Metabolomic responses of indigenous and nonindigenous plants to deer exclosure fencing and deer herbivory in a suburban forest

Janet A. Morrison[1] and Melkamu Woldemariam[1]

[1]The College of New Jersey

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Abstract

Trees and shrubs in suburban forest understories can be subject to chronic herbivory from abundant white-tailed deer. An undocumented consequence of this stress may be shifts in secondary metabolite production associated with defense. We aimed to learn whether plants protected from deer exhibited different metabolomic profiles compared to those exposed to deer. We tested the indigenous species *Nyssa sylvatica* and *Lindera benzoin* and the invasive, nonindigenous species *Rosa multiflora* and *Euonymus alatus* within a suburban forest understory in New Jersey, USA, in unfenced plots and plots fenced for 5.3 years. We did untargeted metabolomics by sampling leaves from three plants of each species per 6-7 fenced and unfenced plots, conducting chloroform-methanol extractions followed by LC-MS/MS, and conducting statistical analysis on Metaboanalyst. We also scored each species for deer browse frequency over eight years, and compared their heights and percent cover between unfenced and fenced plots. The analysis identified 2,333 metabolites. The global metabolome diverged significantly between fenced and unfenced plots pooled across species, but for individual species only *N. sylvatica* exhibited a significant fencing effect. *Nyssa sylvatica* was one of the most browsed species and was the only one with both greater cover and height in fenced plots, suggesting greater susceptibility to deer browsing. The metabolites most responsible for the fenced/unfenced divergence also were affected by the species-fencing combination, with increases in certain species but decreases in others. The most significant metabolites that were upregulated in fenced plants include some involved in defense-related metabolic pathways, e.g. monoterpenoid biosynthesis. Further study of more species in multiple sites is needed to learn how common metabolomic responses to deer are among forest species, how the intensity of deer pressure influences the responses, which types of metabolites are most affected, and if there are ecological consequences at the physiological, population, and/or community levels.

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Janet A. Morrison, Department of Biology, The College of New Jersey, Ewing, NJ 08628

morrisja@tcnj.edu (corresponding author)

Melkamu Woldemariam, Department of Biology, The College of New Jersey, Ewing, NJ 08628

woldemam@tcnj.edu

ORCiD

Janet A. Morrison 0000-0001-5332-9086

Melkamu Woldemariam 0000-0002-0086-0532

ABSTRACT

Trees and shrubs in suburban forest understories can be subject to chronic herbivory from abundant white-tailed deer. An undocumented consequence of this stress may be shifts in secondary metabolite produc-

tion associated with defense. We aimed to learn whether plants protected from deer exhibited different metabolomic profiles compared to those exposed to deer. We tested the indigenous species *Nyssa sylvatica* and *Lindera benzoin* and the invasive, nonindigenous species *Rosa multiflora* and *Euonymus alatus* within a suburban forest understory in New Jersey, USA, in unfenced plots and plots fenced for 5.3 years. We did untargeted metabolomics by sampling leaves from three plants of each species per 6-7 fenced and unfenced plots, conducting chloroform-methanol extractions followed by LC-MS/MS, and conducting statistical analysis on Metaboanalyst. We also scored each species for deer browse frequency over eight years, and compared their heights and percent cover between unfenced and fenced plots. The analysis identified 2,333 metabolites. The global metabolome diverged significantly between fenced and unfenced plots pooled across species, but for individual species only *N. sylvatica* exhibited a significant fencing effect. *Nyssa sylvatica* was one of the most browsed species and was the only one with both greater cover and height in fenced plots, suggesting greater susceptibility to deer browsing. The metabolites most responsible for the fenced/unfenced divergence also were affected by the species-fencing combination, with increases in certain species but decreases in others. The most significant metabolites that were upregulated in fenced plants include some involved in defense-related metabolic pathways, e.g. monoterpenoid biosynthesis. Further study of more species in multiple sites is needed to learn how common metabolomic responses to deer are among forest species, how the intensity of deer pressure influences the responses, which types of metabolites are most affected, and if there are ecological consequences at the physiological, population, and/or community levels.

