Growth and chemical responses of trembling aspen to simulated browsing and ungulate saliva

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Abstract

Aims

Woody plant-browser systems represent an understudied facet of herbivory. We subjected four genotypes of trembling aspen to artificial browsing, similar to that of a large mammalian herbivore, and applied deer saliva to clipped and unclipped trees to assess: (i) the effects of artificial browsing on aspen growth and phytochemistry of leaves and stems, (ii) genotypic variation in responses and (iii) potential alterations of responses by mammalian saliva.

Methods

Potted aspen trees were grown outdoors on the University of Wisconsin-Madison campus. The experiment consisted of a fully-crossed, $2 \times 2 \times 4$ randomized complete block design, with two levels of artificial browsing (unclipped and clipped), two levels of saliva application (no saliva and saliva) and four aspen genotypes. To simulate ungulate browsing damage, we removed the upper 50% of the stem of half of the trees by pinching the stem with needle-nosed pliers and then separating it by tearing. For half of the damaged trees, we immediately swabbed the wound with deer saliva. Trees in the unclipped plus saliva treatment were swabbed with saliva at the 50% height mark. To assess the effects of clipping and saliva application, we harvested all trees after 2 months and measured various growth and chemical properties. Growth measurements included height, vertical growth, mass of leaves, stems and roots, leaf number and area and bud set. Chemical parameters included defensive, nutritional and structural components of both foliage and stems.

Important Findings

Clipping affected most of the growth parameters measured, decreasing tree height, leaf, stem, root and total tree mass and leaf area. Clipped trees had greater vertical growth, more leaves and higher specific leaf area (SLA) than unclipped trees. Deer saliva had little to no effect on plant growth response to the clipping treatment. Terminal budset was delayed by clipping and varied among genotypes but not in response to saliva application. Clipping also affected most of the phytochemical variables measured, reducing defensive compounds (phenolic glycosides and condensed tannins (CTs)) and nutrients (N), but increasing structural components (cellulose and lignin) in both leaves and stems. Saliva had very little effect on tree chemistry, causing only a slight decrease in the amount of CTs in leaves. In general, leaves contained more defensive compounds and nutrients, but much less cellulose, compared with stems. Genotypes differed for all physical and chemical indices, and in tolerance to damage as measured by vertical growth. In addition, for most of the physical and chemical variables measured, genotype interacted with the clipping treatment, suggesting that in natural stands some genotypes will resist or tolerate browsing better than others, affecting forest genetic composition and ultimately forest dynamics.

Keywords: ungulate browsing, deer saliva, defensive chemistry, genetic variation, *Populus tremuloides*, tolerance

INTRODUCTION

Genetics, environment and their interactions strongly influence plant defenses and responses to herbivory (Donaldson and Lindroth 2007; Fritz and Simms 1992; Strauss and Agrawal 1999). Much of this information derives from studies of herbaceous plants and insect folivores, with comparatively little attention directed toward woody plant-browser systems. Due to fundamental differences between these modes of herbivory, however, it is unclear whether insights gained from insect defoliation translate to browsing interactions. Browsing differs from defoliation in several major ways. In contrast to

defoliation, browsing removes woody tissue, including nutrient and carbohydrate reserves (Schutz *et al.* 2011; Woolery and Jacobs 2011) and primary meristems (Cote *et al.* 2004). Removal of woody material has important implications for a tree's ability to tolerate herbivory (Strauss and Agrawal 1999) and for subsequent plant architecture (Whitham and Mopper 1985). In deciduous systems, the timing of browsing can differ from defoliation: folivory occurs only during the growing season, when both insects and leaves are present, but browsers feed year-round (Bryant and Kuropat 1980). Furthermore, depending upon the particular timing of browsing (e.g. early spring vs. winter), tree responses such as regrowth can vary dramatically (Danell *et al.* 1994; Jones *et al.* 2009). While folivorous insects attack plants at all ontological stages and sizes, trees have the possibility to escape many mammalian herbivores *via* vertical growth. Trees have a much better chance of surviving and eventually reproducing once they reach an 'escape height' threshold (Allcock and Hik 2004), where their apical meristems are beyond the reach of ungulate herbivores (Zamora *et al.* 2001). Folivory and browsing also differ from the herbivore's perspective. By definition, folivores consume only leaves, whereas browsers eat both leaves and/or stems (Bryant and Kuropat 1980). Compared with stems, leaves typically contain higher amounts of nutrients (Hagen-Thorn *et al.* 2004) and are less fibrous, but can have higher levels of secondary metabolites (Ruuhola and Julkunen-Tiitto 2000). Therefore, browsers face a poorer quality, less palatable food, which fluctuates in quality and quantity across seasons.

In addition to the physical damage incurred by browsing, plants are also subject to herbivore oral secretions deposited at the wound site (Fig. 1). Early work on the effects of mammalian saliva on plant growth focused mostly on grasses (Detling *et al.* 1980; Dyer 1980; Reardon *et al.* 1974), whereas more recent studies have included woody species. The range of effects for artificial browsing combined with saliva on woody plants includes enhanced branch production (Bergman 2002), increased ratios of aboveground to belowground biomass (Zhang *et al.* 2007), and greater shoot growth (Rooke 2003).

The keystone roles of large ungulate browsers in determining the structure and function of forest ecosystems (Bailey *et al.* 2007; Danell *et al.* 1994; Kaye *et al.* 2005; Myking *et al.* 2011; Pastor and Naiman 1992; Ripple and Beschta 2007) underscores the importance of research on tree-browser interactions. Aspen (*Populus* species) constitute major browse species in many forest systems and browsing by large ungulates often limits recruitment of these trees (Kaye *et al.* 2005; Myking *et al.* 2011; Seager *et al.* 2013). In North American ecosystems, trembling aspen (*Populus tremuloides*) is a dominant species in many early successional and montane forests. While many studies (Donaldson and Lindroth 2008; Stevens and Lindroth 2005; Stevens *et al.* 2007, 2008) have documented aspen responses to insect and/or artificial defoliation, few (Bailey *et al.* 2007) have examined responses to browsing. Trembling aspen provides an ideal system for investigations of plant defenses and responses to mammalian browsing. The secondary chemistry of the species has been well characterized (Lindroth and Hwang 1996). Previous work has demonstrated that allocation to defense exacts a cost to growth in young aspen and that individual genotypes respond differentially to environmental factors such as resource availability and defoliation (Osier and Lindroth 2001, 2004, 2006; Stevens *et al.* 2007, 2008).

