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SPECIAL TOPIC: FROM PLANTS TO HERBIVORES

Genetic variation in aspen phytochemical patterns structures windows of opportunity for gypsy moth larvae

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Abstract

Empirical studies indicate that host-tree bud break will likely advance faster than spring-folivore egg hatch in response to predicted increases in temperature. How these phenological shifts will affect herbivory will depend on temporal patterns of foliar traits that occur during leaf expansion, and their effects on folivore performance. Through fine-scale time series sampling of newly flushed trembling aspen (*Populus tremuloides*) foliage, we observed a previously unknown peak in phenolic glycoside concentrations that coincides with the emergence of sensitive neonates of gypsy moths and rapidly declines soon after bud break. The magnitude and duration of the initial post-bud break peak in phenolic glycosides varied substantially among genotypes. In contrast, foliar nitrogen concentrations declined at a more uniform rate among genotypes throughout leaf expansion. In addition, leaf toughness remained uniformly low throughout these periods of phytochemical change, and did not rise or vary substantially among genotypes until after anticipated windows of climate change-induced shifts between bud break and egg hatch had elapsed. Controlled manipulation of intervals between gypsy moth egg hatch and aspen bud break generated differences in larval performance among hatch cohorts and host genotypes that corresponded with changes in foliar phenolic glycoside and nitrogen concentrations. These findings indicate that the effects of climate change-induced phenological shifts on herbivory will differ among host plant genotypes, and that genetic variation in foliar chemical patterns will strongly influence this heterogeneity.

Keywords Phenology · Bud break · Forest · Insect · Climate change

Introduction

Changes in trophic interactions, and their impacts on disturbance regimes, are critically important yet poorly understood consequences of climate change. Interactions between spring-feeding folivores and trees in temperate forest ecosystems are particularly important, due to their sensitivity to minor temperature changes and potential to trigger ecological cascades. Temperature cues regulate the timing of tightly synchronized phenological events, including host-tree bud break and folivore egg hatch. Recent mesocosm studies indicate that tree bud break will advance faster than folivore egg hatch in response to temperature increases predicted to occur before the end of the 21st century (Schwartzberg et al. 2014; Uelmen et al. 2016). The effects of such shifts on plant–herbivore dynamics, however, remain unclear.

The ecological ramifications of climate change-induced phenological asynchrony will largely depend on underlying temporal variation in foliar chemistry that occurs throughout leaf expansion. Current understanding of these processes stems heavily from Feeny (1970), who proposed a phytochemical model by which foliar quality diminishes throughout the growing season. This pattern is thought to create an ephemeral "window of opportunity" following bud break, during which spring folivores feed optimally on newly

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flushed foliage. In many tree species, foliar nitrogen concentrations are the highest in newly flushed leaves and decline throughout the growing season, thus supporting this model (Feeny 1970; Hunter and Lechowicz 1992; Osier et al. 2000; Barbehenn et al. 2013, 2015, 2017). Recent studies on patterns of secondary metabolite concentrations, however, add further complexity to classic phenological theories. While some studies report that secondary metabolites are the lowest in young leaves and increase with age (e.g., Feeny 1970; Riipi et al. 2002), most report the opposite, in which secondary metabolite concentrations are highest in young leaves and decrease over variable lengths of time (Faeth 1986; Meyer and Montgomery 1987; Wait et al. 1998; Salminen et al. 2004; Koricheva and Barton 2012; Massad et al. 2014).

The concurrent presence of high nitrogen and secondary metabolite concentrations in newly flushed foliage can further confound efforts to predict the consequences of phenological asynchrony. It is unclear whether anticipated shifts in phenology will benefit lepidopteran folivores by allowing larvae to avoid windows of high secondary metabolite concentrations, or hinder performance by forcing larvae to feed on older foliage with lower nitrogen content. While some manipulative hatch studies report that spring-feeding folivores consuming younger foliage perform optimally (Hunter and Lechowicz 1992; Parry et al. 1998; Tikkanen and Julkunen-Tiitto 2003; Jones and Despland 2006; Barbehenn et al. 2013, 2015, 2017), others report optimal performance among larvae that consume older foliage (Meyer and Montgomery 1987; Chilcote et al. 1992; Stoyenoff et al. 1994). Few studies, however, have paired manipulative insect feeding bioassays with fine-scale time series analyses of host-tree foliar chemistry to determine the relationship between phenological synchrony, temporal variation in foliar quality, and insect performance. Information on how herbivore performance will vary within the time scale by which climate change will alter phenological synchrony is particularly lacking.

Trembling aspen (*Populus tremuloides* Michx.) is the most widespread tree species in North America, and is a preferred host for several species of spring-feeding lepidopteran folivores, including gypsy moth (*Lymantria dispar* L.). Aspen produce a well-characterized suite of phenylpropanoid-derived secondary metabolites that consist primarily of phenolic glycosides [a.k.a. salicinoids (Boeckler et al. 2011)] and condensed tannins. Phenolic glycosides serve as the principal anti-herbivore defense compounds in aspen foliage and explain 70–98% of variation in gypsy moth larval survival rate, development time, and weight, when analyzed alongside other foliar parameters such as condensed tannins, total nonstructural carbohydrates, and water content (Hemming and Lindroth 1995; Hwang and Lindroth 1997). While condensed tannins are also abundant in trembling aspen foliage, little evidence supports their role as defensive compounds against these insects (Barbehenn and Constabel 2011). Furthermore, protein-based defenses, such as proteinase-inhibitors, occur at biologically insignificant concentrations in wild-growing, nonjuvenile aspen, and are not induced by insect feeding (Rubert-Nason et al. 2015). We, therefore, focus on phenolic glycosides.

While some studies have reported weak declines in aspen foliar phenolic glycoside concentrations over the course of the growing season (Lindroth et al. 1987; Osier et al. 2000), relatively little is known about fine-scale changes that occur during leaf expansion, or how such rapid changes may affect spring-feeding folivorous larvae. Furthermore, aspen exhibits substantial genetic variation in chemical defense (Donaldson et al. 2006; Smith et al. 2011). It is unknown, however, to what degree temporal patterns in phytochemistry differ among genotypes during leaf expansion, and whether such variation might cause folivores feeding on different host genotypes to respond differently to shifts in phenological timing.

The objectives of this study were to: (1) characterize short-term patterns of temporal variation in aspen foliar chemistry and leaf toughness during leaf expansion, (2) determine the extent to which these foliar patterns vary among aspen genotypes, and (3) assess how genetic variation in aspen foliar chemistry patterns influences performance among gypsy moth larvae hatching at different intervals with respect to bud break. We hypothesized that gypsy moth larval performance would vary in response to phenological asynchrony among aspen genotypes, and that this variation would correspond with heterogeneity in temporal phytochemical patterns.

