Age-Related Shifts in Leaf Chemistry of Clonal Aspen (*Populus tremuloides*)

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Abstract Developmental changes in plant structure and function can influence both mammalian and arthropod feeding preferences for many woody plant species. This study documents age-related changes that occur in the leaf chemistry of trembling aspen (*Populus tremuloides* Michx., Salicaceae) and discusses implications for the herbivore community and ecosystem processes. We collected leaves from replicate ramets from six age classes (1–25+ yr) in each of seven aspen clones growing in south central Wisconsin, USA. Chemical analyses were conducted to determine concentrations of condensed tannins, phenolic glycosides (salicortin and tremulacin), nitrogen, starch, and soluble sugars. Each variable differed significantly among clones and among age classes. On average, condensed tannin concentrations doubled in the first five years and then remained fairly constant among older age classes. Combined phenolic glycoside (salicortin + tremulacin) concentrations were high in the youngest ramets (ca. 19%) and decreased sharply with age. Developmental changes in tannin, salicortin, and tremulacin concentrations exceeded those of nitrogen and carbohydrates. Developmental shifts of this magnitude, and the age-related tradeoff that occurs between condensed tannins and phenolic glycosides, are likely to have significant influence on the herbivore community of aspen and may influence leaf litter decomposition and nutrient cycling.

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Introduction

Plant characteristics, ranging from metabolic processes to morphology, often change through the process of maturation (Jones, 1999). Plant developmental changes can occur rapidly, for example, as leaves mature through a growing season, or slowly, as long-lived perennial plants reach reproductive maturity. These changes can have effects on community interactions. For example, many physical traits contributing to herbivore performance and preference (e.g., thorniness, leaf toughness, resin flow, leaf abscission) change in a predictable manner as plants mature (Brink, 1962; Kozlowski, 1971). Plant chemistry, which can be more important than physical characteristics in determining herbivore host ranges (Schultz, 1988), may also change as plants mature. Comparatively little is known, however, about developmental changes that occur in phytochemical composition as long-lived perennial plants mature.

Plant chemistry is influenced by both genotype and environment and can vary considerably both within species (Lindroth and Hwang, 1996a; Agrawal et al., 1999; Laitinen et al., 2000) and within individual plants (Gill et al., 1995; Orians and Jones, 2001; Pavia et al., 2002). Within-plant differences in chemical defense can occur as a result of induction (Karban and Baldwin, 1997), leaf maturation (Lindroth et al., 1987; Riipi et al., 2002), somatic mutations in meristematic tissues (Suomela and Ayres, 1994; Gill et al., 1995; Tuskan et al., 1996), and through the process of ontogenetic maturation from juvenile to adult phase (Bryant and Julkunen-Tiitto, 1995; Karban and Thaler, 1999; Erwin et al., 2001; Boege and Marquis, 2005; Laitinen et al., 2005.

Within-species variation in plant chemistry can affect ecological interactions and ecosystem processes. For example, primary plant compounds such as protein and water are critical for herbivore growth and development (Awmack and Leather, 2002), and qualitative or quantitative variation in phytochemical defenses within a species also strongly influences herbivore behavior and performance (Crawley, 1983; Schultz, 1988; Shelton, 2000). At the ecosystem level, phytochemical variation can influence litter decomposition and nutrient cycling rates (Schimel et al., 1996; Hättenschwiler and Vitousek, 2000; Kraus et al., 2003; Madritch et al., 2006).

Chemical variation within a plant may have ecological consequences similar to those of within-species variation (Suomela and Ayres, 1994). Whereas relatively little is known about the effects of development on the chemical content of plants, many studies have found that developmentally based variation can significantly influence herbivore behavior and performance (Kearsley and Whitham, 1989; Swihart and Bryant, 2001; Lawrence et al., 2003). Studies that have measured within-year and between-year (Bryant and Julkunen-Tiitto, 1995; Erwin et al., 2001; Boege and Marquis, 2005; Laitinen et al., 2005) developmentally mediated changes in plant chemistry indicate that such changes can be significant and suggest that these chemical shifts are likely to be ecologically important.

