

# Phytochemical traits underlie genotypic variation in susceptibility of quaking aspen (*Populus tremuloides*) to browsing by a keystone forest ungulate

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## Summary

1. Overbrowsing by ungulates is a major cause of poor aspen stand regeneration across North America and Eurasia. In general, factors driving ungulate browser preferences include concentrations of plant secondary compounds and the nutritional composition (non-structural carbohydrates, protein and minerals) of foliage. While each of these phytochemical factors has been shown to independently influence ungulate preference, the relative impact of each factor is unknown, as no study to date has examined them simultaneously.

2. Plant fitness depends not only on the capacity of plants to resist browsing, but also on their capacity to tolerate browsing once it has occurred. Little is known of aspen tolerance to browsing, which inflicts a different form of damage than insect herbivory.

3. We employed multiple aspen genotypes, replicate trees of which were subjected to different soil nutrient treatments, to investigate: (i) the effects of aspen genotype, nutrient treatment and genotype × nutrient interactions on susceptibility to browsing by white-tailed deer, (ii) the phytochemical basis for the patterns observed in (i), and (iii) the effects of genotype, soil nutrients and their interaction on short-term tolerance to deer browsing.

4. Aspen genotypes varied markedly in their vernal susceptibility to deer browsing. Genetic variation in early season levels of non-structural carbohydrates (sugars), protein and multiple macro- and trace minerals had the strongest influence on tree susceptibility to browsing. In contrast, levels of phytochemical defences had minimal effects, although the range of levels expressed in this study was small. Soil nutrient availability did not significantly influence deer preference.

5. The extent of browsing affected post-browse tolerance across genotypes. Soil nutrient treatment had little differential effect on tolerance, and, for the most part, genotypes did not display differential tolerance to browsing, regardless of which soil nutrient treatment they experienced.

6. *Synthesis.* Genetic variation for susceptibility to browsing indicates that ungulate browsers have the potential to be agents of selection in aspen populations. In contrast with previous studies in aspen highlighting the importance of phytochemical defences in shaping preferences of browsing mammals, our results indicate that the nutritional composition of foliage (sugars, protein and mineral concentrations) can have sizable effects on preference. The observed lack of influence of soil nutrient availability on tree browsing tolerance contrasts with predictions of the limiting resource model, the prevailing model for plant tolerance.

**Key-words:** Aspen, browsing, chemical defences, genetic variation, minerals, *Odocoileus virginianus*, phenolic glycosides, plant-herbivore interactions, tannins, tolerance

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## Introduction

Quaking aspen (*Populus tremuloides*) is a widely distributed, early successional, fast-growing species, adapted to many soil types (Perala 1990). Aspen plays prominent roles in the

trophic and ecosystem dynamics of early successional forests throughout much of western and north-temperate North America (Alban & Perala 1992; Pastor & Naiman 1992; Romme, Turner & Wallace 1995), as well as Eurasia (*Populus tremula*; Myking *et al.* 2011). Aspen is a quintessential 'foundation species,' creating and defining ecological communities and ecosystems (Ellison *et al.* 2005) in many forest regions.

Aspen recruitment is in decline throughout much of the United States and Scandinavia, presenting a land management issue of critical importance and public concern. The decline is multifaceted, but overbrowsing by ungulates has been documented as a major cause of poor regeneration in many areas (Romme *et al.* 2005; de Chantall, Lindberg & Kallonen 2009; Myking *et al.* 2011; Rogers, Eisenberg & St. Clair 2013; Seager, Eisenberg & St. Clair 2013; Edenius & Ericsson 2015). Deer and other ungulates are keystone herbivores, affecting forest dynamics and composition (Rooney 2001; Habeck & Schultz 2015). In the United States and parts of Europe, aspen is especially susceptible to browsing by white-tailed deer, mule deer, and cattle in spring, when tender, expanding shoots and leaves become available (DeByle 1985; Howard 1996; Painter *et al.* 2015; R. Lindroth, personal observation). White-tailed deer generally avoid aspen as a major winter food resource (Brown & Doucet 1991; Morrison, Forbes & Young 2002), although other mammals, such as hare and elk, will feed on dormant woody tissues.

Aspen genotypes vary in susceptibility to browsing; browser preferences can alter the genetic and phytochemical composition of aspen populations if damage is severe enough to affect tree fitness (Bailey *et al.* 2007). Factors driving ungulate browser preferences include concentrations of plant secondary compounds and the nutritional composition (non-structural carbohydrates, protein and minerals) of foliage. Each of these factors has been shown to independently influence ungulate preference (Weeks & Kirkpatrick 1976; Villalba & Provenza 1999; Bailey *et al.* 2007; Wooley *et al.* 2008), but the relative impact of each factor is unknown, as no study to date has examined them simultaneously.

The fitness of individual plants depends not only on the magnitude of browsing, but also on their response (tolerance) to browsing. We define tolerance as the extent to which tree fitness is affected by herbivory relative to fitness in the undamaged state (Strauss & Agrawal 1999; Stowe *et al.* 2000). Aspen genotypes differ in their tolerance to insect foliar herbivory (Stevens, Waller & Lindroth 2007). The effects of browsing, however, are fundamentally different from those of insect herbivory. Browsing removes woody tissue as well as terminal and lateral buds, thereby eliminating more of the plant's nutrient and carbohydrate reserves than does foliar herbivory (Landhäuser & Lieffers 2002). Foliar herbivory usually spares new stem growth and allows at least modest photosynthetic productivity, while browsing has more drastic effects on subsequent leaf display, photosynthesis, regrowth and plant architecture (Wu & Hinckley 2001; Keefover-Ring *et al.* 2015). Tolerance to browsing thus likely cannot be predicted based upon tolerance to foliar herbivory.

Finally, both susceptibility and tolerance to browsing may be influenced by resource availability, with variation among genotypes in the magnitude and/or direction of responses to particular resources. Nutrient stress imposed by low soil fertility can alter multiple aspects of foliar quality, including levels of plastic phytochemical defences and nutrients, while also changing patterns of biomass allocation within a plant (Gershenson 1984; Haukioja *et al.* 1998; Donaldson, Kruger & Lindroth 2006a). Tolerance to browsing might also be influenced by soil nutrient availability. The prevailing model for plant tolerance is the limiting resource model, which predicts that if a resource limiting plant fitness (the 'focal resource') differs between soil nutrient treatments, and the type of herbivore damage incurred affects the acquisition or use of that resource, then plant tolerance will be higher in environments with higher soil nutrient availability (Wise & Abrahamson 2005, 2007).

In our study, we employed multiple aspen genotypes, replicate trees of which were subjected to different soil nutrient treatments, to investigate: (i) the effects of aspen genotype, nutrient treatment and genotype  $\times$  nutrient interactions on susceptibility to browsing, (ii) the phytochemical basis for the patterns observed in (i), and (iii) the effects of genotype, soil nutrients and their interaction on short-term tolerance to deer browsing.

## Materials and methods

We investigated the effects of aspen genotype, environment (soil nutrient treatment) and genotype  $\times$  environment interactions on tree susceptibility and short-term tolerance to browsing. Individually potted aspen trees of seven replicated genotypes grown under either low or high soil nutrient availability were deployed to a site frequented by white-tailed deer (*Odocoileus virginianus*). Thirteen days after deployment, when all replicate trees of the most susceptible genotype and soil nutrient availability treatment were heavily browsed, we removed all experimental trees from the browse location and assessed the extent of browse damage to each tree. We related variation in genotypic browse susceptibility to genotype values for phytochemical parameters. Three months after the preference trials, at the end of the growing season, we compared tolerance to browsing among genotypes and soil nutrient treatments.