Methods

Experimental overview

During the springs of 2015 and 2016, we conducted two separate studies on aspen growing in Pine Island Wildlife Area near Portage, Wisconsin, USA. In 2015, our objectives were to characterize patterns of temporal variation in foliar chemistry and leaf toughness among aspen genotypes during leaf expansion, and to identify a subset of genotypes that exhibit distinct foliar quality patterns for use in 2016 experiments. In 2016, our objective was to assess how phenological asynchrony affects performance among gypsy moth larvae feeding on aspen genotypes that exhibit variation in temporal patterns of foliar chemistry. We utilized our phytochemistry data from 2015 to manipulate gypsy moth egg hatch phenology to coincide with windows of high and low foliar quality and infer key dates for foliar sampling and chemical analyses that would explain variation in larval performance.

Aspen genotype selection

In 2015, we identified clonal stands of eight unique trembling aspen genotypes. Genotypes were distinguished based on differences in bud break phenology (Fig. S1) and spatial distribution. To minimize the potentially confounding effects of tree age on phytochemical variation, we selected individuals with 5–8 cm diameters at breast height (DBH) that were approximately 3 m tall. We estimated individuals within this size range to be approximately the same age, which minimizes the potential for ontogenetic differences in foliar chemistry (Donaldson et al. 2006). Within each genotype, we haphazardly selected six replicate ramets for chemical analyses. In 2016, we selected a subset of four of these genotypes that exhibited high, intermediate, and low foliar phenolic glycoside concentrations immediately following bud break. For each genotype, we haphazardly selected 12 ramets (in the same size range as trees selected in 2015) for insect bioassays and chemical analyses.

Leaf collections and chemical analyses

In spring 2015 and 2016, we began leaf collection for phytochemical analysis on the date at which at least one bud broke on three separate branches among all trees within a given genotype. Bud break was defined as the point in which leaf tissue was exposed at the tip of the bud. Following bud break, we collected leaf tissue every 2–3 days for approximately 1 month. During each sampling event, foliage was collected haphazardly from buds on three separate branches per tree, placed in paper bags, and immediately submerged in ice inside larger waterproof plastic bags. Sampled foliage was clipped at the base of the petiole to avoid potential induction effects (Mattson and Palmer 1988). To ensure that a sufficient amount of leaf material was collected for chemical analyses, entire buds were sampled until leaves expanded beyond their bud scales. After this point, three-to-five leaves were collected from each of the three branches per tree during each sampling event. Upon returning to the laboratory, samples were flash frozen in liquid nitrogen, lyophilized, weighed, ground, and stored at – 30 °C until chemical analysis.

Following each leaf sampling event in 2015, we measured leaf toughness on a randomly selected subset of two leaves per tree. Each leaf was pressed between plexiglass sheets containing 1.6 mm diameter holes and punctured with a 0.67 mm^2 McCormick dynamometer (Lindroth et al. 1993). The force (grams) needed to puncture two interveinal portions of each leaf was recorded and the punctured foliage was discarded. Due to minimal changes in leaf toughness among aspen genotypes during realistic windows of gypsy moth egg hatch (see Results), we did not repeat these measurements in 2016.

To assess temporal patterns of aspen foliar quality, we analyzed levels of phytochemical compounds previously demonstrated to strongly influence performance among lepidopteran folivores (Hemming and Lindroth 1995, 2000; Hwang and Lindroth 1997). We quantified foliar concentrations of phenolic glycosides, which are the principal anti-herbivore defense chemicals in aspen, and nitrogen, an indicator of protein. We report levels of phenolic glycosides using salicortin and tremulacin. These phenolic glycosides typically comprise 85–95% of the phenolic glycoside pool in aspen foliage and are substantially more biologically active than other aspen phenolic glycosides against lepidopteran folivores (Lindroth et al. 1988; Lindroth and Peterson 1988). Condensed tannins, though also abundant in aspen foliage, have no clear defensive role against gypsy moths (Hemming and Lindroth 1995; Hwang and Lindroth 1997), and were, therefore, not quantified in this study.

We quantified foliar nitrogen concentrations using a Thermo-Finnigan 1112 Flash Elemental Analyzer. We analyzed foliar phenolic glycosides using ultra-high-performance liquid chromatography (UHPLC) with photodiode array detection (PDA) and negative electrospray ionization-single quadrupole mass spectrometry (MS; Waters Acquity I-Class UPLC and 3100 SQ MS, Milford, MA, USA) following a modified method from Keefover-Ring et al. (2014). Sample preparation included flash freezing foliage soon after collection, followed by lyophilization and grinding with a ball mill. We extracted phenolic glycosides from the powered leaf tissue with cold (4 °C) methanol and sonication in an ice bath (15 min), centrifuged, and used the clear supernatant for analysis. We injected 2 µL of all standards and sample solutions onto the UHPLC and separated peaks with a Waters Acquity CSH C-18 column (2.1 × 100 mm, 1.7 µm) at 40 °C with a flow rate of 0.5 mL min^{-1}, using a gradient of water and acetonitrile, both containing 0.1% formic acid. The PDA was configured to scan from 210 to 400 nm, with 1.2-nm resolution at 20 points s^{-1}. The MS operating parameters were: cone potential, 30 V; capillary potential, 2500 V; extractor potential, 3 V; RF lens potential, 0.1 V; source temperature, 120 °C; desolvation temperature, 250 °C; desolvation gas flow, 500 L h^{-1}; cone gas flow, 10 L h^{-1}; infusion rate, 5 µL min^{-1}; dwell time, 0.025 s.

We used standard curves of methanol solutions of purified salicortin and tremulacin, also containing salicylic acid-d_6 (Sigma-Aldrich, St. Louis, MO, USA) as an internal standard, to calculate concentrations of phenolic glycosides. The salicortin and tremulacin standards had been previously isolated and purified from aspen foliage (Lindroth et al. 1986). Amounts of phenolic glycosides measured were then normalized by the initial sample dry weight.

In 2015, we first analyzed foliage from our earliest and latest timepoints and thereafter analyzed a sequential progression of intermediate timepoints until we identified

the closest possible start and end points of concentration shifts exceeding 3% foliar dry weight. These data informed our decision to analyze timepoints at approximately 0, 50, 100, and 300 degree days after bud break in 2016 (base temperature = 4.4 °C) (Parry et al. 1997). Degree-day data were obtained from NOAA Weather Station GHCND:USC00476718 in Portage, WI.

Insect bioassays

To assess how genetic variation in temporal patterns of aspen phytochemistry influences windows of opportunity for spring-feeding larvae, we applied gypsy moth neonates in two temporally separated hatch cohorts among four unique aspen genotypes during the spring of 2016. These genotypes were predetermined to exhibit a wide range of phenolic glycoside concentrations immediately following bud break. Within each genotype, two groups of six replicate ramets received gypsy moth larvae belonging to one of the two hatch cohorts (4 genotypes × 2 hatch cohorts × 6 replicates = 48 trees). Bud break varied by ~ 50 degree days (5 days) among aspen genotypes (Fig. S1). Within each hatch cohort, larval emergence times were, therefore, normalized with respect to bud break to assure neonates feeding on different aspen genotypes encountered foliage of a consistent development stage. Hatch cohort 1 was deployed 0 degree days after bud break (synchrony), while hatch cohort 2 was deployed 100 degree days (~ 11 to 13 days) after bud break. These timepoints coincide with windows of high and low foliar quality, and represent ecologically realistic larval emergence times, as gypsy moth hatch can vary 2–3 weeks among egg masses (Montgomery 1991), and aspen bud break can vary up to 3 weeks among genotypes growing in a common location (Donaldson and Lindroth 2008).