Key words: ecometabolomics, white-tailed deer, browse pressure, invasive plants, suburban forests

1 | Introduction

Suburban landscapes consist of a mix of human-created infrastructure and fragmented natural communities. In the temperate deciduous forest biome of northeastern North America, small forest stands are common components of the many parks, preserves, and private holdings in suburban areas. In conjunction with the lawns and fields that also commonly occur in suburbia, this landscape provides ideal habitat for white-tailed deer (*Odocoileus virginianus*) . Hunting is very limited in suburbia; consequently, deer densities can be extremely high . The influence of high deer pressure on suburban forest species is of particular interest because of the huge extent of urbanizing landscapes; suburban forests now contain a large share of many regions' biodiversity .

Many consequences of very high deer densities for forest plants and their natural communities are well documented. For example, herbivory by deer causes tissue loss that can lead to increased mortality rates or decreased reproduction; they may eat entire plants in the form of seedlings or seeds like oak acorns; seedlings can be trampled by deer, and deer can otherwise disturb the forest floor as they move across it and bed down at night (reviewed in . These effects vary with the level of deer browse pressure and among plant species . In very severely browsed forests, there may be extremely low abundance of any plants below the browse line, which increases sunlight penetration and recruitment opportunities for less shade tolerant plants, and has a cascade of other indirect effects in the forest community . Defense and stress responses of long-lived, woody plant species also are likely to be strongly affected by high deer browse pressure, but this possibility has not been well studied. Here, we compared the metabolomic profiles of indigenous and nonindigenous woody species that had been exposed to or protected from deer in a forest community in suburban New Jersey, USA.

Woody plants have characteristics particularly attractive and vulnerable to deer. They are chronically exposed to repeated browsing, they have green foliage throughout the growing season or even year-round for evergreens, and their buds provide highly nutritious forage throughout the winter. Thus, high deer browse pressure is associated with depletion of the woody component of forest understories .

Not surprisingly, woody species invest in various mechanical and chemical defenses that deter browsing . Deer browse has been demonstrated to be greater when defense levels are lower , and induced defense responses can occur within individuals in response to deer browse . Additionally, the physiological costs of producing defenses against browse can affect plant fitness , resulting in evolutionary responses at the population level

over time . In some instances, on the other hand, plant chemical defense may be unaffected by deer browse or may even decrease . In suburban forests, where deer browse pressure is particularly high, effects on chemical defenses of long-lived woody plants are likely strong and, as demonstrated for the more commonly studied woody plant defenses against insect herbivory , defenses against deer browse could also influence allocation of resources to plant growth and reproduction .

Advances in metabolomic research have increasingly enabled investigation of metabolome-wide responses of plants to stressors , with growing application of metabolomics to plants in natural ecological communities . There has been limited ecometabolomic research on woody plants so far, but the studies have been wide-ranging, including investigations of abiotic drivers of population or species variation in plant chemistry , the role of plant defenses in phylogenetic diversification , metabolomic niche differentiation among sympatric tropical species , ontological variation in foliar chemistry , and disease resistance . However, there has been very little attention paid to plant metabolomics associated with deer herbivory, with just one study, showing that white-tailed deer browse less frequently on nonindigenous invasive plants that are chemically dissimilar to indigenous plants in the community .