### TREE PROPAGATION AND NUTRIENT TREATMENT

We generated replicate trees of each of the seven aspen genotypes used in this study via tissue culture (Sellmer, McCown & Haissig 1989; Donaldson, Kruger & Lindroth 2006a) from trees of known genotypes maintained in a common garden at the University of Wisconsin-Madison (UW). Genotypes were originally collected from natural aspen stands in south-central Wisconsin, and were confirmed to be unique by microsatellite analysis (Cole 2005). We established micro-cuttings in D-pots (5 cm diameter; 30 cm depth) with a 50:50 mix of sand and potting medium (MetroMix, Conrad Fafard Inc., Agawam, MA, USA) in late July of 2010, thinning trees to one main stem per tree upon planting. Trees were fertilized once per week with soluble J.R. Peters fertilizer (Allentown, PA; 21:8:18 N-P-K) until leaf senescence in mid-September, and were overwintered in the University of Wisconsin greenhouse. We moved the potted trees outside in May

2011. In June 2011, we transplanted the trees into 11.5 L pots with a 1:2 field soil:sand ratio. Trees were paired within a genotype; each genotype pair was randomly positioned within an outdoor growth arena. Within each genotype pair, one tree was assigned to a 'low nutrient availability' treatment, and received 0.5 g of Scott's<sup>®</sup> Osmocote [18:6:12 N-P-K slow release (8–9 months) fertilizer] per 1 L of soil (5.75 g per tree). The other tree of each pair was assigned to a 'high nutrient availability' treatment, and received 4.5 g of Osmocote fertilizer per 1 L of soil (51.75 g per tree). Trees overwintered in the outdoor growth arena and were fertilized again in March 2012. Trees were watered as necessary throughout the experiment.

#### BROWSE PREFERENCE EXPERIMENT

We deployed 144 potted, experimental trees ( $10 \pm 2$  trees representing each genotype  $\times$  soil nutrient treatment combination) to the test site at the University of Wisconsin Arlington Research Station within 3 weeks after budbreak in 2012 (mid-April). At this time, each tree consisted of a single main stem and associated 'short shoots' (proleptic branches) derived from the lateral and terminal stem buds. The following non-destructive physical measurements were taken for each tree prior to deployment: basal diameter ( $d$ ), height ( $h$ , root crown to base of apical bud), number of woody branches, number of short shoots (including expanding buds that would become short shoots in the next 14 days), and average number of leaves per short shoot. Tree heights ranged from 35 to 124 cm; the entire crowns of all trees were thus well within the browse zone for white-tailed deer. We transported trees to the deployment site in a cargo truck with an enclosed bed. Trees were set up at the deployment site in two staggered, adjacent rows (~2.5 m spacing between trees) with a non-experimental tree at the end of each row, thus providing uniform deer access to each tree. Trees were placed in a grass field along the southern edge of a poplar plantation. Habitat types in this portion of the research station included alfalfa, corn and soybean fields, as well as hardwood plantations. We placed game cameras at both ends of the experimental tree rows and recorded multiple deer feeding on the experimental trees.

The natural browsing treatment was terminated 13 days after initial deployment, when all replicate trees of the most susceptible genotype/soil treatment were heavily damaged. Trees were returned to their original growth arena on the UW campus, whereupon we assessed the number of short shoots removed by browsing (number of short shoots pre-browse – number of short shoots post-browse), number of leaves removed by browsing (number of short shoots removed  $\times$  average number of leaves per short shoot for that tree, pre-browse), and presence—absence of leader shoot browsing.

Foliar chemical analyses required destructive harvest of leaves. An additional five trees per genotype  $\times$  soil nutrient treatment were randomly selected to remain at the UW growth arena, rather than be deployed to the test site, for these analyses. We removed multiple entire short shoots from each of the trees 5 days after the experimental trees were deployed, and flash-froze the tissue in liquid nitrogen. Frozen foliar tissue was subsequently freeze-dried, finely ground using a Wiley mill (mesh size #40), and stored at  $-20$  °C.

We analysed short-shoot samples for multiple metrics of foliar quality: concentrations of phytochemical defences (phenolic glycosides and condensed tannins), structural carbohydrates (fiber and lignin), nitrogen (an index of protein), non-structural carbohydrates (sugars and starch) and minerals. We categorize minerals as macrominerals (calcium, phosphorus, potassium, magnesium, sodium and sulphur) and trace minerals (boron, copper, iron, manganese and zinc) (Robbins 1983). Phenolic glycosides (salicin, salicortin and

tremulacin; also known as 'salicinoids') and condensed tannins are the main secondary metabolites in *Populus* (Palo 1984; Lindroth, Hsia & Scriber 1987; Rehill *et al.* 2005, 2006). We assessed phenolic glycosides using ultra performance liquid chromatography (UPLC) with purified aspen phenolic glycosides as standards. Salicin standard was obtained from Sigma-Aldrich, while salicortin and tremulacin were purified from aspen tissue in our laboratory. We report 'total phenolic glycosides' as the sum of the individual components (salicin, salicortin and tremulacin). The acid butanol assay was used to assess condensed tannin concentration (Porter, Hrstich & Chan 1986) with purified aspen condensed tannins as standards. Fibre (acid detergent fiber, ADF; lignin + cellulose) and lignin (acid detergent lignin, ADL) were assayed by sequential extraction in hot acid detergent using an Ankom 200 Digester (ANKOM Technology Corporation, New York, NY, USA). Nitrogen was quantified using a Thermo Finnigan Flash 1112 elemental analyzer (Thermo Finnigan, San Jose, CA, USA). Starch and sugars (sucrose + hexose) concentrations were determined via the dinitrosalicylic acid method as described by Lindroth *et al.* (2002). Elemental analyses to determine mineral concentrations were conducted via Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) by the University of Wisconsin Soil and Plant Analysis Laboratory. We report the results of each chemical assay as percent (%) dry weight or  $\text{mg kg}^{-1}$  dry weight.

#### BROWSE TOLERANCE EXPERIMENT

To assess tree tolerance to deer browsing, we compared fitness (growth) of trees with varying extents of browse damage (0–96% of short shoots removed) within each genotype  $\times$  soil nutrient treatment 3 months after the browse preference trials occurred. To provide a non-damaged basis for comparison, we deployed an extra set of 'control' trees (0% browsed) for this experiment to a similar site at the Arlington research station that was not frequented by deer, simultaneously with deployment of the browse preference trees. After their 13-day deployment, the control trees, along with the browse preference trees, were returned to the UW growth arena for the duration of the study. All trees (control and browsed) were intermixed within the UW growth arena prior to and after their 13-day deployment.

For fitness measurements, we destructively harvested each tree, measuring multiple aspects of growth and biomass allocation: number and dry biomass of all woody branches, leaf number, foliar biomass, number of (new) suckers (root sprouts), aboveground biomass, belowground biomass (root dry mass), aboveground:belowground biomass ratio and total biomass.