Previous work has demonstrated comparatively high levels of allelochemicals in juvenile compared with mature ramets in some species of Salicaceae (e.g., Bryant, 1981; Reichardt et al., 1990; Martinsen et al., 1998; Brian Rehill,

unpublished data), and two studies have indicated that between-year developmental shifts in phytochemistry may occur in aspen (Basey et al., 1988; Erwin et al., 2001). Evidence also suggests that mammals show preferences for tissues from mature vs. juvenile aspen trees (Basey et al., 1988; Swihart and Bryant, 2001) and establishes reasonable expectations for arthropods and pathogens to be affected by developmental shifts in phytochemistry (Lindroth and Hwang, 1996a; Kearsely and Whitham, 1989, 1998).

This research assessed among- and within-clone variation in trembling aspen (*Populus tremuloides* Michx.) leaf chemistry, and identified developmental patterns of allocation to leaf chemical constituents important for growth and defense. By "developmental," we refer to long-term or between-year changes in plant chemistry through the process of maturation. We also assessed how such developmental patterns vary among clones because the rate and magnitude of chemical shifts may significantly affect their potential to influence associated herbivore communities and ecological processes.

Trembling aspen is one of the most widely distributed and genetically variable plant species in North America (Mitton and Grant, 1996). Aspen reproduction is often vegetative, via suckering from previously established root systems. Individual ramets (stems) originating from a common root system often vary widely in age and maturity as the clone expands from the central genet (original seedling). After disturbances, such as windfall, fire, or clear-cutting, aspen produces new ramets from surviving root systems. Depending on disturbance types and frequencies, aspen stand demographics can vary greatly at the local and landscape scales. Thus, developmental-based changes in the chemical attributes of this important and widespread tree species may significantly influence its associated communities.

Methods and Materials

Clone Selection and Sampling Protocols

To assess background levels of among-clone variation in aspen leaf chemistry, we haphazardly selected 20 aspen clones from locations throughout Dane Co., Wisconsin, USA. In early June 2002, four reproductively mature trees (ramets >10 yr of age) from each of the 20 clones were sampled for chemical analyses. Using a pole pruner, we haphazardly collected 5–10 leaves from each of at least three locations in the canopy. These 20 clones were subsequently identified as genetically unique on the basis of microsatellite markers (Cole, 2005).

Seven of the 20 clones sampled were suitable (age-distribution) for extensive sampling from ramets of different age classes. For these seven clones, we haphazardly selected and sampled from three individual ramets within each of the following age classes: 0–1, 2–5, 6–10, 11–15, 16–20, and >20 yr. For one of the clones (clone 11), no trees were available over 20 yr old. Leaf samples and diameter and height measurements were taken for each ramet in June 2002. Samples were collected as described above, i.e., 5–10 leaves from at least three locations within the canopy. To avoid the potentially confounding effect of within year variation in chemistry (Lindroth et al., 1987; Riipi et al., 2002), only fully expanded source leaves were used in chemical analyses. Ramet age was determined by counting annual

growth rings on cut ends or, for larger ramets, by averaging ring counts from two tree cores made with an increment borer. Heights were measured with a measuring tape for felled ramets and a clinometer for large standing ramets. Stem diameters were measured with a hand caliper 2 cm above ground level for small ramets (<3 cm diam) and a diameter measuring tape 10 cm above ground was used for large ramets (>3 cm diam).