Significant quantitative and qualitative variations are commonly observed in defense responses of different plant species; the final defense phenotypes result from genetic factors and/or environmental (light, nutrient availability, geography, etc.) gradients . Additionally, the type of defense strategy that a plant species employs is optimized by the severity of herbivore pressure and the feeding mode of the attacking herbivore (chewing herbivores vs piercing-sucking herbivores) , as well as the plant's mode of growth; i.e. annuals versus perennials or fast-growing versus slow-growing species . Therefore, variation among species' chemical defense responses to abundant deer is expected, and could have important consequences for forest community structure. Species that are less preferred or even avoided by deer altogether may have an ecological advantage beyond avoidance of tissue loss. Many secondary metabolites, especially those rich in nitrogen, are costly for plants to produce , so if upregulation of defense-related chemistry is a consequence of heavy, chronic deer browsing, browsed plants could experience added stress. If severe enough, these metabolic costs could contribute to shifts in species dominance within the community. Since some nonindigenous invasive plant species are less preferred by deer compared to indigenous species , it is even possible that costly, metabolome-wide responses to deer in browsed plants could facilitate invasion by nonindigenous plant species that deer avoid. Indeed, deer are acknowledged as invasion facilitators in some cases , but the possible role of metabolomic responses to deer in this facilitation has not been considered until recently . Despite the multiple hypotheses put forward to explain the variability in defense phenotypes in plant communities and the growth-defense tradeoff , studies that compare the global metabolomic responses and/or constitutive/induced defense responses of indigenous and nonindigenous species are lacking.

A first step in addressing the consequences for plant communities of metabolome-wide responses to high deer pressure is to document the metabolomic profiles of a variety of species in a community. In our study, we tested four long-lived, woody or semi-woody species that occur in one forest preserve, both in plots that had been fenced for over five years to exclude deer and in plots that were open to deer. Two species are indigenous (*Nyssa sylvatica* and *Lindera benzoin*), and two are nonindigenous, invasive species (*Rosa multiflora* and*Euonymus alatus*). The forest is situated in a suburban landscape (more information given in the Materials and Methods section) with very high deer density, and all four species experience deer browse, but at different rates.

We predicted that fenced and unfenced plants would differ in their global metabolite profiles and signaling pathways involved in defense. We also expected that the metabolomic divergence between fenced and unfenced plants would be more pronounced for species that are more highly preferred and affected by deer.

2 | Materials and methods

2.1 | Plant species and deer browse

The four species included in this study were the indigenous tree*Nyssa sylvatica* Marshall, the indigenous shrub *Lindera benzoin* L. Blume, the nonindigenous, invasive shrub *Euonymus alatus* (Thunb.) Siebold,

and the nonindigenous, invasive semi-woody shrub *Rosa multiflora* Thunb. We selected them based on four criteria. First, they were sufficiently abundant in the understory to provide a sample of individuals in both fenced and unfenced plots. Second, they included a mix of indigenous and nonindigenous, invasive species, since both types are common in suburban forests and comparisons of their ecologies is relevant to a broader understanding of plant invasions. Both invasive species are of conservation concern . Third, they include both a tree species and shrubs. The ecological success of both groups is essential for maintaining the physical layers of forest structure and food sources that support a diversity of other forest species . Fourth, the four species were all vulnerable to deer browse, but at somewhat different rates.

Deer browse rates for each species were measured on unfenced and fenced plants in the summer of 2018, the season when we sampled the leaves for metabolomic analysis, and over multiple seasons from 2012-2019, including one fall, two winters, and seven summers. The rates were calculated in each plot by inspecting all individuals of the species in an L-shaped, 0.5 x 7.5 m belt transect that followed two edges of the plot, and recording the presence or absence of the distinctive, tell-tale signs of deer browse: bitten, shredded twig tips . Any browse signs inside the fences should be considered as the error rate of falsely assigning a damaged twig tip to deer browse when it was due to some other cause. For the browse rates presented for the species in this study, the fenced rate on the species was subtracted from its unfenced rate. The browse rates were compared among species for the summer 2018 rates and the rates pooled across years, using G-tests for overall heterogeneity among the species along with pairwise tests with a Bonferroni correction for multiple tests on the same data set. G-tests were done in R v 4.1.2 , with the G.test function in the RVAideMemoire package.