We define tolerance as the extent to which plant fitness (i.e. growth) is affected by browse damage, relative to fitness in the undamaged state. Tolerance reflects the ability of a plant to sustain growth or reproduction following damage. Tolerance must necessarily be measured from a group of related individuals, generally genotype replicates (Fornoni *et al.* 2004) subjected to different levels of damage, as the fitness of a single individual cannot be assessed in both damaged and undamaged states. We measured tolerance as the slope of the function relating plant fitness to level of damage (Strauss & Agrawal 1999; Stowe *et al.* 2000).

#### STATISTICAL ANALYSIS

All data were assessed prior to statistical analyses to confirm that they met assumptions of normality, and were transformed if necessary. Statistical analyses were performed using JMP Pro v.11 statistical software (SAS Institute Inc., Cary, NC, USA) or R (www.r-project.org).

We used a series of general linear models (e.g. analyses of variance) to evaluate the effect of genotype (fixed factor), soil nutrient availability (fixed factor) and their interaction on: (i) the numbers of short shoots and leaves prior to browsing, (ii) phytochemical concentrations, and (iii) deer browse preference (number of short shoots or leaves removed, proportion of short shoots or leaves removed). Chi-square tests were used to assess the effects of genotype and soil nutrient availability on whether tree leader shoots were browsed.

We assessed the relative contributions of phytochemical concentrations to deer browsing preference using partial least squares regression analysis (PLSR; Wold *et al.* 1984; Wold, Sjöström & Eriksson 2001), as described in detail in Couture, Meehan & Lindroth (2012). PLSR is a useful alternative to multiple linear regression when predictor variables, such as our foliar quality parameters, are correlated with one another, violating critical assumptions of multiple linear regression models. We determined the number of latent variables used in the model based on reduction of the predictive residual error sums of squares (PRESS) statistic, employing a random 70/30% calibration/validation data split over 500 permutations. We then assessed coefficient stability, again by using a 70/30% calibration/validation data split and the same number of iterations, producing an error distribution of the influence of the individual variable on deer browsing. Coefficients overlapping zero were considered to have no influence on deer browsing.

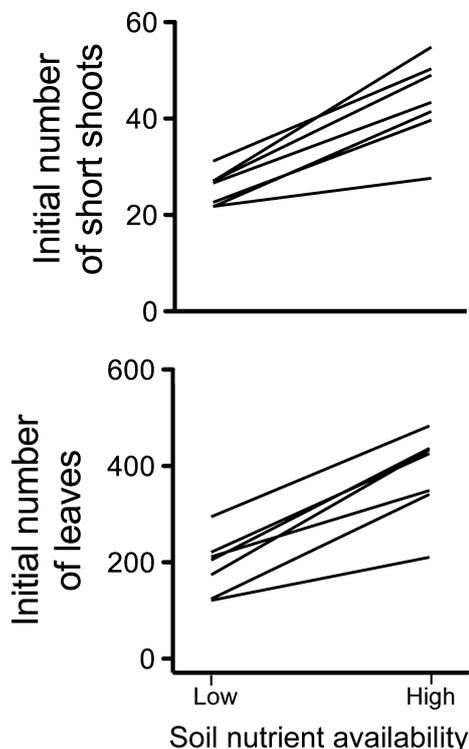
We used a series of general linear models (e.g. analyses of covariance) to determine whether tolerance to deer browsing differed among genotypes or between soil nutrient treatments. We assessed the effects of tree genotype (fixed factor), soil nutrient availability (fixed factor), proportion of short shoots removed by browsing (covariate) and a relevant aspect of tree pre-browse size (covariate), such as the square of basal diameter  $\times$  height ( $d^2h$ ), on each tree growth trait (response variable). We also characterized the effects of the interactions between genotype and browsing (proportion of short shoots removed), soil nutrient availability and browsing, and the three-way interaction between genotype, nutrient availability and browsing on each tree growth trait. In the model for each growth trait, differences in tolerance among genotypes, for example, will manifest as differences among genotypes in the slope of the regression of the extent of browse damage on the growth trait, resulting in a significant interaction between the proportion of short shoots removed and genotype.

## Results

### BROWSE PREFERENCE EXPERIMENT

#### *Tree parameters*

Both genotype and soil nutrient availability influenced the amount of tissue available for browsing (Fig. 1, Tables 1 and S1). Numbers of short shoots varied among genotypes by 1.4 and 2 fold, respectively, under low and high nutrient availability. Similarly, total number of leaves per tree varied among genotypes by 2.2 and 2.1 fold, respectively, under the two nutrient regimes. Averaged across genotypes, the high nutrient treatment increased short shoot production by 2.3 fold and leaf production by 2.2 fold, relative to the low nutrient treatment. Overall, genotypes responded similarly to the



**Fig. 1.** Norm of reaction plots illustrating the amount of tissue available for browsing across soil nutrient treatments. Lines represent genotype means.

nutrient treatments (non-significant genotype  $\times$  nutrient interactions).