To better understand the effects of mammalian browsing on woody species, this research explored the growth and chemical responses of young aspen trees to browsing damage. We subjected four genotypes (genets) of aspen to artificial browsing, similar to that of a large mammalian herbivore, and applied deer saliva to clipped and unclipped trees. We addressed the following questions: (i) Does artificial browsing affect the growth and phytochemistry of aspen, and is chemistry affected similarly in foliage and stem tissue? (ii) Do aspen genotypes vary in their response to damage? and (iii) Does application of saliva from a mammalian herbivore influence growth and phytochemical responses of aspen to damage?

MATERIALS AND METHODS

Aspen genotypes

We micropropagated aspen from root material originally collected from four wild aspen genotypes growing in south-central
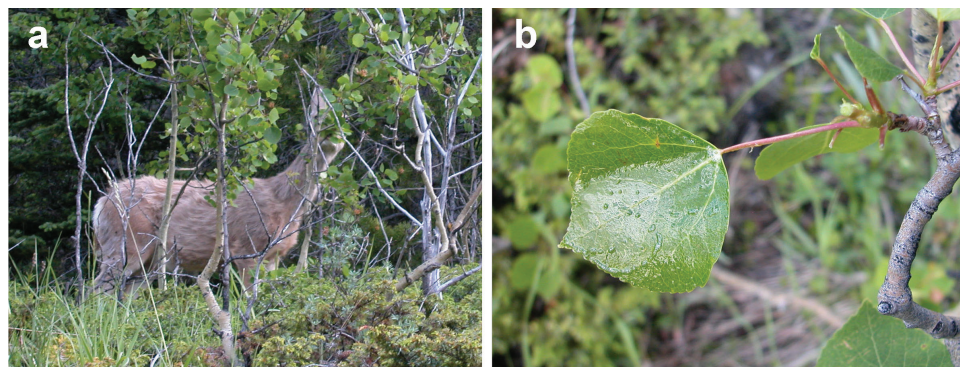
Figure 1: (**a**) mule deer (*Odocoileus hemionus*) browsing on a young aspen tree, Alberta, Canada; (**b**) saliva left on aspen leaves and twigs after browsing.

Wisconsin, USA. For this experiment we used a subset of genotypes from previous studies, identified as Dan 2, PI 12, Sau 3 and Wau 1. Micropropagation allows for the replication of many ramets from a single root source and decreases nongenetic effects (analogous to maternal effects) from source tissues (Wright 1976). Microsatellite DNA markers verified that the genotypes were indeed unique (Cole 2005). We planted the micropropagates outside into 3.8 L pots containing a 2:1 mix of sand:field topsoil (silt-loam) on 8–9 May 2008. All pots received Osmocote (Scotts Miracle-Gro Co., Marysville, OH, USA) 8–9 mo. slow release fertilizer (18:6:12 N-P-K) at a rate of 3.5 g/L of soil. This intermediate level of fertilization provides for excellent growth in aspen (Hemming and Lindroth 1999). We watered all trees, as needed, throughout the experiment.

Experimental design

Potted aspen trees were assembled into an experimental garden in outdoor growing arenas on the University of Wisconsin-Madison campus. The experiment consisted of a fully-crossed, $2 \times 2 \times 4$ randomized complete block design, with two levels of artificial browsing (unclipped and clipped), two levels of saliva application (no saliva and saliva), and four aspen genotypes. All treatment combinations were represented once in each of ten blocks.

Clipping and saliva treatments

Clipping and saliva treatments were applied on 25 June 2008, when trees averaged ~46 cm in height. We measured the height of all saplings, from soil level to the terminal meristem, and then marked the stem at 50% of total height. Using a caliper, we also recorded two orthogonal measurements of basal stem diameter at 1 cm above the soil surface. We used height (h) and average basal diameter (d) to calculate d^2h, a metric of initial tree size (Stevens et al. 2007).

To simulate ungulate browsing damage, we removed the upper 50% of the stem of half of the trees by pinching the stem with needle-nosed pliers and then separating it by tearing. For half of the damaged trees, we immediately swabbed the wound with deer saliva, using a number 2 paint brush. Trees in the unclipped plus saliva treatment were swabbed with saliva at the 50% height mark.

Saliva had been collected 1–2 months earlier from two female adult and one female fawn white-tailed deer (*Odocoileus virginianus*). The deer had been captured and immobilized in the Sandhill Wildlife Area of central Wisconsin as part of an unrelated study. Prior to capture, the deer had fed on natural diets. Saliva was collected separately from the mouths of the deer *via* plastic spoons and syringes and kept frozen at −20°C. Immediately prior to use in this study, the saliva samples were thawed, pooled together and maintained on ice.

Final harvest

We ended the experiment on 25 August 2008 and harvested all trees. Before destructive sampling, we visually inspected all trees for whether they had established a dominant terminal leader and/or had set a terminal bud. We measured tree height as previously described and then cut trees at soil level, separated leaves and stems and placed them into brown paper bags, keeping them on ice in the field. Within ~4 h of sampling we returned the plant material to the lab, measured the leaf area of each tree with a LI-COR LI-3100 area meter (LI-COR Biosciences, Lincoln, NE, USA) and then flash froze both leaves and stems with liquid nitrogen. We stored frozen tissue at −80°C until lyophilization (within 3 weeks). After lyophilization, we obtained separate dry masses for leaves and stems for each tree and used this information, along with root masses (below), to calculate leaf mass ratio (LMR) and stem mass ratio (SMR). Individual tree leaf dry mass, along with total leaf area, were used to compute specific leaf area (SLA). We also calculated vertical growth for all trees, expressed as final height (at time of harvest)—initial height (measured at the time of clipping).

We processed tree roots by depotting them into a basin of water and gently removing most of the soil, followed by further washing in a soil sieve to recover fine roots. We then oven-dried the roots at 60°C, weighed them and used this information, together with aboveground masses, to calculate root mass ratio (RMR).

Tolerance measurements

We used the variables of vertical growth and total tree biomass to calculate tolerance and then tested for differences among genotypes and due to saliva application. We calculated tolerance as the difference between damaged and undamaged ($D - U$) individuals for both vertical growth and tree biomass and also as a proportion of damaged to undamaged (D/U) for tree biomass (Strauss and Agrawal 1999). We paired clipped and unclipped individuals of the same genotype and saliva treatment from the same block to calculate replicate tolerance values and then statistically compared tolerance means for both vertical growth and tree biomass.