2.2 | Study site and plots

The study was conducted in Herrontown Woods Preserve, in Princeton Township in suburban central New Jersey, USA (40.3792, -74.6469). The study site extends 20-45 m from the nearest forest edge, is 0.3 km to the nearest housing community, and is 3.3 km to a town center, Princeton Borough. A recent aerial, infrared drone survey in the region estimated deer density ranging from 35-39 deer/km^2 . The preserve is a 136 ha, second-growth, deciduous forest stand estimated to be at least 150 years old, based on tree ring analysis of the cohort of largest trees in the study site (unpublished data). The most abundant tree species (in descending order) are *Liriodendron tulipifera* ,*Fraxinus pennsylvanica* , *Nyssa sylvatica* , *Carya*spp., *Quercus rubra* , and *Liquidambar styraciflua* . Permanent plots are 4 x 4 m, and those designated for deer exclosure were surrounded since spring 2013 by 5 x 5 m of 2.3 m tall, black plastic fencing with a 4 x 4.5 cm mesh (obtained from deerbusters.com). This type of fencing does not alter light or wind . The fences were staked to the ground, but had three 10 x 30 cm gaps cut at ground level on each side to allow entry by small animals such as rabbits and voles, to ensure that the only excluded vertebrate herbivores were deer. The fences did prevent deer access; the rate of deer browse signs (measured as described above) on all woody species in unfenced plots in this forest was 9.7% (N = 6675 observations), compared to 0.5% (N = 5899) inside fences.

2.3 | Leaf sampling

For each species, we sampled foliage from three juvenile plants in each of six or seven fenced plots and six or seven unfenced plots (six if there were not seven with a sufficient number of plants). In an attempt to limit variation in plant age, we selected individuals that were within the middle 50% of the range of heights for that species in open plots or in fenced plots, based on measurements from fall 2017 in 4 x 1 m belt transects in all plots in the forest. Within the size range, we selected plants with the least amount of visible insect damage. On 26 July 2018, we identified and marked the plants to be sampled and then completed the entire foliage collection the next day, on 27 July. We alternated collection between fenced and unfenced plots, and all collection was completed for one species before proceeding to the next. The youngest fully expanded leaf was collected from the plot's three plants simultaneously, by having three people work together. The three leaves were combined, formed into a single pellet, wrapped in foil and submerged into liquid nitrogen within 30 sec of collection. They were transferred directly into a -80 C freezer within 2 hr of collection, and 4 days later shipped overnight on dry ice to the Boyce Thompson Institute for metabolite extraction and analysis.

2.4 | Extraction and analysis of metabolites

Leaf samples were ground into fine powder in liquid nitrogen using mortar and pestle. Two hundred milligrams of the fine powder were transferred into pre-chilled microcentrifuge tubes that contain two metal beads and homogenized in 1 mL ice-cold extraction buffer (1: 2: 1 chloroform : methanol : water; v/v) for 2 min. The homogenized samples were vortexed for 20 min at 4 °C and centrifuged for 20 min at 15,000 g. Then, 750 µL of the clear supernatants were transferred into new microcentrifuge tubes and dried under vacuum at room temperature. After adding 100 µL 70% methanol (in water; v/v), the tubes were vortexed for 10 min, centrifuged for 10 min at 15,000 g, and 5 µL of the clean supernatants were analyzed on Q-exactive liquid chromatography-tandem mass spectrometer (LC-MS/MS; Thermo Scientific) in negative ionization mode.

2.5 | Pre-processing of mass spectrometric data and statistical analysis

Raw mass spectrometric data from the LC-MS/MS were converted to mzXML format using the MSConvert tool (Version 3.0) of the open-source ProteoWizard software (*http://proteowizard.sourceforge.net/*). Peak peaking, retention time correction and peak grouping were performed using the XCMS package in the R statistical programing language. After annotating the isotopes and adducts using the CAMERA package , the filtered peak lists were normalized by the mass of the leaves used for metabolite extraction. The peak lists were imported to the metaboanalyst platform (*https://www.metaboanalyst.ca/*), filtered using inter-quantile range (IQR) to remove metabolite features that do not provide useful information (e.g. metabolites whose concentrations are close to the background noise, that are constant in all samples and/or have low repeatability), normalized by the median, and log-transformed and scaled to undertake statistical comparison.