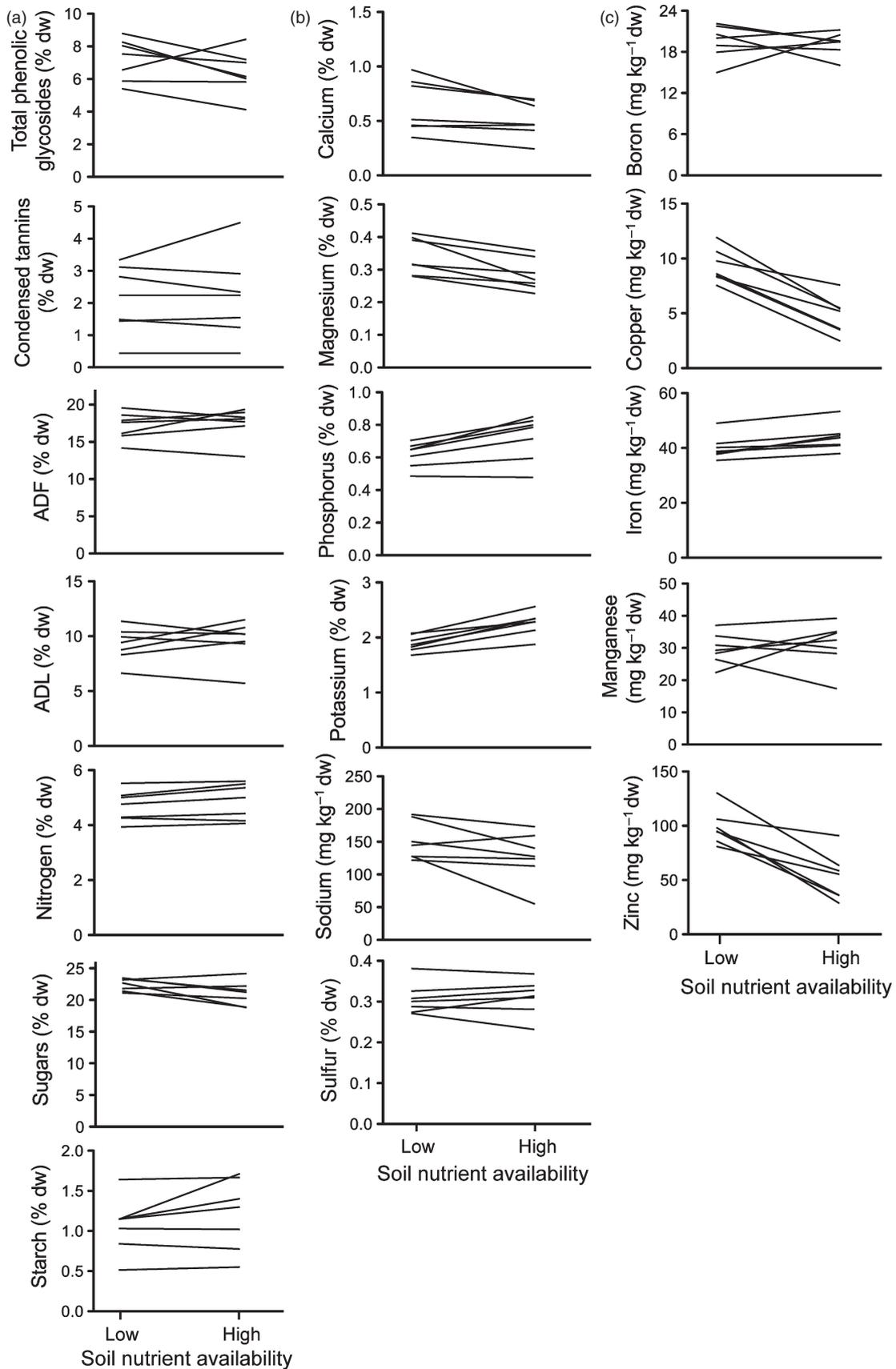
Aspen genotypes differed in multiple aspects of foliage quality, measured as concentrations of phytochemicals in short shoots, while soil nutrient availability had less influence (Fig. 2, Tables 1 and S1). Among genotypes, phytochemical defences (phenolic glycosides and condensed tannins) varied by 2.5–5.5 fold; defences did not differ significantly between soil nutrient treatments. Structural carbohydrates and nitrogen varied by greater than two-fold among genotypes, but were not significantly affected by soil treatment. Non-structural carbohydrates varied by 1.9–6 fold across genotypes, and by 1.1 fold across soil treatments. Macrominerals varied by 2–5.5 fold, and trace minerals by 1.9–3.5 fold, among genotypes. Compared to those in the high nutrient treatment, concentrations of calcium and copper were 1.2- to 2-fold higher in short shoots of trees grown in the low soil nutrient treatment, while levels of potassium were, on average, 20% lower in the low nutrient treatment (Table S1). In general, phytochemical responses to changes in soil nutrient availability were consistent across genotypes. Magnesium and zinc were the only constituents to exhibit significant genotype  $\times$  nutrient interactions.

#### *Deer browse preference*

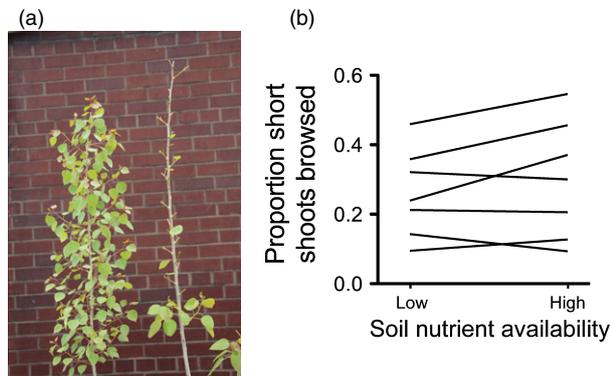
Overall, aspen genotype had a much greater impact on our multiple metrics of deer preference than did soil nutrient

**Table 1.** Results of analyses of variance demonstrating the effects of genotype, soil nutrient availability and their interaction on initial tree traits. Factors with significant effects are in bold type

Trait	Factor	d.f. (numerator, denominator)	F statistic	P
Initial number of short shoots (ss)	<b>Genotype</b>	6, 130	2.94	0.010
	<b>Soil nutrient availability</b>	1, 130	49.36	< 0.001
	Genotype × soil nutrient availability	6, 130	0.92	0.486
Initial number of leaves	<b>Genotype</b>	6, 130	5.07	< 0.001
	<b>Soil nutrient availability</b>	1, 130	60.96	< 0.001
	Genotype × soil nutrient availability	6, 130	0.82	0.557
Total phenolic glycosides	<b>Genotype</b>	6, 55	3.32	0.007
	Soil nutrient availability	1, 55	3.33	0.073
	Genotype × soil nutrient availability	6, 55	1.35	0.251
Condensed tannins	<b>Genotype</b>	6, 55	8.19	< 0.001
	Soil nutrient availability	1, 55	0.61	0.438
	Genotype × soil nutrient availability	6, 55	0.90	0.505
ADF	<b>Genotype</b>	6, 55	5.48	< 0.001
	Soil nutrient availability	1, 55	0.43	0.516
	Genotype × soil nutrient availability	6, 55	1.03	0.414
ADL	<b>Genotype</b>	6, 55	6.84	< 0.001
	Soil nutrient availability	1, 55	0.52	0.476
	Genotype × soil nutrient availability	6, 55	1.29	0.277
Nitrogen	<b>Genotype</b>	6, 55	21.05	< 0.001
	Soil nutrient availability	1, 55	0.56	0.065
	Genotype × soil nutrient availability	6, 55	0.46	0.82
Sugars	<b>Genotype</b>	6, 55	3.81	0.003
	<b>Soil nutrient availability</b>	1, 55	8.78	0.005
	Genotype × soil nutrient availability	6, 55	1.80	0.117
Starch	<b>Genotype</b>	6, 55	16.79	< 0.001
	<b>Soil nutrient availability</b>	1, 55	5.11	0.028
	Genotype × soil nutrient availability	6, 55	2.12	0.066
Aluminium	Genotype	6, 45	0.617	0.716
	Soil nutrient availability	1, 45	0.002	0.965
	Genotype × soil nutrient availability	6, 45	0.919	0.490
Boron	Genotype	6, 45	1.19	0.330
	Soil nutrient availability	1, 45	0.08	0.776
	Genotype × soil nutrient availability	6, 45	1.59	0.172
Calcium	<b>Genotype</b>	6, 45	21.29	< 0.001
	<b>Soil nutrient availability</b>	1, 45	12.55	< 0.001
	Genotype × soil nutrient availability	6, 45	2.08	0.074
Copper	<b>Genotype</b>	6, 45	2.73	0.024
	<b>Soil nutrient availability</b>	1, 45	39.12	< 0.001
	Genotype × soil nutrient availability	6, 45	0.62	0.711
Iron	<b>Genotype</b>	6, 45	3.16	0.011
	Soil nutrient availability	1, 45	2.98	0.091
	Genotype × soil nutrient availability	6, 45	0.12	0.993
Magnesium	<b>Genotype</b>	6, 45	16.75	< 0.001
	<b>Soil nutrient availability</b>	1, 45	37.10	< 0.001
	<b>Genotype × soil nutrient availability</b>	6, 45	2.53	0.034
Manganese	<b>Genotype</b>	6, 45	3.96	0.003
	Soil nutrient availability	1, 45	0.58	0.451
	Genotype × soil nutrient availability	6, 45	2.25	0.056
Phosphorus	<b>Genotype</b>	6, 45	14.01	0.007
	Soil nutrient availability	1, 45	20.89	0.073
	Genotype × soil nutrient availability	6, 45	1.18	0.251
Potassium	<b>Genotype</b>	6, 45	4.56	0.001
	<b>Soil nutrient availability</b>	1, 45	30.87	< 0.001
	Genotype × soil nutrient availability	6, 45	0.67	0.677
Sodium	Genotype	6, 45	2.05	0.075
	Soil nutrient availability	1, 45	1.16	0.286
	Genotype × soil nutrient availability	6, 45	0.25	0.955
Sulphur	<b>Genotype</b>	6, 45	10.85	< 0.001
	Soil nutrient availability	1, 45	0.11	0.744
	Genotype × soil nutrient availability	6, 45	0.93	0.483
Zinc	<b>Genotype</b>	6, 45	8.61	< 0.001
	<b>Soil nutrient availability</b>	1, 45	101.46	< 0.001
	<b>Genotype × soil nutrient availability</b>	6, 45	3.62	0.005