Chemical analyses

We separately analyzed leaves and stems from the August harvest. We quantified levels of the two most abundant and biologically active phenolic glycosides (PGs; salicortin and tremulacin; aka, salicinoids), condensed tannins (CTs), carbon, nitrogen, cellulose and acid-detergent lignin (ADL). In preparation for chemical analyses, lyophilized plant tissues were ground in a Wiley Mill (size 20 mesh screen for leaves and size 2 mm screen for stems).

We measured salicortin and tremulacin concentrations by high-performance thin layer chromatography (HPTLC), using the respective purified aspen phenolic glycosides as standards (Lindroth et al. 1993). We quantified total CT concentrations by a modified acid butanol method (Porter et al. 1986) using purified aspen tannin as a standard. We determined carbon and nitrogen (an index of protein) content with a PerkinElmer EA 1112 Combustion Analyzer (PerkinElmer,

Inc., Waltham, MA, USA) and used these values to calculate the carbon to nitrogen ratio (C:N) for tissues. We employed a gravimetric method and an Ankom 2000 fiber analyzer (Ankom Technology, Macedon, NY, USA) to estimate cellulose and ADL. We calculated all chemical concentrations on a percent dry mass basis.

Statistical analyses

We used SAS (ver. 9.1; SAS Institute 2013) to examine the distributions of all variables, to apply transformations when distributions were non-normal, and for subsequent statistical analyses. We performed analyses of covariance and variance (ANCOVA and ANOVA; PROC GLM) on all physical and chemical variables, with genotype (G), clipping (C) and saliva (S) application designated as fixed effects. We included genotype as a fixed effect because these four genotypes were selected, from among a larger set, for the wide range of traits they express. We used initial height as a covariate for final height, and initial tree size (d^2h) as a covariate for the four metrics of tree mass (leaf mass, stem mass, root mass and tree mass). To test whether tolerance differed due to either genotype or saliva, we ran two-way ANOVAs using the tolerance variables calculated with vertical growth and total tree biomass, using paired damaged and undamaged individuals from the same genotype and saliva treatment. To assess whether the leaves and stems on each tree differed in chemistry, we performed a three-way ANOVA using clipping, genotype and tissue (leaves or stems) as the main effects. Due to the overall lack of effects from saliva application, data were pooled across saliva treatments prior to statistical analysis. Also, because leaves and stem on an individual tree were not independent units, we treated tissue type as a repeated measure (measurements repeated in space rather than in time; PROC MIXED with the REPEATED statement in SAS).

RESULTS

Treatment effects on tree size and architecture

Tree height, vertical growth and the four measures of tissue biomass varied strongly in response to clipping and among genotypes, but little to none due to saliva application (Fig. 2, Table 1, supplementary Table 1). Tree height and stem and root mass decreased due to clipping (14%, 35% and 49%, respectively), and these effects differed across genotypes (significant C × G interactions, Table 1). Conversely, clipped trees exhibited 15% more vertical growth than unclipped trees, with the most rapidly growing genotype gaining 26% more height than the slowest growing genotype. Clipping affected total leaf and tree mass similarly across all genotypes, with decreases of 19% and 35%, respectively. Genotypes with the highest values of tree height and leaf, stem, root and tree mass had 14%, 7%, 38%, 28% and 20% greater values than those with the lowest, respectively, even after correcting for initial plant size. Application of deer saliva did
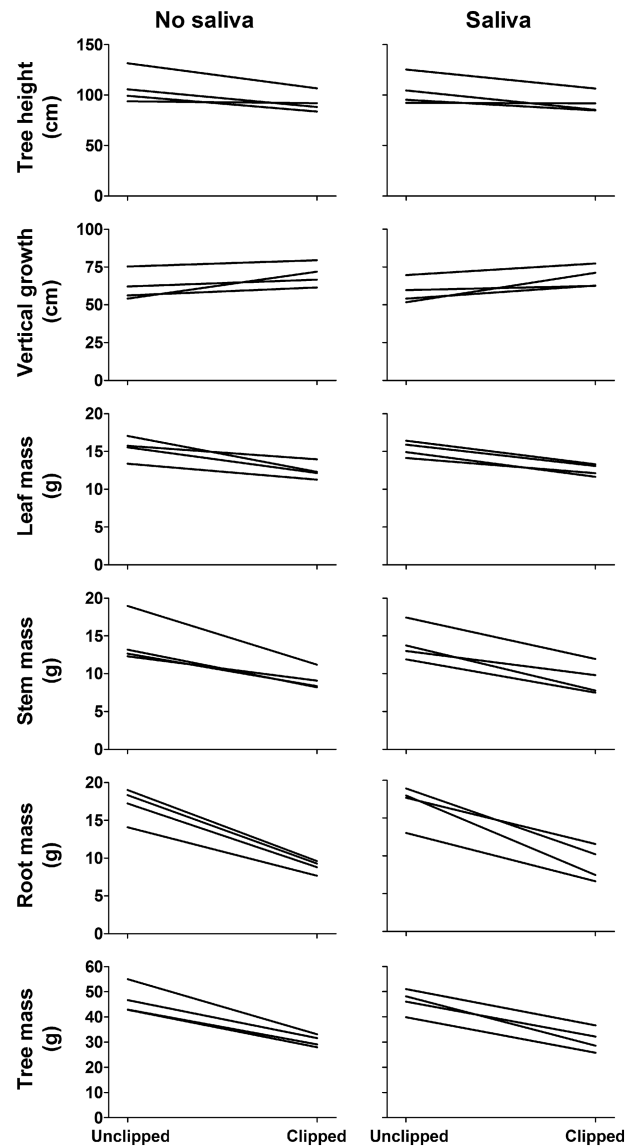

Figure 2: norm of reaction plots for final height, vertical growth and leaf, stem, root and total tree mass. Each line represents the mean response of a single aspen genotype in the unclipped versus clipped (4–5 replicates each) condition, with and without application of deer saliva. Results from statistical analyses are provided in Table 1.

not significantly alter any plant growth response to the clipping treatment (no significant C × S or C × G × S interactions, Table 1).