Gypsy moth egg masses were obtained from the USDA APHIS insect production facility at Otis Air National Guard Base in Buzzards Bay, MA, surface sterilized in a dilute sodium hypochlorite solution to prevent potential nuclear polyhedrosis virus transmission from egg masses to larvae (Havill and Raffa 1999), and incubated in a growth chamber (25 °C, 50–70% humidity, 16:8 L:D) until hatch. Approximately 24 h after hatching, neonate larvae were haphazardly selected from five separate egg masses, placed in microcentrifuge tubes, and transported to the field site. Tubes were fastened to a common branch of intermediate crown position and uncapped, such that 50 neonate larvae from five separate egg masses deployed onto each tree. Groups of larvae were confined to a single branch by securing two layers of nylon mesh sleeves to basal and distal branch ends, with plastic cable ties.

Following deployment, larval mortality and instar were visually assessed every 2–3 days. To account for variation in development rates among individuals feeding on the same ramet, development time was calculated as a weighted mean based on the proportion of individuals that molted at a given degree-day threshold. Final development times were expressed as the total degree days accumulated between egg hatch and the completion of the second instar (L2). When all larvae on a given tree completed L2, branches containing each mesh sleeve were harvested and returned to the lab. Approximately 1 month elapsed from the deployment of the first hatch cohort to the final branch harvest. Upon returning to the lab, we dissected each mesh sleeve and recorded the number of carcasses, the instar of each carcass, and the number of surviving L3 individuals. Survival was expressed as the percent of surviving individuals out of the total number of individuals recovered. We recovered an average of over 46 out of the 50 (range 40–50) individuals deployed per branch sleeve. Recovered L3 larvae were frozen and dried in an oven at 50 °C for 48 h. Ten dried larvae from each replicate tree were selected at random and weighed. Larval weights were then divided by development times (degree days) to calculate growth rates. Final destructive harvest assessments of larval performance closely matched our field estimates of larval performance recorded throughout the experiment.

Statistical analyses

All statistical analyses were conducted using R Statistics. Repeated-measures ANOVAs were conducted using the ‘lme4’ and ‘lmerTest’ packages to analyze the effects of genotype, time, and their interaction on concentrations of foliar phenolic glycosides and nitrogen. F tests were conducted using the Satterthwaite approximation for degrees of freedom. For each genotype, timescales were normalized by degree days after bud break to allow comparability. For analysis of 2015 phenolic glycosides, time was analyzed as a continuous variable to account for variation in sampling times among genotypes. For analysis of 2015 nitrogen, 2016 nitrogen, and 2016 phenolic glycoside concentrations, sampling times were standardized among genotypes, and time was considered a discrete, fixed effect. Genotype was considered a fixed effect in all analyses.

To quantify phytochemistry encountered by larvae throughout their development, we plotted concentrations of phenolic glycosides and nitrogen against accumulated degree days for each tree, fitted these plots with piecewise polynomial curves, and calculated their integrals. We fit these curves using the Fritsch–Carlson method for monotone cubic-spline interpolation (Fritsch and Carlson 1980) via the ‘splines’ package in R Statistics. For phenolic glycoside curves, we added a constant ($c = 1$) to avoid interpolating values less than or equal to zero. Lower and upper limits of integration were assigned to degree-day values at which larvae were deployed and completed L2, respectively. Integral values of phenolic glycoside and nitrogen

concentrations (hereafter referred to as "∫phenolic glycosides" and "∫nitrogen"), therefore, represent a cumulative total concentration of these compounds present throughout a given window of insect development. This approach allowed us to better compare phytochemical patterns that depend on multiple time points with cumulative insect performance metrics. We then used two-way ANOVA to analyze how ∫phenolic glycoside and ∫nitrogen concentrations varied among genotypes, between hatch cohorts, and their interaction. To evaluate significant differences in foliar chemical concentrations encountered by different treatment groups, we applied Tukey's HSD tests at $\alpha = 0.05$.

We used correlation analyses to explore potential trade-offs between early spring phenolic glycoside concentrations and rates of leaf expansion. Pearson's correlation coefficients were calculated to assess relationships between rates of foliar dry weight increase and ∫phenolic glycoside concentrations. Lower and upper limits of integration were assigned to degree-day values at which bud break occurred, and final leaf samples were collected, respectively. Rates of increase in dry leaf weight were calculated as (final dry leaf weight − initial dry leaf weight)/time.

To test for potential induction effects following insect feeding, we compared changes in phenolic glycoside concentrations among genotypes between the first two sampling timepoints using a repeated-measures ANOVA. During this period, insects were feeding on trees that received hatch cohort 1, but absent on trees that received hatch cohort 2, so any measurable induction effects would be apparent.

To compare temporal patterns of gypsy moth performance among aspen genotypes throughout leaf expansion, we analyzed field estimates of larval survival from hatch cohorts 1 and 2 separately. For each hatch cohort, we used repeated-measures ANOVA to analyze the effects of genotype, time, and their interaction on larval survival. Time was measured as the amount of degree days accumulated after larval deployment, and analyzed as a continuous variable to account for variation in observation times among genotypes.

We used two-way ANOVA to analyze variation in final larval performance data among aspen genotypes, hatch cohorts, and their interaction. To evaluate significant differences in larval performance among treatment groups, we applied Tukey's HSD tests at $\alpha = 0.05$. We then used Pearson correlation analyses to evaluate relationships between larval performance and ∫phenolic glycoside and ∫nitrogen concentrations encountered by larvae in each hatch cohort. Phytochemical concentrations encountered by larvae were calculated using the same integration methods described above, but limits of integration were adjusted to correspond with degree-day intervals over which each insect performance metric was measured.

Results

Foliar chemical patterns

In 2015, foliar phenolic glycoside concentrations varied significantly among sampling dates and genotypes (Fig. 1). Among all genotypes, concentrations were generally highest during the approximately first 50 degree days after bud break (~ 4 days), and fell to their lowest levels within the next 150 degree days (~ 15 days). Aside from these general similarities, temporal patterns of phenolic glycoside concentrations varied substantially among genotypes, resulting in a significant time by genotype (T × G) interaction. This variation was most apparent during the first 100 degree days after bud break, in which two divergent patterns emerged. Four genotypes (3, 4, 6, 8) exhibited sharp decreases in foliar phenolic glycoside concentrations < 25 degree days after bud break (~ 2 days). The mean magnitudes of these decreases ranged from 3.5 to 6.7% dry weight (dw) among genotypes. The remaining genotypes (1, 2, 5, and 7) exhibited moderate increases in foliar phenolic glycoside concentrations immediately following bud break, ranging from mean magnitudes of 0.8–2.6% dw. These concentrations peaked approximately 50 degree days after bud break (~ 4 days) at means ranging from 4.1 to 13.4% dw, depending on genotype, and fell to their lowest levels at approximately 175 degree days after bud break. Among all genotypes, the early differences in phenolic glycoside concentrations gradually converged to more similar values as the season progressed.