**Fig. 2.** Norm of reaction plots for phytochemical composition of foliage across soil nutrient treatments. Column (a) includes phytochemical defences, structural carbohydrates, nitrogen and non-structural carbohydrates. Column (b) includes macrominerals, and Column (c) includes trace minerals. Lines represent genotype means.



**Fig. 3.** The effects of genotype on browse damage. Panel (a) shows a lightly browsed tree from a non-preferred genotype (left) and a heavily browsed tree from a preferred genotype (right). Panel (b) illustrates browse damage for genotypes (lines represent genotype means) across soil nutrient treatments.

availability (Fig. 3; Tables 2 and S2). Damage rates varied among genotypes by 7.9 fold and 8.4 fold for numbers of short shoots and leaves browsed respectively. Similarly, damage rates varied by 4.6 fold and 4.0 fold for proportions of short shoots and leaves browsed respectively. High soil nutrient availability increased damage rates by just over 100% for total numbers of both short shoots and leaves removed (number of short shoots removed ranged from 6.9 to 14.2 in the low vs. high nutrient treatments, while number of leaves removed ranged from 63.2 to 134.0), but did not significantly affect proportions of short shoots or leaves removed. Soil nutrient availability did not differentially affect short shoot or leaf removal among genotypes (no significant genotype  $\times$  soil nutrient availability interactions, Table 1). Rates of terminal leader damage varied from 33 to 90% among genotypes ( $\chi^2 = 27.22$ , d.f. = 6, 13,  $P < 0.001$ ), but were unaffected by nutrient availability ( $\chi^2 = 0.26$ , d.f. = 1, 13,  $P = 0.61$ ). Soil nutrient treatment did not differentially affect whether the terminal leader was browsed among genotypes ( $\chi^2 = 8.02$ , d.f. = 6, 13,  $P = 0.24$ ).

We used partial least squares regression to identify factors underlying genotypic differences in browse preference. All independent foliar quality variables that differed among genotypes, as well as one (sodium) that differed marginally among genotypes, were included in our final PLSR model. For the response variable in this analysis, we used the proportion of short shoots removed, as proportions (rather than total numbers) provide the best standardized measure of preference across trees with different amounts of tissue available.

A PLSR model relating the proportion of short shoots removed to phytochemical defences, structural carbohydrates, protein (measured as N), non-structural carbohydrates and minerals produced a significant relationship between the observed and predicted responses (mean calibration  $R^2 = 0.89$ , range across 500 iterations = 0.71–0.99;  $P < 0.001$ ). Levels of nitrogen, and the trace mineral zinc had the strongest negative relationships with deer browse preference (Table S3). Concentrations of sugars, as well as

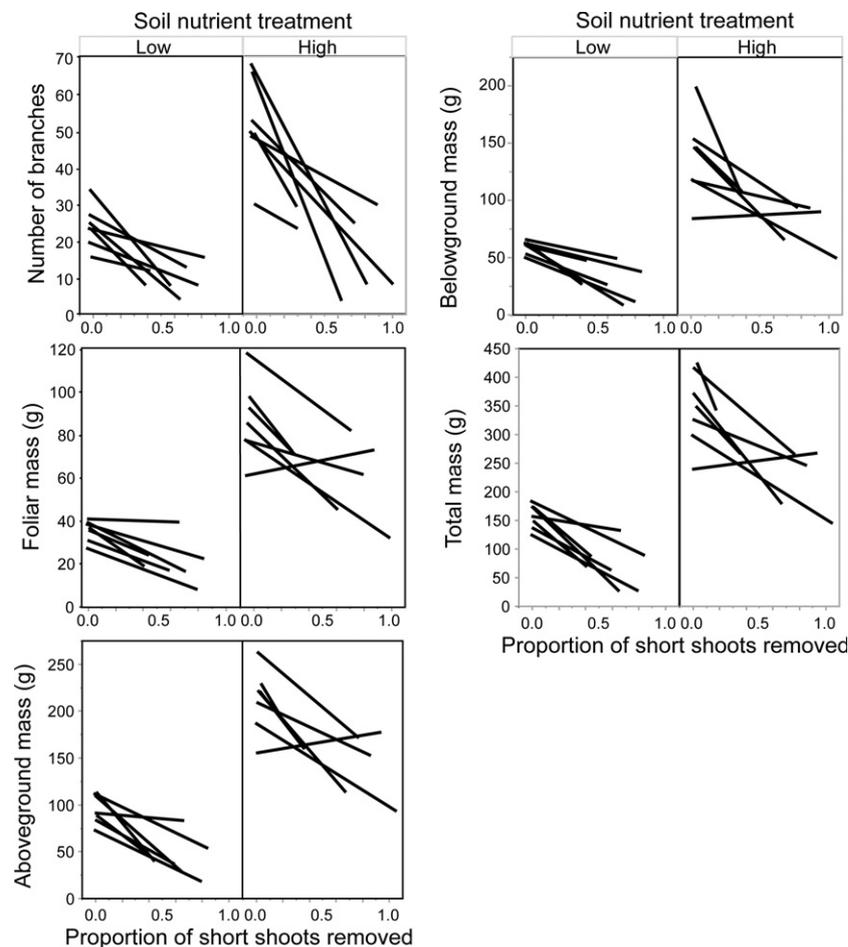
**Table 2.** Results of analyses of variance demonstrating the effects of genotype, soil nutrient availability and their interaction on four metrics of browse preference. Factors with a significant effect on the response variable are in bold type

Trait	Factor	d.f. (numerator, denominator)	<i>F</i> statistic	<i>P</i>
Number of ss browsed	<b>Genotype</b>	6, 130	10.22	< 0.001
	<b>Soil nutrient availability</b>	1, 130	16.89	< 0.001
	Genotype $\times$ soil nutrient availability	6, 130	1.91	0.084
Number of leaves browsed	<b>Genotype</b>	6, 130	10.00	< 0.001
	<b>Soil nutrient availability</b>	1, 130	17.71	< 0.001
	Genotype $\times$ soil nutrient availability	6, 130	1.68	0.130
Proportion of ss browsed	<b>Genotype</b>	6, 130	8.12	< 0.001
	Soil nutrient availability	1, 130	1.01	0.316
	Genotype $\times$ soil nutrient availability	6, 130	0.44	0.864
Proportion of leaves browsed	<b>Genotype</b>	6, 130	7.22	< 0.001
	Soil nutrient availability	1, 130	0.02	0.884
	Genotype $\times$ soil nutrient availability	6, 130	0.68	0.664

the macrominerals calcium, magnesium, potassium, and sulphur, and the trace mineral iron, showed the strongest positive relationships with deer browse preference (Table S3).