Leaf number, area per leaf, LMR and RMR all differed with clipping, genotype, and the interaction between these two factors (Fig. 3, Table 2, supplementary Table 2). Overall, clipped trees had more (58%) but smaller (27%) leaves than unclipped trees. Genotypic differences in leaf number and leaf area were strongly affected by clipping with a greater than 5-fold difference among genotypes for leaves and almost an 8-fold difference for area. Clipping increased LMR by 10%, while decreasing RMR by 22%. Clipping also caused an

Table 1: *F*-ratios and *P*-values from a three-factor ANCOVA assessing the effect of clipping (C), genotype (G) and saliva (S) and their interactions, on aspen height, vertical growth, leaf mass, stem mass, root mass and total tree mass, using initial height as a covariate for tree height and initial size (d^2h) as a covariate for all mass measurements

Source	df	Tree height		Vertical growth		Leaf mass		Stem mass		Root mass		Tree mass	
		F	P	F	P	F	P	F	P	F	P	F	P
Clipping	1, 141	77.0	**<0.001**	28.8	**<0.001**	78.6	**<0.001**	219.8	**<0.001**	491.8	**<0.001**	304.0	**<0.001**
Genotype	3, 141	8.6	**<0.001**	19.5	**<0.001**	4.4	**0.005**	15.1	**<0.001**	17.5	**<0.001**	6.1	**0.001**
Saliva	1, 141	2.7	0.104	2.0	0.160	0.1	0.725	0.6	0.452	0.3	0.588	0.4	0.538
C × G	3, 141	7.7	**<0.001**	4.3	**0.007**	1.8	0.160	5.4	**0.002**	3.8	**0.012**	1.9	0.130
C × S	1, 141	0.1	0.759	0.3	0.591	0.0	0.964	0.0	0.866	0.1	0.763	0.0	0.843
G × S	3, 141	1.3	0.296	1.15	0.330	1.0	0.394	1.5	0.207	3.1	**0.028**	2.0	0.125
C × G × S	3, 141	0.2	0.900	0.2	0.892	0.8	0.511	1.2	0.307	1.4	0.238	1.4	0.256
Initial size	1, 141	60.5	**<0.001**	—	—	9.0	**0.003**	11.9	**0.001**	7.2	**0.008**	11.6	**0.001**

Corresponding data are shown in Fig. 2. Boldface *P*-values indicate significance at alpha = 0.05.

average 33% increase in SLA, with some genotypes responding more strongly than others (marginally significant C × G interaction). Genotypes differed for SMR, while the clipping treatment had only a minimal effect. Finally, the only tissue allocation metric to respond to saliva was RMR. Although the average response of RMR was a mere 1% decrease (nonsignificant), genotypes varied in their magnitude (significant G × S interaction).

Regardless of treatment, all trees established a dominant terminal leader by the time of harvest in August. Formation of terminal buds differed with both clipping and genotype, but not with saliva application (Table 3). Nearly half of unclipped trees produced a terminal bud, compared with only one clipped tree that did so.

Tolerance measurements: vertical growth and total biomass

Tolerance values for vertical growth were positive for all genotypes, highlighting the greater vertical growth of clipped over unclipped trees. ANOVA results for vertical growth revealed genotypic differences in tolerance ($F = 4.6$, $P = 0.005$), but not with saliva application ($F = 0.2$, $P = 0.673$). The *post hoc* analysis indicated that genotype PI 12 had greater tolerance in terms of vertical growth than the other three genotypes, which responded similarly (18.7 ± 3.2 cm for PI 12, vs. 7.4 ± 2.7, 4.0 ± 2.9 and 5.8 ± 3.3 cm for Dan 2, Sau 3 and Wau 1, respectively [mean ± SE]). Tolerance measurements using tree biomass, calculated as either a difference or a proportion, did not differ among genotypes or in response to application of saliva.

Treatment effects on tree chemistry

Clipping affected levels of the PGs salicortin and tremulacin in a similar fashion, causing greater reductions in stems than leaves (Fig. 4, Table 4, supplementary Table 3). Overall, genotypic variation in both leaf and stem PGs greatly exceeded the variation due to clipping and saliva, with genotypes differing by 2-fold or more. Although the

magnitude of effects was small, the levels of both compounds in both tissues varied among genotypes in response to clipping, and this variation itself was modestly affected by saliva treatment (two- and three-way interactions). Clipping reduced leaf CTs by 16–49% among genotypes and while the main effect of clipping was not significant for stem CTs, clipping interacted with both the genotype and saliva treatments to influence levels of CTs in stems (Fig. 4, Table 4). Saliva caused a slight decrease (11%) in the amount of CTs in leaves, making tannins the only chemical constituent influenced by the main effect of saliva.

In general, nitrogen (N) responded similarly in both leaves and stems, with slight reductions due to clipping, differences among genotypes, and interactions between genotype and both clipping and saliva (Fig. 5, Table 5, supplementary Table 4). C:N ratios increased by 15% in the stems of clipped trees. Genotypic variation, though significant, was minimal for N in both leaves and stems. Genotypes also differed only slightly for C:N in leaves, but showed greater levels of genotypic variation in stems. Finally, genotype by saliva interactions indicated a slight effect of saliva on both N and C:N in leaves and stems.

Cellulose and ADL concentrations differed with clipping and genotype in both leaves and stems (Fig. 5, Table 6, supplementary Table 4). Clipped trees had increased cellulose and ADL in both leaves and stems, with 8% and 10% more in leaves and 11% and 13% more in stems, respectively. Genotypes varied minimally for both cellulose and ADL in leaves and stems. Genotype and clipping interacted for both leaves and stems for cellulose, but only in stems for ADL. Saliva application had no effect on either of these constituents.