Temporal patterns of foliar phenolic glycoside concentrations were generally consistent between 2015 and 2016 (Fig. 1). As in 2015, phenolic glycoside concentrations varied significantly among sampling dates and genotypes, and exhibited a significant T × G interaction. Rank orders of mean phenolic glycoside concentrations present at bud break were consistent among genotypes across both years, although mean concentrations were 2.7–4.4% dw higher in 2016. Genotypes 3 and 5 exhibited observable differences in post-bud break foliar phenolic glycoside patterns between 2015 and 2016. Immediately following bud break, phenolic glycoside concentrations in genotype 3 decreased by 6.7% dw in 2015, but decreased by only 2.4% dw in 2016. Conversely, genotype 5 exhibited a 2.6% dw decrease in phenolic glycosides in 2015 immediately following bud break, but increased by 2.6% dw in 2016. ∫phenolic glycoside concentrations were positively correlated with rates of leaf weight increase among genotypes ($r = 0.54$, $P < 0.001$).

In 2015 and 2016, foliar nitrogen concentrations varied significantly over time, among genotypes, and exhibited a significant T × G interaction (Fig. 1). Sampling date,

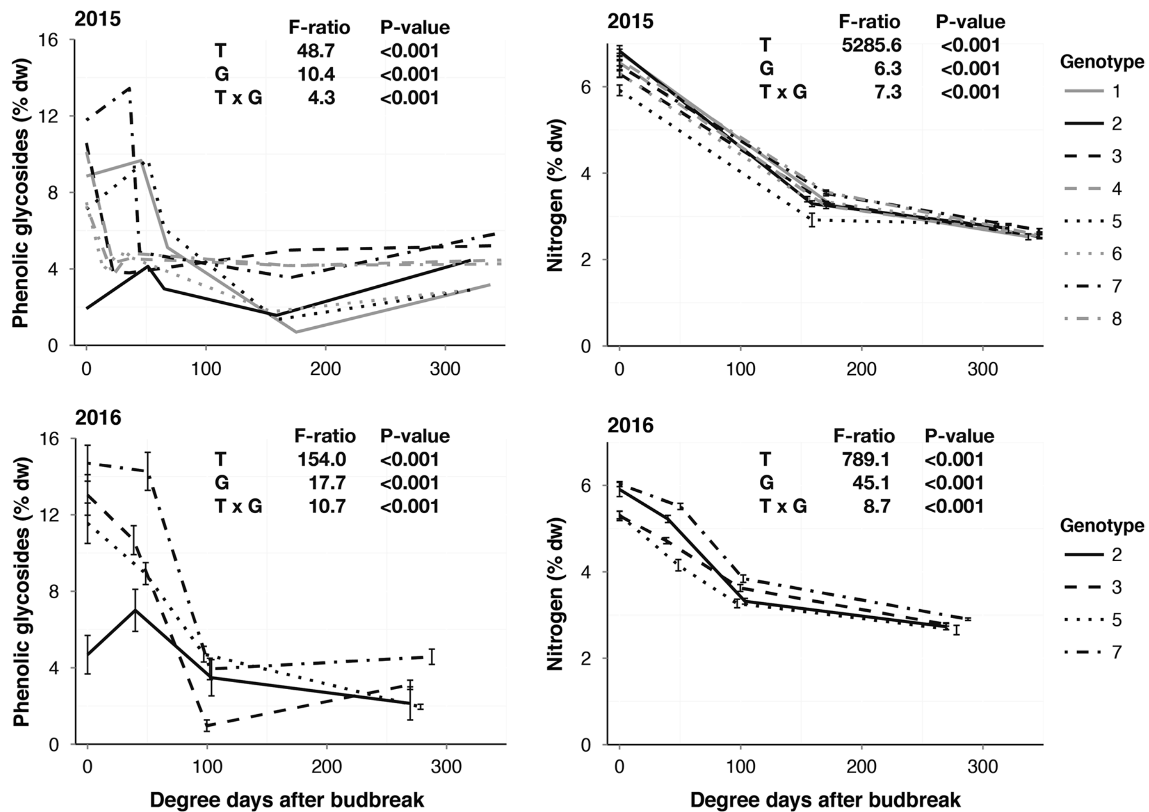

Fig. 1 Variation in concentrations (% dry weight) of phenolic glycosides and nitrogen among aspen genotypes ("G") over time ("T") during leaf expansion in 2015 and 2016. Black lines represent genotypes included in both 2015 and 2016 studies, while gray lines represent genotypes included in only the 2015 study. Timescales were normalized by degree days after bud break to allow direct comparisons among genotypes and years. Vertical lines represent ± 1 SE. For 2015 phenolic glycoside data, SE values ranged from 0.09 to 1.71, but vertical lines are not included to allow clearer comparisons of patterns among genotypes. In 2015, $n = 6$ ramets per genotype. In 2016, $n = 11–12$ ramets per genotype. F ratios and P values indicate the results of repeated-measures ANOVA

however, accounted for much more variation than any other explanatory variable. In both years, nitrogen concentrations were the highest in newly flushed foliage, with mean maximum levels of approximately 6.5% dw in 2015, and 5.6% dw in 2016. These concentrations then decreased to approximately half their initial levels by ~ 300 degree days after bud break (base temperature = 4.4 °C).

Leaf toughness

In 2015, leaf toughness varied significantly over time and exhibited a significant T × G interaction (Fig. 2). Mean leaf toughness varied by approximately 57% among genotypes, although these differences were only marginally significant. Leaf toughness remained uniformly low (< 50 g) among all genotypes until approximately 150–200 degree days after bud break, at which time means increased to approximately 100–200 g, depending on genotype. These increases began to level off at approximately 300 degree days after bud break.

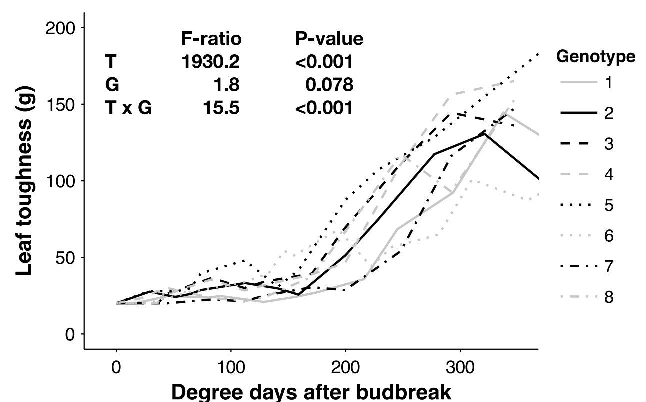

Fig. 2 Variation in leaf toughness among aspen genotypes ("G") over time ("T") during leaf expansion in 2015. Black lines represent genotypes included in both the 2015 and 2016 studies, while gray lines represent genotypes included in only the 2015 study. Timescales were normalized by degree days after bud break to allow direct comparisons between genotypes. SE values ranged from 0.00 to 16.63, but vertical lines are not included to allow clearer comparisons of patterns among genotypes. $N = 6$ ramets per genotype. F ratios and P values indicate the results of repeated-measures ANOVA

Foliar chemistry encountered by gypsy moth larvae

Throughout larval development, ∫phenolic glycoside concentrations varied among aspen genotypes by up to 85% (Fig. 3). Larvae that hatched in synchrony with bud break (hatch cohort 1) encountered foliage with more than 67% higher ∫phenolic glycoside concentrations than did larvae that were deployed 100 degree days after bud break (hatch cohort 2), although this difference was significant at only $P=0.096$.