Phytochemical Analyses

Leaf samples were kept on ice while in the field and returned to the laboratory daily. Samples were flash-frozen in liquid nitrogen, vacuum dried, ground with a Wiley mill through a number 40 mesh, and stored at −20°C. We analyzed leaf tissues for chemical constituents likely to be important in influencing general host or litter quality (condensed tannins, phenolic glycosides, total nitrogen, starch, and sugars). Condensed tannins were assayed using a modified butanol–HCL method described by Porter et al. (1986). Purified aspen condensed tannins served as standards. The phenolic glycosides salicortin and tremulacin were quantified by HPLC as reported by Lindroth et al. (1993), using purified phenolic glycosides as standards. Samples were initially screened for salicin and tremuloidin, but contained little or none of those two compounds. Thus, the value for total phenolic glycosides is the sum of salicortin and tremulacin concentrations. Samples were analyzed for total N (an index of protein) with an elemental analyzer (LECO, St. Joseph, MI, USA). Glycine-*p*-toluenesulfonate was used as a standard in N analyses. Starch and sugar (sucrose + hexose) concentrations were assayed by using enzymatic hydrolysis and the dinitrosalicylic acid method as in Lindroth et al. (2002). All phytochemical variables are reported in the text and figures as percent dry weight.

Statistical Analyses

Statistical analyses were performed by using JMP IN version 4.0.4 (SAS, 2001) and SAS version 8.2. Because this study was conducted on naturally occurring aspen clones, it was not possible to replicate clones. Estimates of within-clone error were achieved by measuring chemistry of multiple ramets ("trees") from within a clone, either from 1) four mature ramets within a clone (Fig. 1), or 2) multiple trees from within an age class (Figs. 2 and 3).