Leaf versus stem chemistry

Chemistry comparisons between leaves and stems from the same tree, with the genotype and clipping treatments pooled across the saliva treatment, revealed substantial differences between these two tissues for most chemical variables measured (Figs 4 and 5, Table 6). The presence of two- and
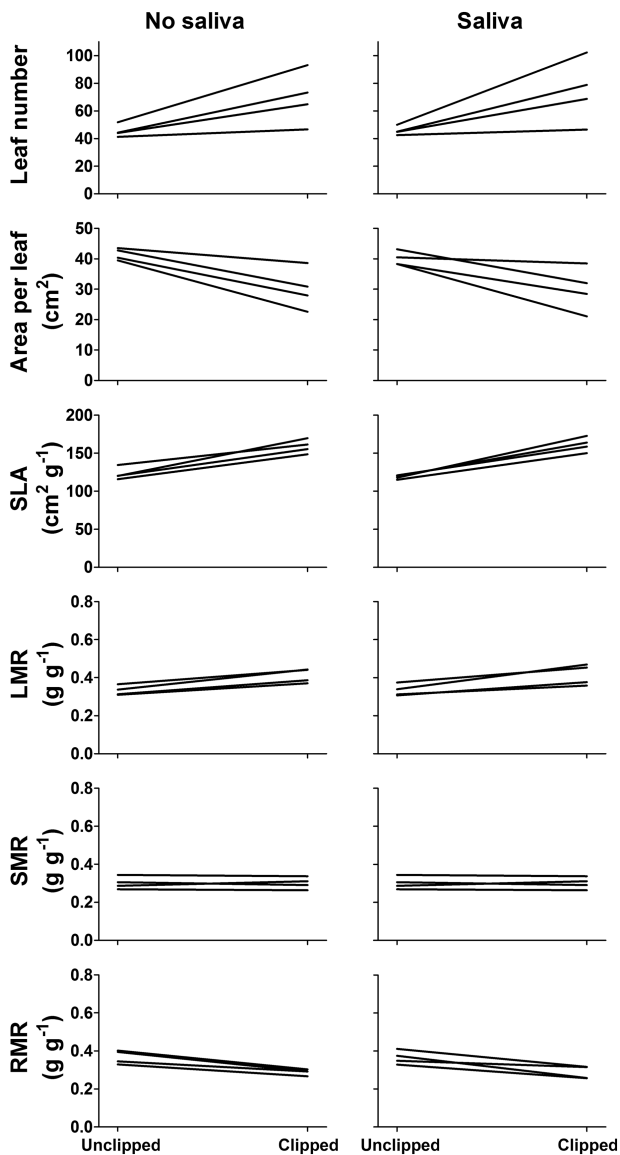
Figure 3: norm of reaction plots of leaf number, area per leaf, SLA, LMR, SMR and RMR. Each line represents the mean response of a single aspen genotype in the unclipped versus clipped (4–5 replicates each) condition without and with application of deer saliva. Results from statistical analyses are provided in Table 2.

three-way interactions of tissue type with clipping and genotype for most chemical components demonstrated that tissue differences were mediated by both of these factors. Overall, leaves contained more defensive compounds than stems with almost one and a half times more salicortin, two and a half times more tremulacin and 5-fold greater levels of CTs than stems. Leaves were more nutritious than stems with nitrogen concentrations about two and three quarters higher than stems, resulting in an opposite pattern in C:N ratio. Tissue comparisons also showed that stems contained 273% more cellulose than leaves, while ADL levels differed minimally between the two tissues.

DISCUSSION

Mammalian browsing differs from insect folivory mainly due to removal of twigs and stems in addition to leaves. Consequently, browsers' diets are generally lower in nutrients, higher in fiber (cellulose) and in the case of aspen, lower in defense compounds than are diets of folivores. When we subjected aspen of different genotypes to simulated browsing and deer saliva, physical and chemical parameters of trees responded primarily to the clipping and genotype treatments and little, if at all, to saliva application.

Effects of artificial browsing on aspen growth, biomass allocation and phytochemistry

Rapid vertical grow can allow trees to escape apical meristem damage by mammalian herbivores, often resulting in greater survival and reproduction (Allcock and Hik 2004). At the termination of our experiment, clipped aspen, although still shorter than unclipped aspen, had closed the height differential by growing faster. Moreover, clipped trees had set a much lower proportion of terminal buds than had unclipped trees, indicating a shift to growing later into the field season. While prolonged indeterminate growth may offset some of the consequences of browsing damage, this shift in phenology comes with the attendant risks of frost damage (Jonsson and Óskarsson 2007) or late season browsing (Danell *et al.* 1994; Jones *et al.* 2009), both of which have been shown to be detrimental to subsequent plant performance. In addition, the normal formation of a late-season terminal bud is a nutrient loading strategy for aspen, allowing trees to store assimilated nutrients and carbon rather than using them for growth (Schott *et al.* 2013).

We also found that aspen responded to browsing with large reductions in tree biomass, driven by losses in all tissues, but with the greatest declines in roots. Previous artificial and natural defoliation experiments with aspen have shown similar patterns. Using multiple *P. tremuloides* genotypes (including the genotypes used here), Stevens *et al.* (2007, 2008) reported decreased relative growth and lower allocation of biomass to stems and roots with defoliation. Studies with other tree species have also documented decreases in root growth or biomass as a result of clipping. Ruess *et al.* (1998) found that browsing of four *Salix* species by moose and snowshoe hares reduced fine root growth, and in both *Quercus robur* (Kullberg and Welander 2003) and *Pinus sylvestris* (Hester *et al.* 2004) root mass decreased with simulated browsing. Millard and Grelet (2010) argue that in most systems, nitrogen, rather than carbon, limits tree growth and that deciduous tree roots are the main sources for nitrogen remobilization after disturbances such as browsing. Thus, the loss of root biomass as a result of browsing has important implications both for recovery from future herbivore damage and for resistance to environmental stressors, such as drought, that are mediated *via* root function.

The changes in aspen due to simulated browsing should have consequences for herbivores feeding after the initial

Table 2: *F*-ratios and *P*-values from a three-factor ANOVA assessing the effect of clipping (C), genotype (G) and saliva (S) and their interactions on aspen leaf number, stem, area per leaf, SLA, LMR, SMR and RMR

Source	df	Leaf number		Area per leaf		SLA		LMR		SMR		RMR	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Clipping	1, 141	251.3	**<0.001**	124.4	**<0.001**	335.0	**<0.001**	318.1	**<0.001**	0.1	0.717	214.8	**<0.001**
Genotype	3, 141	54.5	**<0.001**	18.9	**<0.001**	5.8	**<0.001**	193.3	**<0.001**	79.3	**<0.001**	22.6	**<0.001**
Saliva	1, 141	1.7	0.189	0.8	0.373	0.2	0.636	0.7	0.418	0.2	0.641	0.1	0.759
C × G	3, 141	23.1	**<0.001**	9.0	**<0.001**	2.6	0.054	11.4	**<0.001**	8.2	**<0.001**	7.8	**<0.001**
C × S	1, 141	1.4	0.247	0.3	0.614	2.3	0.134	0.1	0.808	0.1	0.757	0.0	0.995
G × S	3, 141	0.1	0.936	0.2	0.930	2.0	0.117	1.0	0.392	2.2	0.092	3.0	**0.034**
C × G × S	3, 141	0.5	0.689	0.2	0.908	1.4	0.245	0.9	0.443	0.3	0.825	0.5	0.693

Corresponding data are shown in Fig. 3. Bold face *P*-values indicate significance at alpha = 0.05.