During the first 100 degree days after bud break, phenolic glycoside patterns did not differ significantly among aspen receiving hatch cohorts 1 or 2. This result suggests little to no herbivore-induced defense responses occurred. Trees within a given genotype, therefore, exhibited similar patterns of foliar chemistry, regardless of which hatch cohort they received.

In addition, throughout larval development, ∫nitrogen concentrations varied significantly among genotypes and between hatch cohorts (Fig. 3). ∫Nitrogen concentrations varied among genotypes by up to 24%. Gypsy moth neonates that were deployed in synchrony with bud break (hatch cohort 1) encountered foliage with over 30% higher ∫nitrogen concentrations than did larvae that were deployed 100 degree days after bud break (hatch cohort 2). Nitrogen concentrations varied more than 30% among genotypes throughout the duration of hatch cohort 1 development, but varied less than 23% among genotypes throughout the duration of hatch cohort 2 development (significant G × HC interaction).

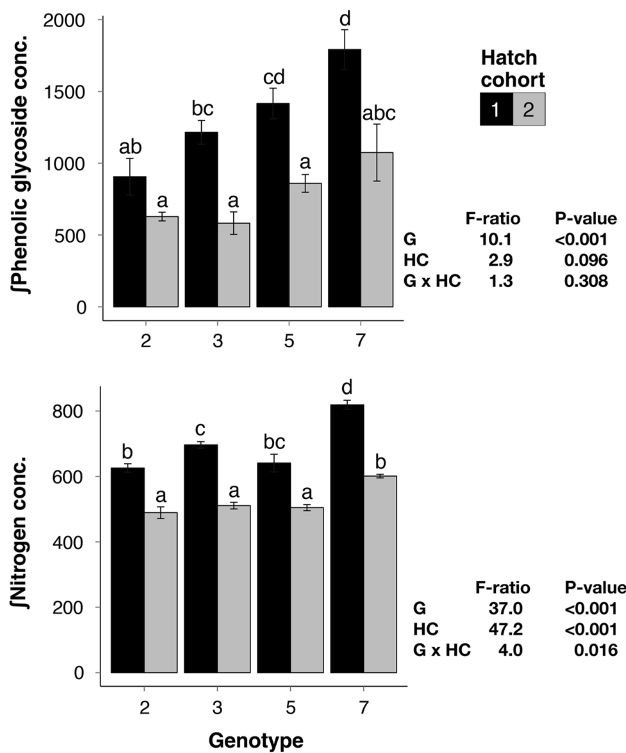

Fig. 3 ∫Phenolic glycoside and ∫nitrogen concentrations (% dry weight integrated over time) encountered by gypsy moth neonates deployed in hatch cohort ("HC") 1 (synchrony with bud break) and hatch cohort 2 (100 degree days after bud break), among aspen genotypes ("G") in 2016. Lower and upper limits of integration were assigned to degree-day values at which larvae hatched, and completed L2, respectively. Error bars represent ±1 SE. $n=5$–6 ramets per genotype. F ratios and P values indicate the results of a two-way ANOVA. Columns with different letters are significant at $P<0.05$ (Tukey's HSD test)

Gypsy moth larval performance

Field estimates of gypsy moth performance revealed that larval survival varied significantly over time and among aspen genotypes for larvae that hatched in synchrony with bud break (hatch cohort 1, Fig. 4). Mean survival generally declined during the first 100 degree days (~ 11 to 13 days) after hatch, and stabilized throughout the remainder of the experiment. Magnitudes and rates of decline in larval survival, however, varied among genotypes (significant T × G interaction). Among larvae that hatched 100 degree days after bud break (hatch cohort 2), survival varied significantly over time and among aspen genotypes. Mean survival declined slightly among all genotypes within the first 50 degree days (~ 5 to 6 days) after hatch, but remained above 96% throughout the duration of the experiment.

Final destructive harvests of branch sleeves indicated that first instar (L1) survival varied significantly among genotypes (Fig. 5). L1 survival was an average of 12.7% lower among larvae that hatched in synchrony with bud break (hatch cohort 1) than larvae that hatched 100 degree days after bud break (hatch cohort 2), although this difference was

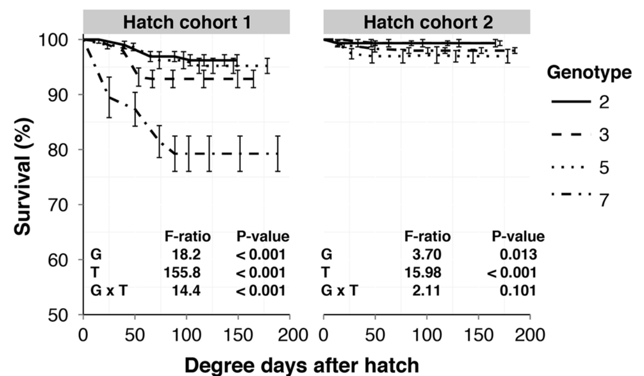

Fig. 4 Field estimates of survival rates among gypsy moth larvae feeding on four unique aspen genotypes ("G") over time ("T") during leaf expansion in 2016. Larvae were deployed in synchrony with bud break (hatch cohort 1) or 100 degree days after bud break (hatch cohort 2). F ratios and P values indicate the results of repeated-measures ANOVA. Within each hatch cohort, n = 5–6 ramets per genotype. Error bars represent ±1 SE