#### BROWSE TOLERANCE EXPERIMENT

We assessed tolerance as the extent to which tree fitness (i.e. multiple aspects of biomass growth and allocation) was affected by browse damage (proportion of short shoots removed) (Fig. 4; Tables 3 and S4). The proportion of leader shoots browsed was correlated with proportion of short shoots removed, and thus yielded similar tolerance results (data not shown). Overall, browsing reduced the number of branches, as well as foliar mass, above- and belowground mass and total biomass. The responses to browsing were similar among genotypes (non-significant interaction terms for proportion of short shoots removed  $\times$  genotype, Table 3) for all tolerance traits except production of woody branches. Trees in the two soil nutrient treatments responded similarly to browsing (non-significant interaction terms for proportion of short shoots removed  $\times$  soil nutrient treatment, Table 3), for all traits except number of branches, indicating that there was little differential tolerance to browsing between the nutrient treatments. Moreover, the effect of soil treatment on tolerance did



**Fig. 4.** Norm of reaction plots for multiple metrics of tolerance to browse damage across soil nutrient treatments. Lines represent genotype means.

not differ *across* genotypes, as indicated by the lack of significant browse  $\times$  genotype  $\times$  soil nutrient interactions (Table 3).

## Discussion

Aspen genotypes varied markedly in their susceptibility to deer browsing. Genotypic variation in the levels of non-structural carbohydrates (sugars), protein, and multiple macro- and trace minerals had the strongest influence on tree susceptibility to deer browsing, while early season levels of phytochemical defences had minimal effects. Soil nutrient treatment did not significantly influence deer preference. The magnitude of deer browsing influenced tree growth across genotypes, as did soil nutrient availability. However, for the most part, genotypes did not display differential tolerance to browsing, regardless of soil nutrient availability, and soil nutrient treatment generally did not differentially affect tolerance.

### EFFECTS OF GENOTYPE, ENVIRONMENT AND GENOTYPE $\times$ ENVIRONMENT INTERACTIONS ON PLANT TRAITS

We found genetic variation for concentrations of phytochemical defences, structural and non-structural carbohydrates, nitrogen and most minerals. With the exception of minerals, for which genetic variation in aspen has not been previously

characterized, these results are consistent with previous studies (e.g., Hwang & Lindroth 1997; Donaldson, Kruger & Lindroth 2006a; Donaldson *et al.* 2006b; Osier & Lindroth 2006; Stevens, Waller & Lindroth 2007; Keefover-Ring *et al.* 2015). However, the range of concentrations for phenolic glycosides, condensed tannins, sugars, and starch in our study is considerably smaller than in previous studies, which used some of the same genotypes. In addition, concentrations of phenolic glycosides, condensed tannins, and starch in our study are on the low end of the ranges found previously for aspen of a similar age, while our nitrogen values are on the high end of those ranges. Sugar levels are comparable with previous work (Donaldson, Kruger & Lindroth 2006a; Donaldson *et al.* 2006b; Osier & Lindroth 2006; Stevens, Waller & Lindroth 2007). Aspen phytochemistry exhibits seasonal variation; temporal changes in aspen phenolic glycoside concentrations are strongly dependent upon genotype, while levels of condensed tannins increase and levels of nitrogen decrease across genotypes as a season progresses (Osier, Hwang & Lindroth 2000). The difference between this study and most previous studies was leaf age; with the exception of the first sampling date in Osier, Hwang & Lindroth (2000), previous aspen work was conducted on fully expanded leaves. Results from this study indicate that genetic variation in levels of chemical defences may be reduced in young, expanding leaves, relative to mature leaves, of aspen.

**Table 3.** Results of analyses of covariance demonstrating the effects of genotype, soil nutrient availability, the proportion of short shoots (prop. ss) removed by deer browsing, and their interactions, on tree tolerance traits measured 3 months after browsing. Initial trait values (initial  $d^2h$ , branch number, leaf number) relevant to the response variable are included as covariates. Factors with a significant effect on the response variable are in bold type. Significant interaction terms for proportion of short shoots removed  $\times$  genotype indicate differential tolerance to browsing among genotypes for a particular fitness trait; significant proportion of short shoots removed  $\times$  nutrient availability interactions indicate differential tolerance to browsing between the nutrient treatments

Trait	Factor	d.f. (numerator, denominator)	F statistic	P
Number of woody branches	<b>Genotype</b>	6, 102	2.81	0.014
	<b>Soil nutrient availability</b>	1, 102	63.79	< 0.001
	<b>Initial # woody branches</b>	1, 102	15.66	< 0.001
	<b>Proportion short shoots (prop. ss) removed</b>	1, 102	31.75	< 0.001
	Genotype $\times$ soil nutrient availability	6, 102	0.82	0.558
	<b>Prop. ss removed <math>\times</math> genotype</b>	6, 102	2.39	0.033
	<b>Prop. ss removed <math>\times</math> soil nutrient availability</b>	1, 102	7.45	0.008
	Prop. ss removed $\times$ genotype $\times$ soil nutrient availability	6, 102	0.42	0.864
Foliar mass	<b>Genotype</b>	6, 116	14.56	< 0.001
	<b>Soil nutrient availability</b>	1, 116	180.37	< 0.001
	<b>Initial number of leaves</b>	1, 116	34.44	< 0.001
	<b>Proportion short shoots (prop. ss) removed</b>	1, 116	23.92	< 0.001
	<b>Genotype <math>\times</math> soil nutrient availability</b>	6, 116	4.12	< 0.001
	Prop. ss removed $\times$ genotype	6, 116	1.26	0.284
	Prop. ss removed $\times$ soil nutrient availability	1, 116	1.43	0.234
	Prop. ss removed $\times$ genotype $\times$ soil nutrient availability	6, 116	1.78	0.110
Aboveground biomass	Genotype	6, 92	1.83	0.102
	<b>Soil nutrient availability</b>	1, 92	34.11	< 0.001
	<b>Initial <math>d^2h</math> (log)</b>	1, 92	34.71	< 0.001
	<b>Proportion short shoots (prop. ss) removed</b>	1, 92	19.17	< 0.001
	Genotype $\times$ soil nutrient availability	6, 92	1.97	0.078
	Prop. ss removed $\times$ genotype	6, 92	0.94	0.468
	Prop. ss removed $\times$ soil nutrient availability	1, 92	2.79	0.098
	Prop. ss removed $\times$ genotype $\times$ soil nutrient availability	6, 92	0.70	0.647
Belowground biomass	<b>Genotype</b>	6, 104	3.85	0.002
	<b>Soil nutrient availability</b>	1, 104	15.95	< 0.001
	<b>Initial <math>d^2h</math> (log)</b>	1, 104	50.00	< 0.001
	<b>Proportion short shoots (prop. ss) removed</b>	1, 104	23.45	< 0.001
	<b>Genotype <math>\times</math> soil nutrient availability</b>	6, 104	2.52	0.025
	Prop. ss removed $\times$ genotype	6, 104	1.04	0.403
	Prop. ss removed $\times$ soil nutrient availability	1, 104	2.66	0.106
	Prop. ss removed $\times$ genotype $\times$ soil nutrient availability	6, 104	0.91	0.488
Total biomass	<b>Genotype</b>	6, 92	2.21	0.049
	<b>Soil nutrient availability</b>	1, 92	29.05	< 0.001
	<b>Initial <math>d^2h</math> (log)</b>	1, 92	40.11	< 0.001
	<b>Proportion short shoots (prop. ss) removed</b>	1, 92	20.86	< 0.001
	Genotype $\times$ soil nutrient availability	6, 92	1.51	0.182
	Prop. ss removed $\times$ genotype	6, 92	0.97	0.447
	Prop. ss removed $\times$ soil nutrient availability	1, 92	0.98	0.325
	Prop. ss removed $\times$ genotype $\times$ soil nutrient availability	6, 92	1.01	0.423