Phytochemical differences among the 20 mature clones were evaluated by an analysis of variance (ANOVA). The effects of plant age on phytochemical attributes were assessed in a mixed model (Proc Mixed; SAS, 1999). Ageclass was fitted as a fixed effect, and clone was fitted as a random effect. *Post hoc* means comparisons were made for phytochemical concentrations among age classes by using *t* tests and Bonferroni-adjusted probabilities (SAS, 1999). Two different analyses were used to determine whether developmental patterns varied among clones. First, we compared −2 log-likelihoods for models run with and without the random variable "clone." Chi square tests were used to assess the significance of the clone effect (Zar, 1999). Second, an analysis of covariance (ANCOVA) was used to compare the trajectory (slopes) with which concentrations of phytochemicals differed as plants aged. Tree age (years) was used as the covariate. A significant clone by age interaction indicated that clones showed different developmental trajectories in allocation to phytochemical variables. In our assessments of age-related variation, a
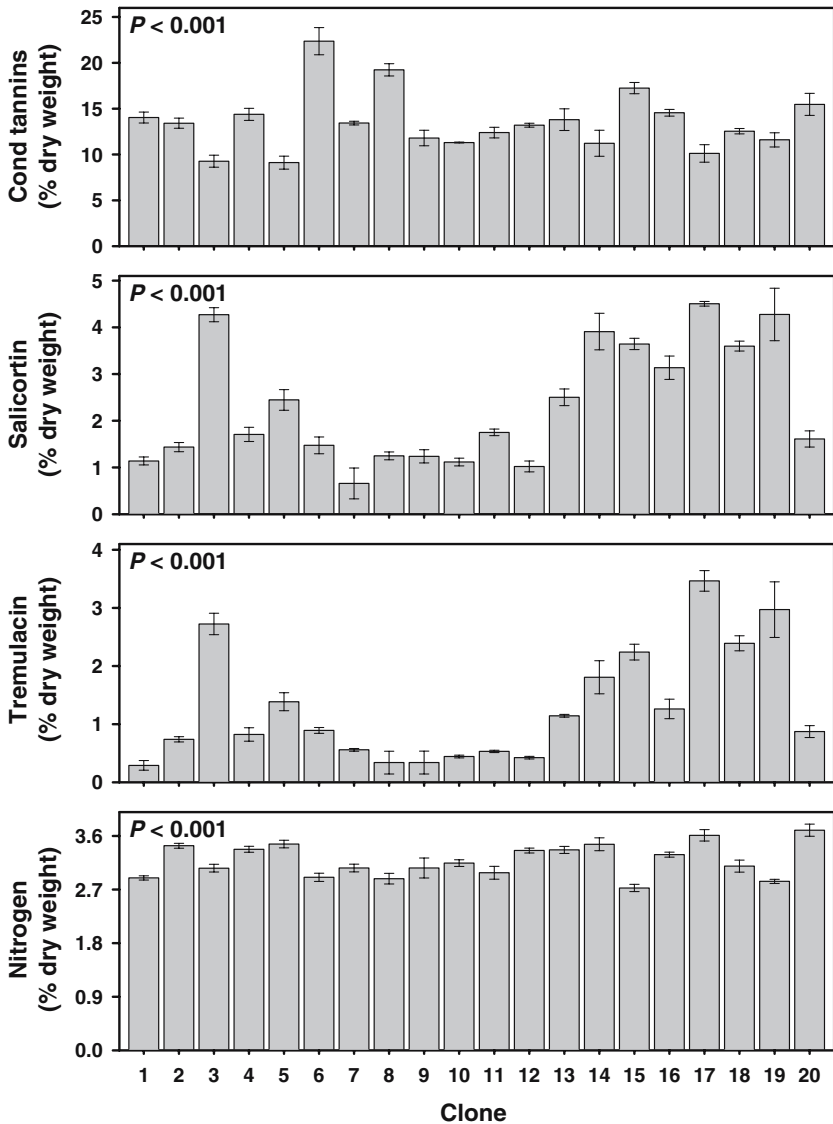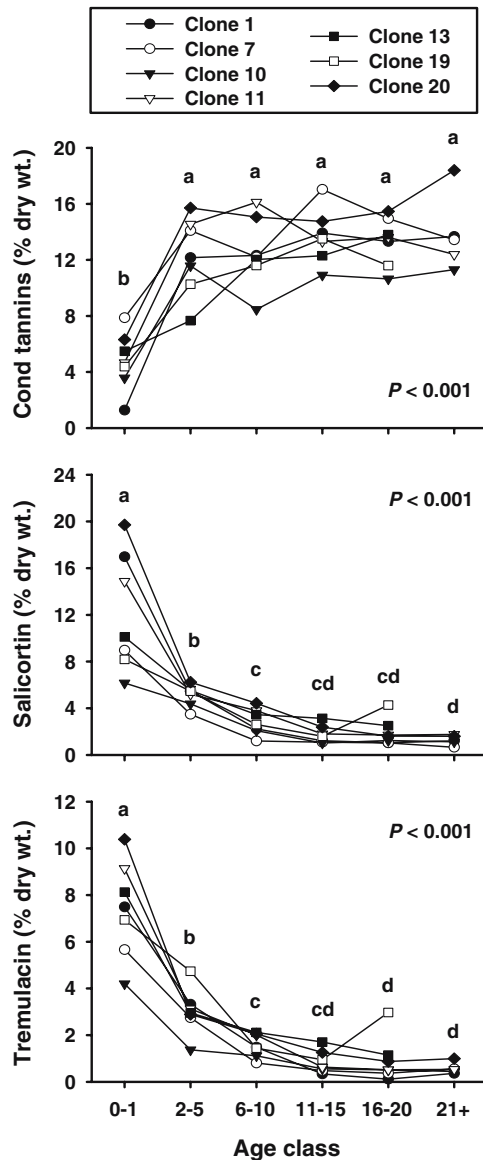
Fig. 1 Leaf concentrations of condensed tannins (cond tannins), salicortin, tremulacin, and nitrogen from mature trees of 20 aspen clones haphazardly selected throughout Dane Co., Wisconsin. Bar heights represent averages of four mature ramets per clone. Error bars indicate ±1 SE

Box-Cox transformation was applied to salicortin and tremulacin values prior to statistical tests to improve normality and homogeneity of variance (Zar, 1999).

Pairwise correlations were examined to identify relationships among phytochemical constituents, size, and age variables (SAS, 2001). Correlations were assessed by using mean values for age classes within clones. The relationship between condensed tannin and phenolic glycoside concentrations was described using quadratic regression.