Principal component analysis (PCA) was conducted using the normalized peak areas to assess the overall relationship of the samples in an unsupervised manner. To identify the 15 top metabolite features that accumulate significantly differently among the plant species and/or treatments, we conducted partial least squares-discriminant analysis (PLS-DA). We also conducted hierarchical cluster analysis (HCA) to identify the top 25 significant metabolite features and followed that with Pearson correlation to find out the relatedness of these samples. To probe and visualize the metabolite-based relationship of the samples, we constructed dendrogram using Ward's clustering algorithm and Pearson correlation.

2.6 | Putative identification of the significant metabolites

Metabolite features that are unique to each species or are shared among some/all of them were identified using a Venn Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/). To obtain putative prediction of the metabolic pathways that the identified metabolite features are assigned to, we compared the accurate masses of these features against the annotated metabolite database of*Arabidopsis thaliana* using the "Functional Analysis" module and the Gene Set Enrichment Assay (GSEA) tool of MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/MetaboAnalyst/ModuleView.xhtml). Using these predictions, we compared the metabolic pathways that are shared by some/all of the species or are unique to each.

2.7 | Measurements of the effect of deer exclosure on plant percent cover and height

Percent cover and maximum height of the four species were measured in fall 2019, after 6.5 years of the fencing or no-fencing treatment. Percent cover was estimated in each of the sixteen 0.25 m^2 subplots per plot, formed by blindly tossing a quadrat frame into each 1 m^2 section of the 16 m^2 plot. Percent cover was recorded as 0, >0-10%, >10%-20%, etc. The midpoints of the ranges were averaged to provide one cover score per 16 m^2 plot. Maximum height was recorded on all individuals of the species in a 0.5 m x 4 m belt transect in each plot.

For each species, a one-tailed t-test for the difference between percent cover or mean heights was conducted using the Analysis Toolpak in Excel 2019.

3 | Results

3.1 | Deer browse rates on the four species

Browse rates on unfenced plants scored during the summer of 2018 (from 20 July-20 August) varied slightly among the species, with the highest rates on *N. sylvatica* and *E. alatus* and the lowest on*R. multiflora* **(Figure 1)** , but the difference among the four was not statistically significant (G = 1.1, df = 3, P = 0.8). Deer browse can be patchy, so any one sample in a season may be rather idiosyncratic, whereas browse measured over multiple seasons offers a fuller picture of deer preference. The overall browse rates based on the scores pooled from 2012 through 2019 displayed significant differences among the four species (G = 22, df = 3, P < 0.0001). Bonferroni-corrected pairwise tests indicated that the indigenous species *L. benzoin* had significantly greater browse rates than the nonindigenous invasive species *E. alatus* (P = 0.0006) and*R. multiflora* (P = 0.004). The indigenous species *N. sylvatica* also was significantly more browsed than *E. alatus* (P = 0.02) and was somewhat less browsed than *R. multiflora* (P = 0.06). The two indigenous species were browsed at similar rates, as were the two nonindigenous species **(Figure 1)** .

3.2 | Metabolomic comparison of the four species in fenced and unfenced conditions.