Our results showed a general lack of response of phytochemical defences, structural carbohydrates, and nitrogen to soil nutrient treatment, and a variable response of minerals to nutrient treatment. Nutrient augmentation led to only marginal increases in foliar nitrogen and decreases in total phenolic glycosides, and did not affect condensed tannin concentrations. We attribute the minimal effect of nutrient availability on foliar nitrogen to the mobilization of stored N into new leaf tissue, captured by our early sampling date. Although the nutrient treatment had strongly affected prior tree growth (Fig. 1), foliar levels of N were uniformly high across genotypes and nutrient treatments in young, expanding shoots. Corresponding with this general lack of plasticity, we also

found few genotype  $\times$  environment interactions for phytochemical traits. These results contrast with previous published studies of aspen, where nitrogen and condensed tannins were generally plastic in response to soil nutrient treatments similar to those we used (Donaldson, Kruger & Lindroth 2006a; Osier & Lindroth 2006; Stevens, Waller & Lindroth 2007).

No prior work has addressed the effects of genotype and soil nutrient availability on mineral levels in aspen. To confirm that results we obtained reflect levels in natural aspen stands in the study region, we analysed foliar minerals in six aspen clones from southern Wisconsin. The natural range of concentrations for each mineral overlapped strongly with the range in our potted trees (unpublished data).

#### INTRA- AND INTER-SPECIES VARIATION IN BROWSE PREFERENCE

Our observation of genetic variation for aspen susceptibility to browsing is similar to that of two previous studies with aspen (Bailey *et al.* 2007; Wooley *et al.* 2008), although foliar parameters corresponding to browser preference differed between those studies and ours. Both Bailey *et al.* (2007) and Wooley *et al.* (2008) explored the influence of phytochemical defences on browse preference and, in contrast to our results, found strong negative relationships between levels of phenolic glycosides and ungulate browser preference. In those studies, however, concentrations of phenolic glycosides were much higher than in ours, and feeding deterrence was minimal at levels less than 15% dry weight (Wooley *et al.* 2008). In addition, neither of the previous studies found a significant effect of condensed tannin concentrations on browser preference, and neither assessed foliar nutritional quality (structural and non-structural carbohydrates or minerals). Studies of within-species genetic variation on browse susceptibility in other plant species have focused on the effects of phytochemical defences rather than nutritional quality, as well (Bryant *et al.* 1991; Vourc'h, Russell & Martin 2002; Kimball, Russell & Ott 2012).

Several studies of inter-species variation in ungulate browse susceptibility have tracked species distributions and foliar traits of plants found inside and outside of long-term browser exclosures (e.g., Wardle, Bonner & Barker 2002; Mason *et al.* 2010; Wigley *et al.* 2014). As with studies of browse susceptibility within species, phytochemical defences have been the traits most commonly measured, often along with nitrogen and phosphorus. A comparison of vegetation density and litter traits inside and outside of fenced exclosures in New Zealand demonstrated that both red deer (*Cervus elaphus scoticus* Lonnberg) and feral goats (*Capra hircus* L.) preferred plant species with relatively lower concentrations of phenolics, condensed tannins and lignin, while levels of nitrogen and phosphorus did not affect browse preference (Wardle, Bonner & Barker 2002). Likewise, study of vegetative traits inside and outside of exclosures in southern African savannas showed trends for elevated levels of total phenolics and lignin outside of the exclosures, compared to inside them (Wigley *et al.* 2014). This study was one of few that assessed minerals other than phosphorus, but no differences were found in concentrations of any constituent (calcium, magnesium, phosphorus, potassium and sodium) between the interior and exterior of the exclosures (Wigley *et al.* 2014).

#### FACTORS INFLUENCING BROWSER PREFERENCE

An abundance of evidence suggests that ungulates feed selectively, with selection based on the quality (rather than merely abundance) of forage materials, as determined by foliar taste, odour and appearance (Weckerly 1994; Provenza *et al.* 2003; Iason & Villalba 2006; Burney & Jacobs 2013). Foraging theory predicts that browsers will minimize intake of toxic compounds and/or digestibility reducers such

as phytochemical defences and structural carbohydrates, while maximizing intake of highly nutritional components such as non-structural carbohydrates and protein (Geist 1982; Jones & Weeks 1985; Hanley 1997; Provenza *et al.* 2003; Burney & Jacobs 2013). On the other hand, foraging theory has tended to minimize the role that minerals may play in mammalian browser preferences, generally focusing on phytochemical defences, carbohydrates, and protein content in predictions of browse preference (Palo & Robbins 1991; Bryant, Reichardt & Clausen 1992). We know of no previous study that simultaneously evaluates the effects of phytochemical defences, structural and non-structural carbohydrates, protein, and macro- and trace minerals on browser preference.

In our study, phytochemical defences and structural carbohydrates had little impact on browse preference. Non-structural carbohydrates, multiple macrominerals and the trace mineral iron had the strongest positive relationship with preference, while nitrogen and the trace mineral zinc had the strongest negative relationships with deer browse preference. While phytochemical defences, structural and non-structural carbohydrates, and protein all have clear influences on deer preference, as predicted by foraging theory and confirmed by numerous empirical studies, experimental results (such as ours) do not always show a straightforward pattern. We attribute the weak relationship between phytochemical defences/structural carbohydrates and deer preference to the relatively low levels of these compounds in the young, expanding leaves of our experimental trees. Seasonal changes in factors affecting foliar quality, as well as changes in the nutritional needs of mammalian browsers, can interact to influence browser preferences (Nelson & Leege 1982; McArthur *et al.* 1993; Burney & Jacobs 2011, 2013).

Protein and non-structural carbohydrates (sugars and starch) are some of the most important nutritional components of ungulate foods (Robbins 1983; Nolte 2004; Burney & Jacobs 2013). Our results show that sugar and starch concentrations were positively correlated with deer preference, as predicted. However, protein concentrations (as measured by total N) had a negative association with browse preference. This counter-intuitive result is concordant with a previous study of white-tailed deer foraging in the winter and early spring in Canada, where deer preferred diets lower in protein when energy content was held constant (Berteaux *et al.* 1998). The protein needs of mature white-tailed deer, including those of males during antler growth, are fairly low, although gestating or lactating female deer do have higher requirement (11–13% of diet, Fulbright & Ortega-Santos 2013). Protein levels in our trees were quite high across all experimental treatments, relative to deer dietary needs [3.9–5.6% dry weight for nitrogen represents 24–35% protein, using the conventional conversion factor of 6.25 (Magomya *et al.* 2014)]. Neighbouring alfalfa and clover crops were also high in protein. Thus, deer likely had more than adequate protein supply, regardless of tree choice. Protein is likely more important in driving browser preference under conditions in which its availability is limited.