Fig. 2 Developmental shifts in aspen leaf concentrations of condensed tannins (cond tannins) and phenolic glycosides (salicortin and tremulacin). Symbols represent the average concentration for replicate ramets ($n = 3$) within each of six age classes for the seven clones, respectively. *P* values refer to the main effect of age class (Proc Mixed). Age classes with different letters are significantly different (Bonferonni-adjusted $P < 0.05$)


Results

Among-Clone Phytochemical Variation

Leaf chemical constituents of mature ramets varied among the 20 aspen clones (Fig. 1). Condensed tannin concentrations varied 2-fold among clones ($P < 0.001$). Concentrations of salicortin and tremulacin were highly correlated and more variable among clones than were tannins. Salicortin concentrations varied almost 7-fold and tremulacin varied 12-fold among clones ($P < 0.001$). Nitrogen

Fig. 3 Developmental shifts in aspen leaf concentrations of nitrogen (N), sugars, and starch. Symbols represent the average concentration for replicate ramets (*n* = 3) within each of six age classes for the seven clones, respectively. *P* values refer to the main effect of age class (Proc mixed). Age classes with different letters are significantly different (Bonferroni-adjusted *P* < 0.05)
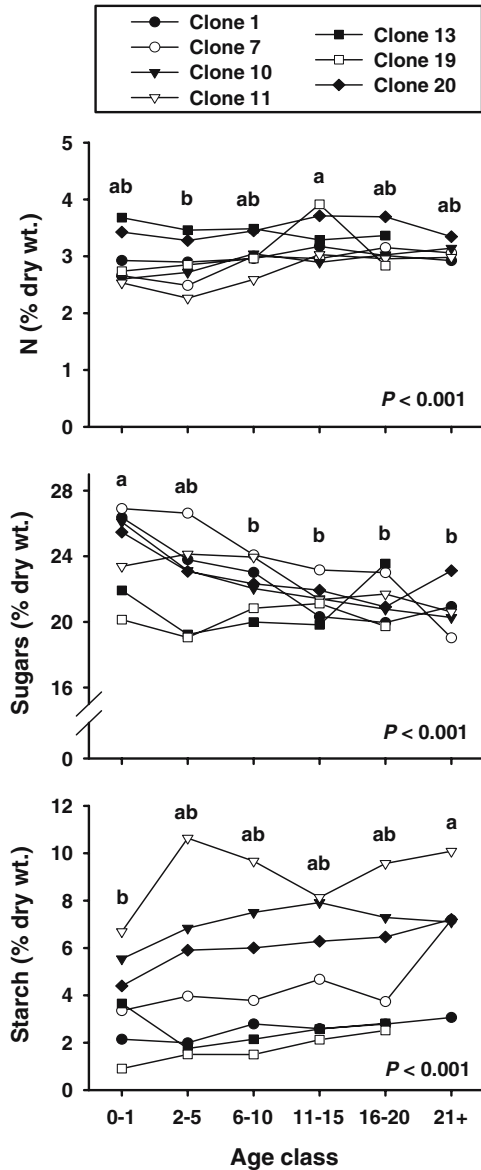
(N) concentrations were less variable among clones, ranging from 2.8% to 3.6% (*P* < 0.001).

Developmental Patterns Within and Among Aspen Clones

Variation in Growth

Equal-aged ramets from the seven clones differed considerably with respect to height and diameter. An ANCOVA with tree age confirmed differences (height,

$P < 0.001$; diameter, $P < 0.001$). In general, tree size and growth trajectories varied among clones. Separate plots of height and diameter show similar patterns (data not shown).

Variation in Secondary Metabolites

Condensed tannin concentrations increased sharply with age (Fig. 1). Developmental changes occurred primarily between the "0–1" and "2–5" yr age classes, where condensed tannin concentrations increased from 4.5% to an average of almost 14% dry weight (Fig. 2). Phenolic glycoside concentrations also varied among age classes (Table 1), but in contrast to tannins, salicortin and tremulacin concentrations decreased with age, from an average of almost 13% to 1%, and 7% to less than 1%, respectively (Fig. 2). The developmental changes occurred in the first 10 yr, as evidenced by the lack of differences among age classes greater than 10 yr old. Among-clone variation for salicortin and tremulacin was much greater for juvenile than for mature ramets (Fig. 2).