To compare the global metabolome of the four species under fenced and unfenced conditions, we conducted untargeted metabolomic analysis, which resulted in the identification of 2,333 metabolite features. A significant portion (84.3%) of these metabolite features were unique to each species: 950 metabolites in *E. alatus* (19.99%), 1,190 metabolites in *R. multiflora* (25.04%), 849 metabolites in*N. sylvatica* (17.87%) and 1,017 metabolites in *L. benzoin*(21.40%). While some metabolites were shared by two or more species (**Appendix Table 1**), only 27 metabolite features (1.1%) were commonly found in all the four species (**Figure 2A**). Principal component analysis (PCA) conducted to assess the overall relatedness of the samples with respect to the 2,333 metabolite features generated four distinct clusters, each cluster corresponding to a species; the first two principal components PC1 and PC2 explained 25.4% and 23% of the total variability among the samples, respectively (**Figure 2B**). The same pattern of relatedness among the samples is also observed on the dendrogram constructed using Pearson correlation and Ward's clustering algorithm. The dendrogram grouped the two nonindigenous species (*R. multiflora* and *E. alatus*) in the same clade, indicating that the nonindigenous species are the closest to each other with respect to their metabolite profiles. For most species, samples collected from the fenced (indicated by red color) and unfenced (indicated by green color) plants did not form distinct subclades on the dendrogram; however, *N. sylvatica* samples were grouped into two distinct subclades that correspond with the presence/absence of fence (**Figure 2C**).

Using the metabolite features, we conducted functional analysis, which putatively assigned the metabolite features to 61 metabolic pathways; 14 (22.9%) of these metabolic pathways were found in all the four species while a few were shared by two or three species. Among the pairwise comparisons, the two indigenous species (*L. benzoin* and *N. sylvatica*) shared the largest numbers of predicted metabolic pathways (30 metabolic pathways, 49.2%) (**Figure 2D**). The complete list of predicted metabolic pathways that are unique to each species or shared among any of them is given in **Appendix Table 2** .

To identify metabolites that differentiate the samples based on species and/or treatment (fence vs. no fence), we conducted partial least squares-discriminant analysis (PLS-DA), which resulted in two main clusters corresponding to treatment (presence or absence of fence) and four subclusters within each of these clusters that correspond to species (**Figure 3A**). The accumulation of the top 15 most important features that contributed to the separation of the samples into the two main PLS-DA clusters is influenced mainly by the presence or absence of fences (**Figure 3B**). However, close inspection of the relative accumulation of some of the top metabolite features (m/z: 149.0597; 176.8251; 188.0995; 215.0810 and 187.0967) indicates that their abundance was influenced both by species and/or treatment (**Figure 3C**).

Among the four species and fencing treatments, the accumulation of metabolite 1 (m/z = 149.0597) was significantly lower in fenced *N. sylvatica* and fenced and unfenced *L. benzoin* plants (P < 0.05; Tukey HSD). Within species, we observed no significant differences in the accumulation of this metabolite between the fenced and unfenced samples for *R. multiflora* , *L. benzoin* and*E. alatus* , but unfenced *N. sylvatica* accumulated significantly more of this metabolite (P = 0.03; ANOVA). Fenced *N. sylvatica* plants also accumulated significantly lower (P < 0.05; Tukey HSD) amount of metabolite 2 (m/z = 176.8251) than the other species, while unfenced *L. benzoin* samples had significantly more (P < 0.05; Tukey HSD). Comparing fenced and

unfenced plants within species, we found that the accumulation of metabolite 2 increased significantly ($P < 0.05$; Tukey HSD) in *N. sylvatica* and*L. benzoin* samples from unfenced plots. On the other hand, the accumulation of metabolite 3 (m/z = 188.0995) did not vary much among or within the species in both fenced and unfenced conditions. The exception was *N. sylvatica* , which, under fenced conditions, accumulated a significantly lower amount than all other species except for unfenced*L. benzoin* ($P < 0.05$; Tukey HSD). With respect to metabolite 4 (m/z = 215.0995), fenced *N. sylvatica* , *L. benzoin* and *E. alatus* plants accumulated significantly lower amounts compared to *R. multiflora* ($P < 0.05$; Tukey HSD), whereas in unfenced conditions, *L. benzoin* and *E. alatus* accumulated significantly lower amounts ($P < 0.05$; Tukey HSD). Within species, comparison of fenced and unfenced samples revealed a difference only for *N. sylvatica* , with significantly more metabolite 4 in the unfenced plants ($P < 0.05$; Tukey HSD). Unfenced *R. multiflora* , *N. sylvatica* , and *E. alatus* plants accumulated significantly lower amounts of metabolite 5 (m/z = 187.0967) compared to *L. benzoin* ($P < 0.05$; Tukey HSD). Under unfenced conditions, the accumulation of this metabolite feature was not significantly different among *R. multiflora* , *N. sylvatica* and *E. alatus* , but *L. benzoin* plants accumulated significantly more of this metabolite ($P < 0.05$; Tukey HSD). Comparing fenced and unfenced plants within species, we observed that unfenced *R. multiflora* , *N. sylvatica* and *E. alatus* plants had significantly reduced accumulation of metabolite 5 ($P < 0.05$; Tukey HSD) (**Figure 3C**).