Table 3: contingency tables and χ^2 statistics testing for terminal bud set differences between the clipping, genotype and saliva treatments

Treatment	Terminal bud		df	χ^2	*P*
	Yes	No			
Clipping					
°Unclipped	35	41	1	43.6	**<0.001**
°Clipped	1	78			
Genotype					
°Dan 2	15	22	3	16.7	**0.001**
°PI 12	13	27			
°Sau 3	6	33			
°Wau 1	2	37			
Saliva					
°No saliva	19	59	1	0.0	0.882
°Saliva	18	59			

Boldface *P*-values indicate significance at alpha = 0.05.

damage. For instance, the increase in SLA after clipping may make trees more attractive to herbivores, since this functional trait was found to have a positive relationship with both ungulate feeding (Lloyd *et al.* 2010) and insect palatability (Schädler *et al.* 2003). In addition, the lower amounts of PGs we found in clipped trees, especially in stems, should make trees more edible to subsequent mammalian browsers, given their sensitivity to these compounds (Wooley *et al.* 2008). Conversely, leaf beetles should benefit from feeding on previously browsed aspen, due to the decreased levels of CTs in aspen leaves (Donaldson and Lindroth 2004).

Effects of artificial browsing on aspen genotypes

All of the physical and chemical parameters measured in this study differed among the aspen genotypes. For instance, both final tree height and tolerance, measured as a differential in vertical growth, differed among genotypes. Thus, aspen populations may exhibit genotypic variation in employing escape as an anti-herbivore strategy (Allcock and Hik 2004). Genotypes also differed for both leaf number and area per leaf, which was especially dramatic after clipping. The inverse relationship of these two parameters (trees with lower area per leaf grew more leaves) demonstrated that genotypes use different strategies to maintain total leaf area after damage.

Most leaf and stem chemical traits measured in this study also showed large genotypic differences, but overall, leaves had a much greater range of variation, especially in PGs and CTs. Chemistry differences among aspen clones have proved to be important drivers in natural selection. For instance, PGs have been shown to be important factors in the consumption of aspen by elk (*Cervus canadensis*; Wooley *et al.* 2008), resulting in large and rapid phytochemistry shifts in natural populations (Bailey *et al.* 2007). Given the differences in chemistry between leaves and stems in this study, the strength of this selective force may possibly also vary with season. Specifically, browser choice should be more pronounced during the time of year that aspen have leaves (high and variable PG levels) and less so when they are feeding only on stems (low PG levels). Temporal differences in plant chemistry should influence the timing and intensity of browsing, both of which have been shown to alter growth outcomes in natural stands of *P. tremuloides* (Jones *et al.* 2009).

Effect of deer saliva on aspen growth and phytochemistry

We found very little influence of saliva treatment on aspen growth and chemistry. Of all variables measured, we observed only a slight decrease (11%) in foliar CTs with saliva application. Whether the overall minor effects of saliva are unique to our study system remains unclear. Previous artificial browsing studies involving other woody plants and large mammal saliva did not focus on defensive chemistry, but detected much larger effects for physical parameters. Bergman (2002) found that *Salix caprea* grew more branches with clipping plus moose saliva, as opposed to clipping only. Sheep saliva, combined with clipping, enhanced aboveground over belowground productivity for a semi-shrub
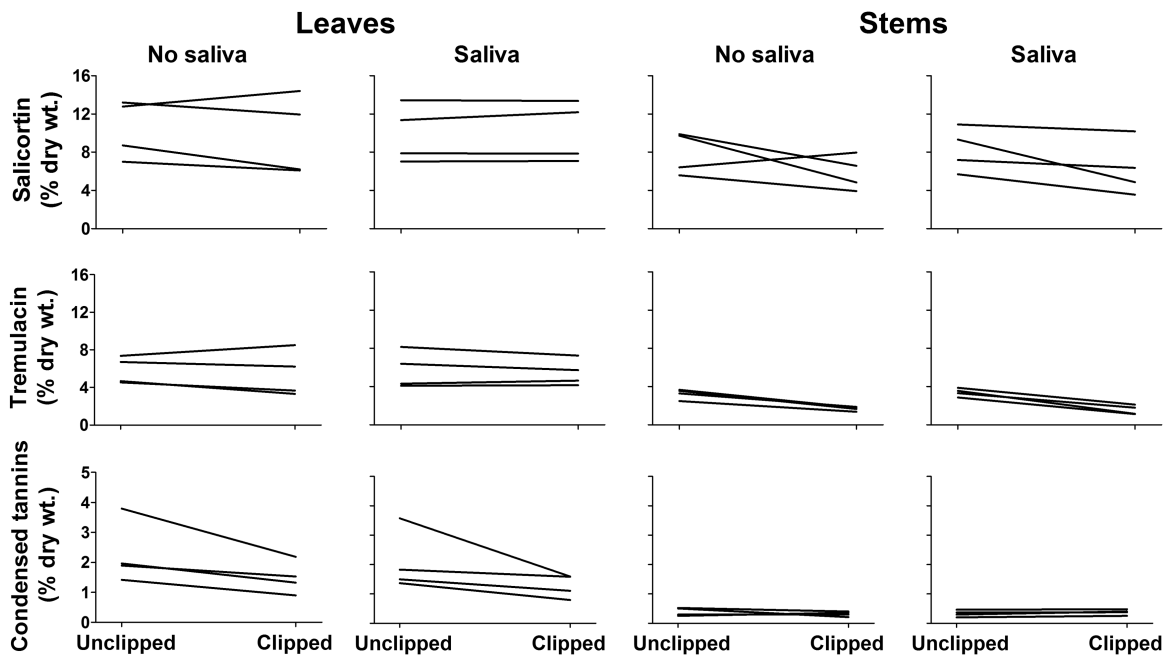
Figure 4: norm of reaction plots of foliar and stem concentrations of phenolic glycosides (salicortin and tremulacin) and CTs. Each line represents the mean response of a single aspen genotype in the unclipped versus clipped (4–5 replicates each) condition without and with application of deer saliva. Results from statistical analyses are provided in Table 3.