Experimental evidence suggests that deer can discriminate among both macro- and trace minerals in forage material, according to their nutritional requirements (Ceacero *et al.* 2010a,b, 2015; Estevez *et al.* 2010). For example, in a study of plant species-level preferences of red deer (*Cervus elaphus hispanicus*) in Mediterranean ecosystems, deer avoided species with high levels of K, Ca, S, Mg and Zn, along with lower levels of protein (Ceacero *et al.* 2015). Numerous studies have shown a sodium 'drive' in browsing mammals (reviewed in Belovsky & Jordan 1981). Regional mineral deficiencies for red deer (*Cervus elephus*; copper) tule elk (*Cervus elaphus nannodes*; copper and phosphorus), black-tailed deer (*Odocoileus hemionus columbianus*; selenium) and mule deer (*Odocoileus hemionus*; selenium) have been well-documented (Oliver *et al.* 1990; Flueck 1994; Grace & Wilson 2002; Johnson, Bleich & Krausman 2007).

While relatively little is known of hypocalcemia and hypomagnesemia (low blood serum levels of calcium and magnesium respectively) in non-domesticated ungulates, they are a common and serious problem in domesticated cattle and sheep in the springtime (Schonewille 2013; Silk 2013). Studies with semi-domesticated reindeer have also shown that hypomagnesemia is common in the late winter and early spring (Hoff *et al.* 1993; Ropstad *et al.* 1997). Thus, the positive correlation between levels of calcium and magnesium and browse preference in our study is possibly an effect of hypocalcemia and hypomagnesemia (Jones & Weeks 1985; H.P. Weeks, Purdue University, personal communication). These deficiencies, in turn, may have been exacerbated by pregnancy. In southern Wisconsin, adult female deer are approaching parturition by mid-May, so are under heavy demands to provide minerals for foetal skeletal growth. Explanations for the observed positive relationships between iron and potassium and browse preference, and the negative relationship between zinc concentrations and browse preference are less clear. It is possible that interactions among elements prevent absorption of critical spring nutrients. Female moose (*Alces alces*) in Alaska were found to have springtime iron deficiencies, and in other mammals, including humans, excess intake of zinc can reduce absorption of copper and iron (Crofton *et al.* 1989; Lind *et al.* 2003).

Soil nutrient availability did not have a significant effect on deer preference, when measured as the proportion of tissue removed. This result contrasts with those of a number of studies demonstrating that fertilization of woody plant species (other than aspen) can increase the probability of ungulate browsing (reviewed in Burney & Jacobs 2013). This pattern is associated with widespread increases in foliar nitrogen concentrations with fertilization (Burney & Jacobs 2013). As nitrogen was probably not limiting to deer in our study, however, it is not surprising that fertilization did not affect deer preference. Responses of phytochemical defences to fertilization are more complex and variable, although there is a general trend for a decrease in condensed tannin concentrations with fertilization (Haukioja *et al.* 1998; Forkner & Hunter 2000; Osier & Lindroth 2006). Prior studies of the effects of soil fertilization on carbohydrate and mineral concentrations

in foliage of deciduous trees have generally shown minimal to no response (Balsberg Pahlsson 1992; Giertych *et al.* 2005).

#### TOLERANCE TO DEER BROWSING

Tolerance is a common defence strategy in plants, such as aspen, that have rapid growth rates, substantial storage capacity, and physiological plasticity (Rosenthal & Kotanen 1994; Strauss & Agrawal 1999). In environments with severe browsing pressure (e.g., large domestic or wild ungulate populations), tolerance may increase plant fitness to a greater extent than would resistance (Donaldson & Lindroth 2008; Lindroth & St. Clair 2013). We found that the magnitude of deer browsing influenced tree growth across genotypes, as did soil nutrient availability. However, genotypes did not generally display differential tolerance to browsing, regardless of soil nutrient availability.

Previous studies of tolerance to foliar herbivory in aspen and other woody tree species have generally demonstrated genetic variation for tolerance (Mattson *et al.* 1991; Byington, Gottschalk & McGraw 1994; Stevens, Waller & Lindroth 2007, but see Anttonen *et al.* 2002; Prittinen *et al.* 2003). To our knowledge, no previous study has investigated genetic variation in tolerance to browsing in a woody plant species. The type of damage incurred by browsing removes more of a plant's nutrient and carbohydrate reserves than does foliar herbivory (Wu & Hinckley 2001; Landhäusser & Lieffers 2002). We hypothesize that the amount of browsing damage across genotypes was sufficiently high to overwhelm tree stem and root reserves, leading to similar responses among genotypes. Browsing in spring and early summer, when much of a woody plant's stored nutrient and carbohydrate reserves have been allocated to new shoot growth, may have stronger impacts on plant fitness than browsing during the dormant season, when resource levels are typically high in non-browsed organs (Canham, McAninch & Wood 1994; Woolery & Jacobs 2011, 2014).

Tolerance to browsing was not generally influenced by soil nutrient treatment in our study, contrary to predictions of the prevailing model for tolerance, the limiting resource model (Wise & Abrahamson 2005, 2007). However, we assessed only short-term tolerance to browsing. Short-term response to browsing may be based more heavily on immediately accessible stem and root reserves than would be longer term (i.e. more than one growing season) responses. In the long term, differences in the ability of genotypes to acquire critical resources between the soil nutrient treatments might lead to differences in tolerance to browsing, as predicted by the limiting resource model. Studies of long-term tolerance to browsing in woody plants are needed to test this hypothesis.

#### Conclusions

Aspen genotypes were differentially susceptible to deer browsing, indicating that ungulate browsers have the potential to be agents of selection in aspen populations. Differences in

browse susceptibility at our early season assessment point were best explained by genotypic variation in non-structural carbohydrate and macro- and trace mineral concentrations, rather than by phytochemical defences or structural carbohydrates. Our results contrast with previous studies illustrating the influence of phytochemical defences on susceptibility to browsing in aspen. This contrast highlights the importance of seasonal variation in phytochemistry for browse preference; the relative availability of critical phytochemicals is likely to differ across seasons, possibly shifting browse preferences.

Tolerance to browsing did not differ substantially among genotypes. In contrast to predictions of the limiting resource model, tolerance to browsing was not affected by soil nutrient treatment. Our results demonstrate that tolerance to browse damage does not necessarily correspond to patterns of tolerance to insect foliar herbivory. Additional studies of both susceptibility and tolerance to browse damage are needed to determine whether our results for susceptibility to browsing hold across seasons and experimental species, and whether results for short-term tolerance are concordant with longer term responses to browsing damage.

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## Data accessibility

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.8856h> (Holeski et al. 2016).

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Means (top number in each cell) and standard errors (bottom number in each cell) for initial tree traits of each tree genotype and soil nutrient availability treatment. 'L' and 'H' indicate low and high soil nutrient treatments, respectively.

**Table S2.** Summary statistics for browse damage experienced by genotypes within soil nutrient availability treatments.

**Table S3.** Standardized coefficients calculated by partial least squares regression (PLSR) characterizing the relationship between deer browse preference (estimated as proportion of short shoots removed from a tree) and phytochemical traits.

**Table S4.** Mean values for multiple metrics of tree tolerance to browse damage across soil nutrient availability treatments.