3.3 | Metabolomic comparison of fenced and unfenced*N. sylvatica* plants

Comparison of the global metabolome of fenced and unfenced *N. sylvatica* plants resulted in the identification of 1,025 metabolite features. To assess the overall metabolomic accumulation pattern of the samples from the fenced and open areas, PCA was conducted using the 1025 metabolite features, which clustered the *N. sylvatica* samples into two separate groups that correspond with the treatment (presence or absence of fence); the first two principal components (PC1 and PC2) explained 47.5% of the total variability (**Figure 4A**). The top 15 metabolite features that contributed to the separation of the samples into the two distinct clusters were identified using PLS-DA (**Figure 4B**), and their accumulation pattern varied based on treatment (fence or no fence) (**Figure 4C**). Hierarchical cluster analysis (HCA) was conducted to identify the 25 top significant metabolite features and to cluster the samples based on the top features (**Figure 4D**). The PLS-DA (**Figure 4C**) and HCA (**Figure 4D**) analyses identified similar metabolite features whose accumulation patterns were consistent with the presence/absence of fences (**Figure 4D**).

We obtained putative prediction on the chemical identity of the 1,025 metabolites and their associated metabolic pathways using the GSEA functional analysis tool. Among the identified metabolic pathways are the pentose phosphate pathway ($P = 0.01$), starch and sucrose metabolism ($P = 0.02$), pyrimidine metabolism ($P = 0.054$), cyanoamino acid metabolism ($P = 0.054$), riboflavin metabolism ($P = 0.054$) and monoterpenoid biosynthesis ($P = 0.058$) (**Appendix Table 3**).

Out of the identified metabolic pathways, the monoterpenoid biosynthetic pathway produces metabolites that mediate indirect defenses in many plant species , while the cyanoamino acid pathway is implicated in detoxification .

3.4 | Metabolomic comparison of fenced and unfenced*L. benzoin* plants

We identified 1,225 metabolite features in all *L. benzoin* samples by conducting untargeted metabolomics analysis. PCA did not indicate clear treatment- or species-based subgrouping of the samples (**Figure 5A**). Hence, we followed a supervised approach (PLS-DA and HCA) to probe for metabolites that varied among the samples based on treatment (fence vs no fence) (**Figure 5B** , **D**). Both methods (PLS-DA and HCA) identified similar significant metabolite features with similar patterns of accumulation between the fenced and unfenced plots (**Figure 5C, D**).

We used the metabolite features that we identified to obtain putative prediction of the chemical identities of the 1,225 metabolite features and the metabolic pathways that they are assigned to using the GSEA functional analysis tool. Our analysis identified glyoxylate and dicarboxylate metabolism ($P = 0.01$) and pentose phosphate pathway ($P = 0.02$) as significantly overrepresented pathways (**Appendix Table 4**). The glyoxylate and dicarboxylate pathway is not involved in plant defense directly; however, the possible

role of the pentose phosphate pathway in pathogen defense has been shown in *Arabidopsis thaliana* . .

