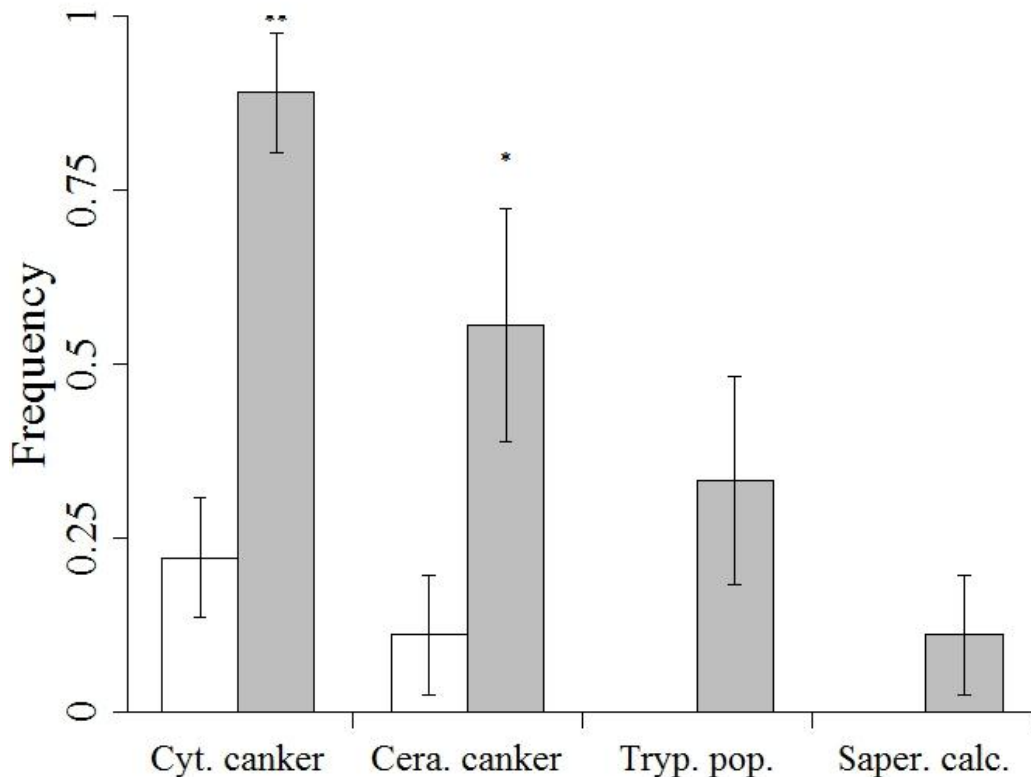


Figure Five: Frequency (Mean \pm SE) of fungus or insect attacks in control (white) and defoliated (gray) ramets one year following defoliation for *Cytospora canker* (*Cytospora chrysosperma*), black canker (*Ceratocystis populicola*), poplar borer (*Saperda calcarata*), and aspen bark beetle (*Trypophloeus populi*).

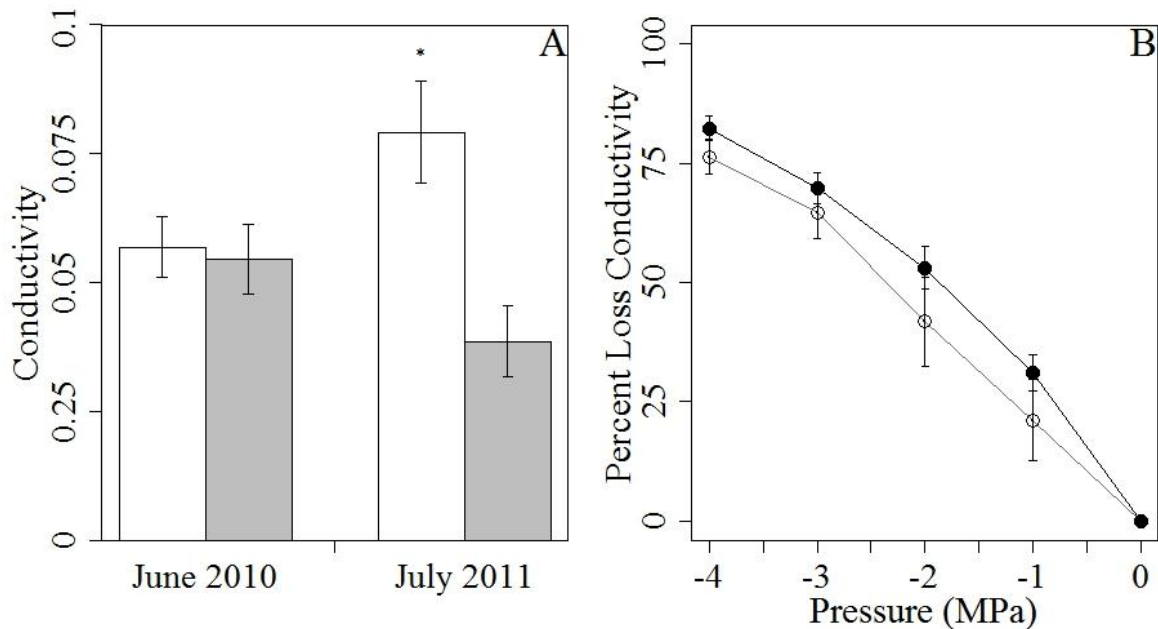


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