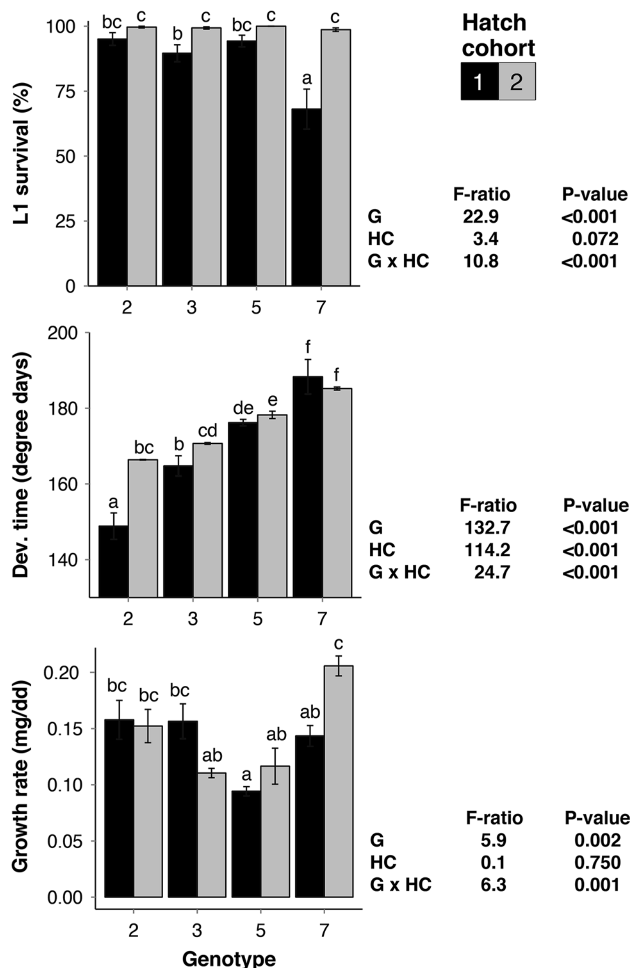

Fig. 5 First instar (L1) survival (%), development (dev.) time (degree days), and growth rate (mg/degree day) among aspen genotypes ("G") and hatch cohorts ("HC") in 2016. Development time and growth rate correspond to the period between egg hatch and completion of L2. Error bars represent ± 1 SE. For each column, $n = 5$–6. *F* ratios and *P* values indicate the results of a two-way ANOVA. Columns with different letters are significant at $P < 0.05$ (Tukey's HSD test)

only marginally significant. L1 survival varied substantially among genotypes in hatch cohort 1, but was uniformly high among genotypes in hatch cohort 2 (significant G × HC interaction). L1 survival was inversely correlated with $\int$phenolic glycoside concentrations between hatch cohorts 1 and 2, and with $\int$nitrogen concentrations in hatch cohort 1 (Fig. 6). L2 survival rates were greater than 98% among all genotypes and between hatch cohorts, although differences among genotypes were statistically significant.

Development time varied significantly among genotypes and between hatch cohorts (Fig. 5). Larvae in hatch cohort 1 completed L2 an average of over 6.4 degree days faster than those in hatch cohort 2. Genotype, however, explained over 63% of the variation in development times, while hatch cohort explained approximately 18%. Larvae in hatch cohort 1 completed L2 either at the same time as, or significantly faster than larvae in hatch cohort 2, depending on genotype (significant G × HC interaction). Development time was positively correlated with $\int$phenolic glycoside concentrations between hatch cohorts 1 and 2, and with $\int$nitrogen concentrations in hatch cohort 1 (Fig. 6).

Growth rate varied by up to 65% among genotypes (Fig. 5). Larvae grew at similar rates between both hatch cohorts, with the exception of those feeding on genotype 7, for which larvae in hatch cohort 1 developed 43% slower than those in cohort 2 (significant G × HC interaction). Growth rate was not correlated with $\int$phenolic glycoside concentrations between either hatch cohort, but was positively correlated with $\int$nitrogen concentrations in hatch cohort 2 (Fig. 6).

Discussion

Our results demonstrate that rapid, genetically variable changes in aspen phytochemistry occur immediately after bud break, and structure windows of opportunity for an important spring-feeding folivore. We found that phenolic glycoside concentrations were highest immediately after bud break and decreased after only a few days (Fig. 1). Gypsy moth larvae that hatched in synchrony with bud break encountered peak phenolic glycoside concentrations, and exhibited lower survivorship than larvae that hatched 100 degree days (11–13 days) later (Figs. 1, 5). Significant genetic variation in the magnitude and duration of these initial concentrations corresponded with variation in larval survivorship among aspen genotypes. In contrast to phenolic glycosides, nitrogen concentrations declined at a gradual, relatively uniform rate regardless of genotype (Fig. 1). High $\int$nitrogen concentrations seemed to partially offset the sublethal effects of high $\int$phenolic glycoside concentrations, as larvae that hatched in synchrony with bud break developed as fast as, or faster than, larvae that hatched 100 degree days after bud break, depending on host genotype (Fig. 5).

These findings elucidate some important new components of how phenological synchrony influences interactions between plants and insect herbivores. Gypsy moth neonates that hatched in synchrony with bud break encountered 30% higher $\int$nitrogen concentrations than larvae that hatched 100 degree days after bud break, supporting the prediction that synchrony between host bud break and spring-folivore emergence maximizes foliar protein availability (Feeny 1970; Hunter and Lechowicz 1992; Osier et al. 2000). However, the early peak in phenolic glycoside concentrations immediately after bud break is not consistent with the classic view that phenolic defense chemicals are lowest at bud break and accumulate over time (Feeny 1970). Some other studies have likewise reported moderate declines in secondary metabolite