Variation in Nitrogen, Sugars, and Starch

Aspen nitrogen and carbohydrate concentrations also differed among age classes, but the developmental patterns were weak relative to those of tannins and phenolic glycosides (Fig. 3). Nitrogen differed among clones and increased only slightly in more mature ramets (Fig. 3). Average sugar concentrations decreased from 24% in the youngest trees to 19.6% in mature trees. Average starch concentrations were highly variable among clones, ranging from 1.5% to over 8%, and tended to increase in mature trees (Fig. 3).

Variation in Developmental Trajectories

The effects of plant age on phytochemical concentrations varied among aspen clones. For each variable tested, removing the effect of "clone" from the mixed model significantly decreased the goodness of fit (χ^2 tests, $P < 0.001$ for each variable). Looking specifically at the slopes, or rates of concentration change in phytochemical variables, we found that the trajectories of developmental shifts in aspen secondary metabolites were consistent among clones for tannins, but not for

Table 1 Correlation matrix (Pearson) of mean age and aspen phytochemicals, including condensed tannins, total phenolic glycosides (box-cox transformed salicortin + tremulacin), nitrogen (N), sugars, and starch

	Ramet age	Condensed tannins	Phenolic glycosides	N	Sugars
Condensed tannins	0.60***				
Phenolic glycosides	−0.73***	−0.74***			
N	n.s.	n.s.	n.s.		
Sugars	−0.50***	n.s.	0.45**	−0.36*	
Starch	n.s.	n.s.	n.s.	n.s.	n.s.

n.s. = not significant.

n.s.: $P > 0.05$; *$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$.

Fig. 4 The negative relationship between condensed tannin (cond tannins) and phenolic glycoside (salicortin + tremulacin; phen glycosides) concentrations in aspen leaves from ramets of different ages. Each point represents a single ramet. Quadratic regression provided the best fit ($P < 0.001$)
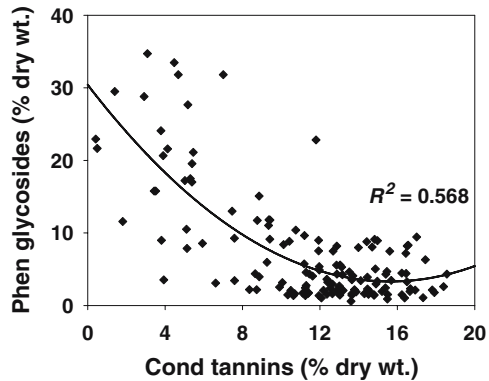
phenolic glycosides. Condensed tannin concentrations increased with age similarly for the seven clones (i.e., nonsignificant age × clone interaction; ANCOVA). Clones that contained higher relative tannin concentrations in juvenile ramets also had higher concentrations in mature ramets. Again, the differences occurred in the first five years, after which tannin concentrations showed no consistent pattern of change. The developmental trajectories of salicortin and tremulacin, however, were markedly different among the clones (i.e., significant clone by age interactions; $P = 0.005$ and $P = 0.018$, respectively). The differences in trajectory were largely due to the variability in levels of phenolic glycosides in juvenile ramets. For example, juvenile ramets of clone 20 (0–1 age class) had almost 30% total phenolic glycosides (salicortin + tremulacin), compared with ca. 10% in clone 11. However, in both clones, ramets greater than 20 yr old had less than 2% total phenolic glycosides.

Correlations

Nitrogen was not correlated with tannins or total phenolic glycosides but was negatively correlated with sugar concentrations (Table 1). Sugar concentrations were positively correlated with total phenolic glycosides. A particularly strong negative correlation occurred between tannin and total phenolic glycoside concentrations when all age classes were considered together. The shape and strength of this relationship are described by a quadratic regression in Fig. 4 ($R^2 = 0.568$; $P < 0.001$).

Discussion

Our survey of phytochemical variation among aspen clones in south central Wisconsin indicates that clones occupying similar habitats and growing under similar conditions produce varying concentrations of condensed tannins, phenolic glycosides, and nitrogen. These patterns are consistent with those found in other natural aspen populations growing in northern lower Michigan (Lindroth and Hwang, 1996b) and in northern Wisconsin (Donaldson, 2005).