Table 4: *F*-ratios and *P*-values from a three-factor ANOVA assessing the effect of clipping (C), genotype (G) and saliva (S) and their interactions on total on two phenolic glycosides, salicortin and tremulacin and CT in aspen leaves and stems

		Salicortin				Tremulacin				CTs			
		Leaves		Stems		Leaves		Stems		Leaves		Stems	
Source	df	F	P	F	P	F	P	F	P	F	P	F	P
Clipping	1, 141	2.6	0.111	46.9	**<0.001**	8.4	**0.004**	280.9	**<0.001**	75.9	**<0.001**	0.5	0.504
Genotype	3, 141	193.0	**<0.001**	33.8	**<0.001**	221.1	**<0.001**	13.8	**<0.001**	41.0	**<0.001**	25.1	**<0.001**
Saliva	1, 141	0.1	0.743	1.5	0.222	0.1	0.740	0.0	0.972	4.8	**0.031**	0.0	0.980
C × G	3, 141	3.4	**0.021**	8.1	**<0.001**	2.1	0.097	0.8	0.509	6.9	**<0.001**	5.1	**0.002**
C × S	1, 141	5.1	**0.026**	0.0	0.874	0.1	0.727	2.4	0.122	0.1	0.772	13.3	**<0.001**
G × S	3, 141	1.7	0.175	9.1	**<0.001**	2.1	0.104	1.6	0.185	4.5	**0.005**	4.2	**0.007**
C × G × S	3, 141	3.7	**0.013**	4.3	**0.006**	11.0	**<0.001**	2.9	**0.037**	0.4	0.732	8.8	**<0.001**

Corresponding data are shown in Fig. 4. Boldface *P*-values indicate significance at alpha = 0.05.

(Zhang *et al.* 2007). Finally, Rooke (2003) measured greater shoot growth and diameter, and more leaves, on African red bushwillow trees with clipping plus goat saliva, compared with clipping alone. These studies, with disparate herbivores and plant species, may indicate species-specific or context-dependent effects of mammalian saliva. Elk, moose and domestic cattle regularly browse aspen (Bailey and Whitham 2002; De Jager *et al.* 2009; Kaye *et al.* 2005) and their saliva may cause responses different from those elicited by deer saliva. In southern Wisconsin, however, where these genotypes were originally collected, and in large parts of North America, white-tailed deer represent the dominant native large browser (Hale *et al.* 2008).

Browsing versus defoliation

While browsing and insect defoliation share many characteristics, their fundamental differences warrant separate investigation. Browsers feed year-round, removing both foliage and woody tissue, and leave saliva at wound sites, all of which have implications for plant regrowth and phytochemistry. A browsing feeding strategy exposes animals to the chemistry of both leaves and stems. Our comparison of the chemistry of both tissues demonstrates that a browser, simultaneously eating leaves and stems or stems alone, acquires a different mix of nutrients and defense compounds than a folivore. Leaves contained more than twice the nitrogen, and only a third of the cellulose, than stems, making them much higher quality
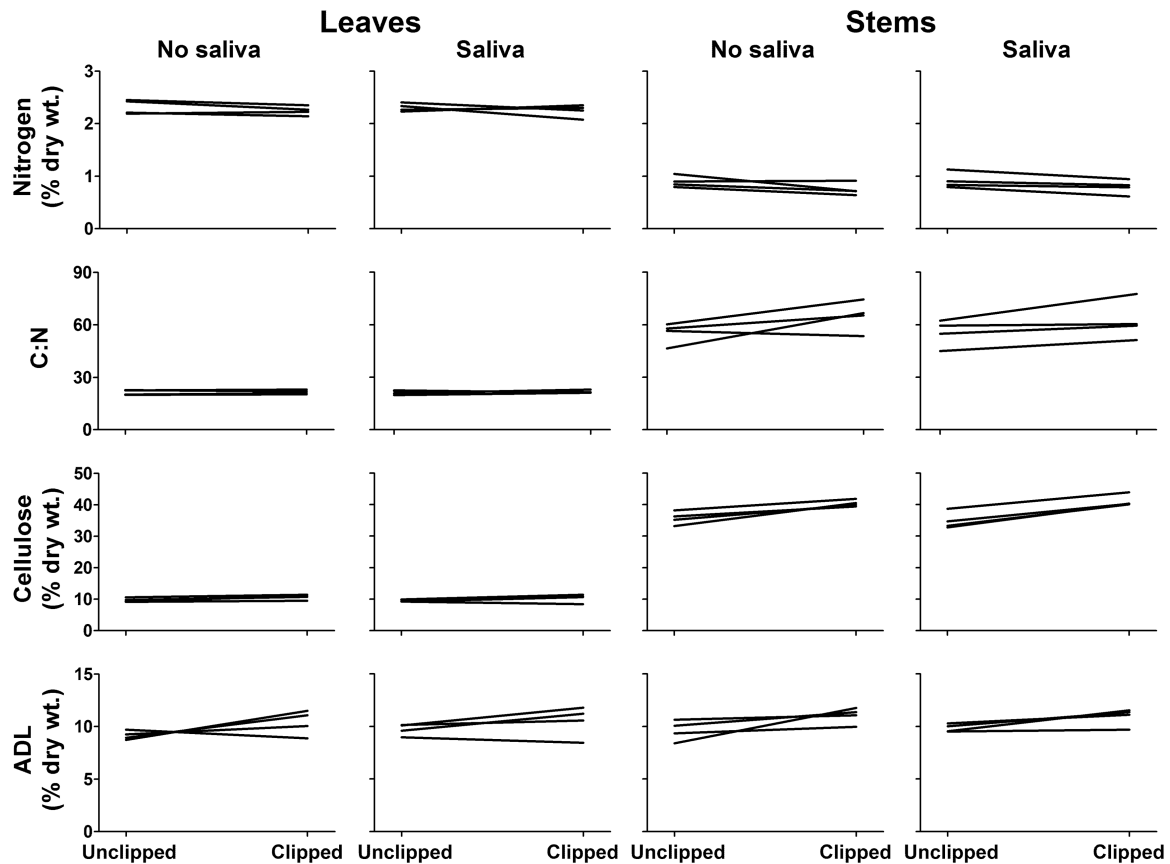
Figure 5: norm of reaction plots of foliar and stem of nitrogen (N) concentrations, carbon to nitrogen ratios (C:N), cellulose, and acid-detergent lignin (ADL). Each line represents the mean response of a single aspen genotype in the unclipped versus clipped (4–5 replicates each) condition without and with application of deer saliva. Results from statistical analyses are provided in Table 5.