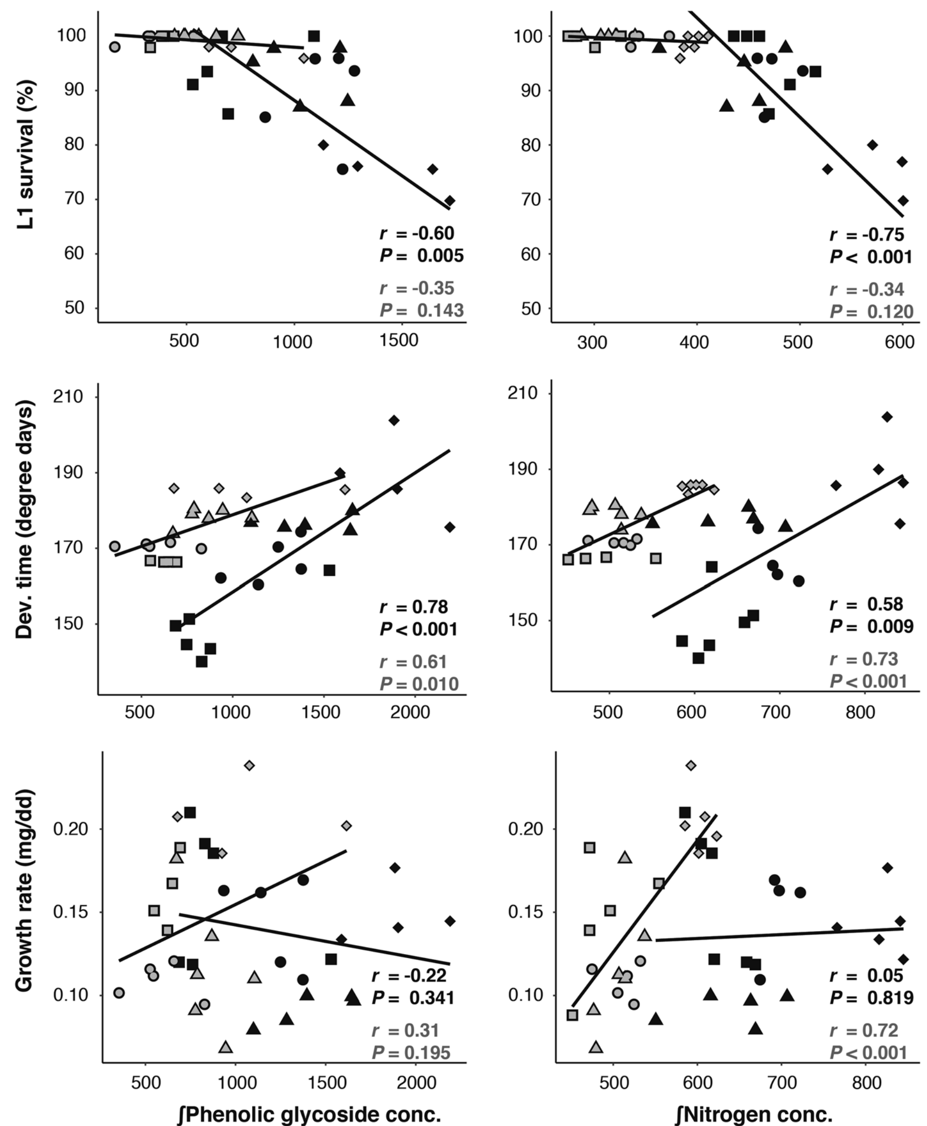

Fig. 6 Regressions of ∫phenolic glycoside and ∫nitrogen concentrations (% dry weight integrated over time) against first instar (L1) survival, development (dev.) time, and growth rate in 2016. Square, circle, triangle, and diamond symbols represent ramets from genotypes 2, 3, 5, and 7, respectively. Black and gray symbols represent hatch cohorts 1 (synchrony with bud break) and 2 (100 degree days after bud break), respectively. Limits of integration correspond with degree-day intervals over which L1 survival, development time, and growth rate were measured. Development time represents total degree days accumulated between egg hatch and completion of L2

concentrations over the course of a growing season (Salminen et al. 2004; Barber and Fahey 2015; Chernyak et al. 2016), or between immature and mature stages of foliage (Meyer and Montgomery 1987; Wait et al. 1998; Koricheva and Barton 2012). Our data, however, indicate that substantial declines in aspen phenolic glycoside concentrations can occur in as little as 48 h during aspen leaf expansion (Fig. 1), a time interval that falls within empirically predicted windows of climate change-induced phenological shifts (Schwartzberg et al. 2014), and affect the performance of insects hatching synchronously and asynchronously with bud break.

As anticipated, ∫phenolic glycoside concentrations were positively correlated with development time within both hatch cohorts (Fig. 6). Furthermore, ∫phenolic glycoside concentrations were negatively correlated with survival among neonates in hatch cohort 1 but not 2 (Fig. 6). This difference in survival trends between hatch cohorts supports previous findings that lepidopteran folivore performance decreases when phenolic glycoside concentrations surpass a minimum threshold (Lindroth and Hemming 1990; Lindroth and Bloomer 1991), as neonates in hatch cohort 1 encountered a higher range of phenolic glycoside concentrations than did those in hatch cohort 2.

Spring-feeding folivores appear to face a trade-off between maximizing protein consumption and minimizing exposure to chemical defense, as seasonal concentrations of both chemical groups are highest shortly following bud break in most tree species (Koricheva and Barton 2012). In contrast to phenolic glycosides, high concentrations of dietary nitrogen typically improve larval performance (Lindroth and Bloomer 1991; Stockhoff 1992; Lindroth et al. 1997). Paradoxically, we observed the opposite relationship, as both ∫nitrogen and ∫phenolic glycosides were negatively

correlated with L1 survival and positively correlated with development time (Fig. 6). Because high nitrogen concentrations are unlikely to negatively impact larvae, we attribute these trends to high collinearity between $\int$nitrogen and $\int$phenolic glycoside concentrations ($r=0.77$, $P<0.001$; Fig. S2). The negative effect of high phenolic glycoside concentrations appears to have overridden the otherwise beneficial effect of high nitrogen concentrations on larval performance. Artificial diet bioassays have reported similar outcomes: gypsy moth larvae fed high nitrogen, high phenolic glycoside diets perform poorly relative to those fed low nitrogen, and low phenolic glycoside diets (Lindroth and Hemming 1990; Hemming and Lindroth 2000). Consistent with this interpretation, growth rate was not correlated with $\int$nitrogen concentrations among larvae in hatch cohort 1, when $\int$phenolic glycoside concentrations were high, but was strongly positively correlated with $\int$nitrogen concentrations in cohort 2, when $\int$phenolic glycoside concentrations were low (Fig. 6).

The extent to which chemical defenses can offset the benefits of high nitrogen concentrations in newly flushed foliage depends on genotype-specific patterns of post-bud break secondary chemistry. For example, larvae that hatched in synchrony with bud break on genotypes with high phenolic glycoside concentrations in newly flushed foliage exhibited longer development times and lower survivorship than larvae that hatched later (Fig. 6). In this scenario, the negative effects of high phenolic glycoside consumption outweighed the positive effects of a high nitrogen food. On aspen genotypes with lower phenolic glycoside concentrations in newly flushed foliage, however, larvae that hatched in synchrony with bud break benefitted from high nitrogen concentrations and developed faster than those that hatched 100 degree days after bud break. Optimal egg hatch timing can, therefore, differ substantially among plant genotypes based on differences in underlying temporal phytochemical patterns.

Spring-feeding folivores are assumed to have evolved to hatch in synchrony with ephemeral windows of high nitrogen concentrations in newly flushed foliage, but few studies have considered the possibility that seasonal patterns of secondary chemistry may have evolved as counter adaptations to annual threats of spring herbivory. Here, we demonstrate that temporary windows of high, early season defense chemical concentrations can offset the benefits of high nitrogen concentrations for larvae feeding on newly flushed foliage by increasing mortality and development time (Fig. 6). By doing so, some aspen genotypes avoided offering a substantial "window of opportunity" to gypsy moth larvae. This strategy restricts high chemical defense concentrations to periods in which foliage is most susceptible to herbivory, potentials for long-term photosynthetic loss are greatest (Harper 1989), and larvae are most sensitive to defense chemistry (Lindroth and Hemming 1990; Zalucki et al. 2002). These ideas entail an extension of the optimal defense hypothesis (McKey 1974; Rhoades 1979), which suggests that plants adaptively prioritize defense investment at the expense of growth in high value tissues that are subject to frequent herbivory. Although phenolic glycoside biosynthesis and leaf tissue development each demand newly assimilated carbon (Kleiner et al. 1999; Massad et al. 2014), we found that $\int$phenolic glycosides were positively correlated with rates of leaf weight increase among genotypes ($r=0.54$, $P<0.001$).

Recent studies suggest that host bud break will advance faster than folivore egg hatch in response to warming temperatures (Schwartzberg et al. 2014). Host-specific bud break phenology will influence whether this shift increases or decreases synchrony between egg hatch and bud break. Our data, however, indicate synchrony, alone, cannot predict how phenological shifts will affect folivore performance; variation in temporal patterns of phytochemistry must also be considered. Aspen bud break can vary up to 3 weeks among genotypes (Donaldson and Lindroth 2008). Among early flushing genotypes, egg hatch will likely occur later with respect to bud break under future conditions. As a result, larvae feeding on genotypes that are poorly defended during leaf expansion may miss valuable windows of high nitrogen concentrations, while larvae feeding on genotypes that are well defended during leaf expansion may avoid windows of high defense. Among late-flushing genotypes, however, egg hatch will likely occur in closer synchrony with bud break under future conditions. In this scenario, larvae feeding on genotypes that are poorly defended during leaf expansion will gain access to windows of high nitrogen concentrations, while larvae feeding on genotypes that are well defended during leaf expansion will emerge in synchrony with windows of high defense.