In common gardens, genetically based differences in condensed tannins and phenolic glycosides among clones are similar in magnitude to those observed here (Lindroth and Hwang, 1996a; Stevens and Lindroth, 2005; Donaldson et al., 2006). Nonetheless, in this study, we are unable to infer what proportion of the observed

variation may be due to genetics rather than to environment. However, phenolic glycosides (the most variable compounds) show relatively little phenotypic plasticity in common gardens (Hwang and Lindroth, 1997; Osier and Lindroth, 2001). Thus, most of the observed variation in concentration among clones may be due to genotype.

Our survey of developmentally based phytochemical variation indicates that stand demographics may add to the overall magnitude of spatial and temporal chemical variation occurring in aspen populations (Erwin et al., 2001). The strong inverse relationship between the developmental trajectories of tannins and phenolic glycosides in aspen creates strikingly different chemical phenotypes among mature and juvenile ramets within a clone that are likely to influence herbivory (Lindroth and Hwang, 1996a; Osier and Lindroth, 2001) and other chemically mediated processes (Lindroth et al., 2002; Madritch et al., 2006). The trend of increasing tannins with age is difficult to explain because we lack sufficient understanding of their functions in aspen (Lindroth and Hwang, 1996a). The biological activity of phenolic glycosides, however, is well established (Lindroth and Hwang, 1996a; Osier and Lindroth, 2001). The fact that leaves of young ramets contain much higher concentrations of these compounds compared with mature ramets may indicate that they have historically experienced more intense selective pressure than have mature ramets (discussed below).

The magnitude of developmentally based phytochemical shifts in condensed tannin and phenolic glycoside concentrations varied among the clones surveyed (i.e., clones showed different developmental trajectories). If this variation is genetically based and heritable, it would suggest that the same evolutionary factors involved in selecting for (or against) chemical traits in aspen may influence the degree to which developmental shifts occur. Spatial or temporal variation in costs and benefits of allocation to tannins and phenolic glycosides among clones and among age classes may explain the observed patterns. For example, temporally variable population densities of insect herbivores or browsing mammals, or spatially variable resource availability, all can potentially affect the relative costs and benefits of producing foliar allelochemicals (Donaldson et al., 2006; Osier and Lindroth, 2006).

Developmental patterns are also visible but far less pronounced for nitrogen, starch, and sugars. This comparative lack of change in primary compounds may reflect that their role in plant physiology is less variable over developmental time than is that of "secondary" compounds.

We recognize that the developmental patterns observed in this study cannot with certainty be attributed solely to ontogeny (genetically programmed developmental changes). Some portion of the variation between juvenile and mature ramets may be due to differences in their respective environments. For example, resource (light, soil nutrients) availability may differ across space occupied by a clone. As discussed above, the short stature of younger ramets may predispose them to browsing (historically and recently). However, in our selection of 0- to 1-yr-old ramets, we specifically selected individuals that were new root sprouts and that had not been previously browsed. Therefore, the most extreme differences between juvenile and mature clones appear not to be a function of recent browsing. Previous defoliation studies also argue against induction as a proximate explanation. Contrary to the patterns we observed, studies have failed to find substantial induction of phenolic glycosides, but do find significant induction of tannins (Osier and Lindroth, 2004; Stevens and Lindroth, 2005).

Another consideration we must make is that our data derive from differently aged ramets of clonally integrated plants. The importance of clonal integration for the chemical composition of ramets may be significant, as ramets may exchange photosynthate, nutrients, and perhaps even compounds signaling herbivore damage (Magyar et al., 2004). Clonal integration, therefore, may influence the costs and benefits of allocation to defense and contribute to both spatial and temporal variation in the chemical profiles of aspen populations. However, considering the number of studies that report strong herbivore preferences for (Basey et al., 1988; Swihart and Bryant, 2001) and phytochemical differences among (Erwin et al., 2001) juvenile and mature trees in this and other systems, we believe that the developmental changes evident in aspen tannin and phenolic glycoside concentrations may be largely a result of ontogenetic shifts in allocation.