Table 5: *F*-ratios and *P*-values from a three-factor ANOVA assessing the effect of clipping (C), genotype (G) and saliva (S) and their interactions on nitrogen (N), carbon to nitrogen ratio (C:N), cellulose and ADL in aspen leaves and stems

| | | N | | | | C:N | | | | Cellulose | | | | ADL | | | |
| | | Leaves | | Stems | | Leaves | | Stems | | Leaves | | Stems | | Leaves | | Stems | |
Source	df	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
Clipping	1, 141	4.6	**0.033**	36.9	**<0.001**	0.6	0.453	31.0	**<0.001**	18.8	**<0.001**	158.5	**<0.001**	7.2	**0.008**	59.9	**<0.001**
Genotype	3, 141	3.6	**0.016**	24.3	**<0.001**	8.9	**<0.001**	22.4	**<0.001**	12.0	**<0.001**	17.4	**<0.001**	3.0	**0.034**	10.5	**<0.001**
Saliva	1, 141	0.0	0.864	1.6	0.209	0.0	0.855	0.8	0.368	1.7	0.193	0.0	0.858	0.8	0.384	0.4	0.529
C × G	3, 141	3.7	**0.014**	4.7	**0.004**	3.1	**0.028**	5.3	**0.002**	3.4	**0.019**	2.7	**0.047**	2.4	0.072	9.7	**<0.001**
C × S	1, 141	0.0	0.857	0.6	0.424	0.1	0.807	1.0	0.317	0.2	0.665	3.1	0.078	0.3	0.574	1.3	0.248
G × S	3, 141	3.5	**0.017**	3.5	**0.018**	2.9	**0.037**	2.9	**0.037**	0.5	0.708	1.2	0.328	0.8	0.479	1.0	0.397
C × G × S	3, 141	0.6	0.601	1.9	0.126	1.1	0.360	2.4	0.070	1.1	0.360	0.3	0.835	0.8	0.475	2.3	0.083

Corresponding data are shown in Fig. 5. Boldface *P*-values indicate significance at alpha = 0.05.

nutritionally. Conversely, foliage had considerably higher levels of defensive compounds, including high amounts of both PGs, with greater amounts of the more toxic tremulacin, and much more CTs. Browsers face a similar situation with other tree species. Hagen-Thorn *et al.* (2004) found that the leaves of a variety of tree species, including five deciduous species and an evergreen, contained much higher

macronutrient levels than stems. In addition, Ruuhola and Julkunen-Tiitto (2000) found twice the amounts of PGs in the leaves of another salicaceous species, *Salix myrsinifolia*, compared with stems. Our results, however, did show that young aspen stems may actually represent a relatively digestible food for browsers. Although lignin percentages in mature poplar wood can range from ~20% to 30% by dry mass (Sannigrahi

Table 6: *F*-ratios and *P*-values from a three-factor repeated measures ANOVA assessing the effect of clipping (C), genotype (G) and tissue (T; leaves or stems) and their interactions on various chemical variables between aspen leaves and stems

Source	df	Salicortin		Tremulacin		CTs		Nitrogen		C:N		Cellulose		ADL	
		F	P	F	P	F	P	F	P	F	P	F	P	F	P
Clipping	1, 296	28.3	**<0.001**	145.1	**<0.001**	51.4	**<0.001**	11.1	**0.001**	31.3	**<0.001**	175.4	**<0.001**	31.0	**<0.001**
Genotype	3, 296	22.5	**<0.001**	77.0	**<0.001**	39.6	**<0.001**	4.08	**0.007**	19.4	**<0.001**	20.7	**<0.001**	7.0	**<0.001**
Tissue	1, 296	188.7	**<0.001**	1365.1	**<0.001**	1545.3	**<0.001**	20.8	**<0.001**	2388.3	**<0.001**	13431.0	**<0.001**	6.6	**0.011**
C × G	3, 296	3.6	**0.014**	2.4	0.069	7.2	**<0.001**	2.1	0.095	5.1	**0.002**	0.8	0.500	6.1	**<0.001**
C × T	1, 296	17.5	**<0.001**	65.0	**<0.001**	41.5	**<0.001**	1.7	0.190	27.6	**<0.001**	92.1	**<0.001**	0.23	0.630
G × T	3, 296	127.2	**<0.001**	155.6	**<0.001**	26.8	**<0.001**	3.54	**0.015**	20.3	**<0.001**	12.2	**<0.001**	1.1	0.360
C × G × T	3, 296	7.4	**<0.001**	0.5	0.654	3.3	**0.021**	3.8	**0.011**	5.2	**0.002**	5.0	**0.002**	1.0	0.410

Saliva treatment results were pooled for these statistical analyses. Comparison data are shown in Figs 4 and 5. Boldface *P*-values indicate significance at alpha = 0.05.

et al. 2010), we found that young aspen stems contained relatively low amounts of lignin, similar to other poplar species of the same age (Harding *et al.* 2009). Since ungulates can readily process cellulose but not lignin, new aspen stems should be a palatable food for browsers, especially for winter feeding (Bryant and Kuropat 1980).

CONCLUSIONS

We found that artificial browsing, similar to that of a large ungulate herbivore, affected both the growth and phytochemistry of aspen, and differentially so among genotypes. Deer saliva, however, showed either no or negligible effects on these parameters. Clipped trees were shorter but grew faster and had lower final biomass values with disproportionally more loss in root tissue. In addition, clipping reduced the levels of defensive compounds in leaves (CTs) and stems (PGs) and increased stem C:N, mostly due to more cellulose in clipped trees. These results show that damage caused by browsing mammals has major effects on young aspen that will influence both the developmental trajectory of individual trees and future herbivory patterns. The numerous differential genotypic responses observed also indicate that browsing can act as a strong force of natural selection, with implications for the genetic composition of aspen forests.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Plant Ecology* online.

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