Understanding how these plant-level responses will affect herbivore population-level responses in a warming climate requires integration with stand composition. Tikkanen and Julkunen-Tiitto (2003) demonstrated that stand-level variation in host-tree phenology can prevent folivores from adapting to a consistent bud break date. Variation in temporal patterns of phytochemistry may affect herbivory similarly by preventing folivores from adapting to a consistent phytochemical pattern. Inter- and intra-specific variation in both temporal patterns of foliar chemistry and bud break phenology may, therefore, contribute to stand-level resilience under future herbivory regimes. Our data suggest that trembling aspen, in particular, exhibits substantial intra-specific variation in these traits, which may prevent spring-feeding folivores from easily adapting to climate change-induced shifts in phenological relationships.

Responses of other spring-folivore species to temporal variation in aspen phytochemistry could differ from those of gypsy moth. As a generalist, gypsy moth is more susceptible

to phenolic glycosides than some aspen specialists, such as the Canadian swallowtail butterfly (*Papilio canadensis* Roths. and Jordan) (Lindroth et al. 1986). Among specialists, the benefits of high nitrogen concentrations in newly flushed foliage likely outweigh the negative effects of high phenolic glycoside concentrations. These specialists may, therefore, exhibit more consistent decreases in performance if warming temperatures drive egg hatch to occur later with respect to bud break, as nitrogen concentrations decline at relatively uniform rates among aspen genotypes (Fig. 1) and host-tree species (Hunter and Lechowicz 1992). In addition, stand-level variation in bud break phenology likely hinders specialist performance to a greater extent than stand-level variation in phytochemical patterns, as the former influences nitrogen availability to a stronger degree.

Our results demonstrate that short-term, temporal patterns of foliar chemistry comprise an additional dimension to how phenological timing will mediate plant–herbivore interactions within a climate change context. These patterns can vary substantially among host genotypes and strongly impact performance among spring-folivores hatching at different intervals relative to bud break. Future work should evaluate the extent to which these traits vary within and among other host-tree species, and assess their impacts on the performance of other insect folivore species. Evaluating how these temperature-dependent factors affect herbivory will further our understanding of plant–herbivore dynamics under both current and future conditions.

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Author contribution statement MAF, RLL, KKR, and KFR contributed to overall question and experimental design. MAF and KKR performed chemical analyses. MAF performed bioassays and statistical analyses. MAF wrote the manuscript and all co-authors provided editorial advice. KFR and RLL secured research funding.

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Supplemental Figure 1: Variation in dry weight concentrations of phenolic glycosides and nitrogen among aspen genotypes throughout leaf expansion in 2015 and 2016. For each genotype, timepoint 1 represents the accumulated degree day thresholds at which budbreak took place. In 2015, n=6 ramets per genotype. In 2016, n = 11-12 ramets per genotype.

Supplemental Figure 2: Regressions of ∫nitrogen concentrations against ∫phenolic glycoside in 2016. Square, circle, triangle, and diamond symbols represent ramets from genotypes 2, 3, 5, and 7, respectively. Black and gray symbols represent hatch cohorts 1 (synchrony with budbreak) and 2 (100 degree days after budbreak), respectively. Limits of integration correspond with degree days accumulated between egg hatch and completion of L2.

Figure S1.

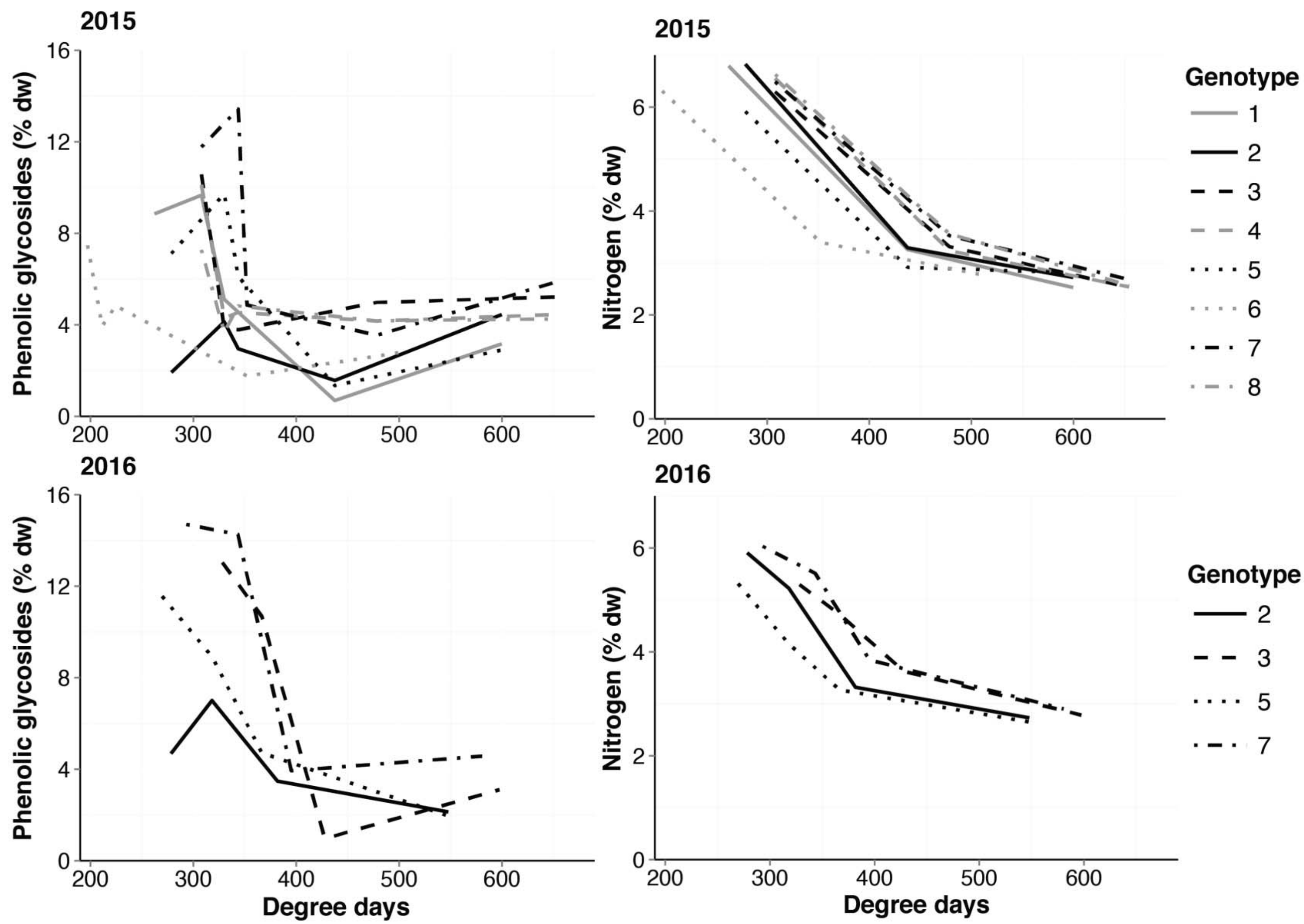

Figure S2.