Our findings have several potentially important evolutionary and ecological implications that require further investigation. The fact that leaf tissues of young aspen trees are better defended (i.e., higher levels of phenolic glycosides) suggests a greater selective pressure for defense in young trees compared with mature trees. Browsing pressure from mammals is a likely explanation for such patterns. Mammals exert tremendous selective pressure on aspen, particularly on resprouts and seedlings that are accessible to browsers (Swihart et al., 1994; Kay and Bartos, 2000). Furthermore, because the impacts of herbivory on plant survival, growth, and reproduction may be particularly strong during the juvenile phase (Watkinson, 1986), optimal defense theory (Rhoades, 1979) predicts that this selective pressure leads to relatively higher levels of defense in juvenile compared with mature tissues. Indeed, a meta-analysis conducted by Swihart and Bryant (2001) indicates that mammals almost always prefer mature tissues to juvenile tissues of forest trees, including aspen. Our data suggest that mammals may be a primary source of selection explaining the quantitative distribution of phenolic glycosides in aspen.

The most significant arthropod herbivores of aspen, eruptive outbreak species such as forest tent caterpillars, large aspen tortrix, and gypsy moths, generally cause the greatest damage in advanced even-aged stands. During outbreaks, trees are defoliated regardless of their level of commitment to chemical defenses (Mattson et al., 1991; Donaldson, 2005). As a result, defoliating arthropods may present less selective pressure for chemical defenses than previously thought. In fact, this study and other recent work suggest that advanced stands of aspen in the Great Lakes region generally have lower concentrations of phenolic glycosides than previously thought (Fig. 1; Donaldson, 2005), although levels in advanced stands of western aspen are typically high (Donaldson, Lindroth, and Wooley, unpublished data).

Regardless of the evolutionary outcomes of aspen–arthropod interactions, phenolic glycoside concentration is the best predictor of insect performance both among aspen clones (Lindroth and Hwang, 1996a) and among species of Salicaceae (Edwards, 1978; Tahvanainen et al., 1985). Given that trees undergo developmental shifts in phenolic glycoside concentrations that are greater in magnitude than the genotypic variation observed among adult clones, it is likely that these age-related changes have significant impacts on insect behavior, distributions, and populations (Kearsley and Whitham, 1989, 1998; Waltz and Whitham, 1997).

Explaining the marked increase in condensed tannins as aspen trees mature in terms of selective pressure exerted by herbivory is complicated. In fact, the role of condensed tannins in aspen (and many other plant species), although long assumed

to be defensive, is still not well understood (Ayres et al., 1997). Most studies have failed to find a relationship between aspen tannin concentrations and mammalian or arthropod preferences or performance (Lindroth and Hwang, 1996a). Tannins may play diverse "alternative" roles in plants, from defenses against photooxidative stress (Close and McArthur, 2002) and microbial pathogens (Kellam et al., 1992), to modifying local soil processes and nutrient cycling rates (Hättenschwiler and Vitousek, 2000). For example, variation in tannin concentrations can alter leaf litter quality (Lindroth et al., 2002; Madritch et al., 2006). Concentrations of both nitrogen and phenolics (especially tannins) play important roles in litter decomposition and soil nutrient dynamics in forest soils (Schimel et al., 1996; Northup et al., 1998; Hättenschwiler and Vitousek, 2000; Kraus et al., 2003; Schweitzer et al. 2004). In fact, an alternative hypothesis to the role of tannins as antiherbivore defenses posits that they may be adaptive in their capacity to regulate proximal nutrient cycling (Northup et al., 1998).

In summary, developmental shifts in chemical defenses within aspen clones appear to be greater in magnitude than the variation observed among mature clones. We believe that these developmental changes have likely been shaped by evolutionary factors related to herbivory and ecosystem function and hold importance for diverse ecological interactions today. Developmental shifts in chemical composition in this dominant clonal species are likely to contribute to temporal and spatial mosaics of host quality and to influence patterns of community structure and ecosystem function.

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