3.5 | Metabolomic comparison of fenced and unfenced *R. multiflora* plants

We conducted untargeted metabolomics analysis on all *R. multiflora* samples and identified 1,350 metabolite features. Even though the samples tend to separate from each other on the PCA plot based on treatment (fence vs no fence), the PCA did not group the samples into distinct clusters (**Figure 6A**). To inspect the treatment-based differences in the metabolite profile of the plants, we conducted PLS-DA (**Figure 6B**) and identified the top 15 features that varied significantly across the treatments (**Figure 6C**). We followed this analysis with HCA to obtain a filtered set of 25 metabolites that consistently and significantly varied between the fence and no fence samples. The heatmap generated from the HCA analysis indicates that the top 25 metabolites clearly differentiate the fence and no fence samples (**Figure 6D**). The GSEA tool in Metaboanalyst was used to identify some putative metabolic pathways that the metabolomic features are associated with, including the pentose phosphate pathway (P = 0.04) and carbon fixation in photosynthetic organisms (P = 0.04) (**Appendix Table 5**); though carbon fixation is not directly related with plant defense, the pentose phosphate pathway is implicated with stress responses in plants .

3.6 | Metabolomic comparison of fenced and unfenced *E. alatus* plants

We identified 1,153 metabolite features from the untargeted metabolomic analysis of all *E. alatus* plants. The PCA that we conducted using these features did not produce clearly distinct clusters based on treatment (**Figure 7A**). Using PLS-DA, we identified the top 15 metabolite features that separate these samples into two groups based on treatment (**Figure 7 B, C**). Similarly, the HCA identified the top 25 significant metabolite features that differed significantly among whole group and whose accumulation pattern clustered the 12 samples into two nearly distinct groups: fenced and unfenced (**Figure 7D**). Among the top metabolic pathways predicted by the GSEA are glutathione metabolism (P = 0.02), pentose phosphate pathway (P = 0.04), and alanine, aspartate and glutamate metabolism (P = 0.04) (**Appendix Table 6**). All of the significantly overrepresented pathways are involved in stress response and/or detoxification of defensive related metabolites .

3.7 | Percent cover and height of the four woody tree species in fenced and unfenced plots

After 5.3 years of fencing, three species increased in percent cover and/or maximum height in fenced compared to unfenced plots. *Nyssa sylvatica* , the indigenous tree species, had significantly greater cover ($P<0.01$) and height ($P<0.0001$) in fenced plots. The indigenous shrub *L. benzoin* had greater height in fenced plots ($P<0.05$) and a trend of greater cover, and the nonindigenous, invasive shrub *E. alatus* had greater cover in fenced plots ($P<0.05$). In contrast, the nonindigenous, invasive shrub *R. multiflora* had greater maximum height in unfenced plots, compared to the plots that had been fenced for 5.3 years ($P<0.01$) **(Figure 8)** .

Discussion

In this study, we investigated how long-term protection from herbivory by overabundant white-tailed deer affected the metabolomic profiles of long-lived woody or semi-woody plant species in a suburban forest understory. Furthermore, by studying two indigenous and two nonindigenous, invasive plant species that are subject to different deer preferences, we could consider how metabolomic responses to deer browse may affect the ecology of invasion in suburban forests. Below, in light of the results, we discuss our hypotheses that 1) the metabolomic profiles of fenced and unfenced plants diverge, and 2) this divergence is more pronounced for species most affected by deer in our site. First, we discuss the overall metabolomic variation among the species.

We studied four diverse species, including the indigenous tree species *N. sylvatica* , the indigenous shrub *L. benzoin* , the nonindigenous shrub *E. alatus* , and the nonindigenous semi-woody shrub *R. multiflora* . All four species are potentially subject to severe negative effects from deer herbivory; they are all long-lived and are therefore exposed to chronic deer browse in from overabundant deer while growing in the deer browse zone. Their shared understory forest environment has very limited light , so losing photosynthetic tissue to deer herbivory can be a serious problem for any of these species. Although the four species

share these morphological and ecological similarities, they are rather distant phylogenetically. All belong to different plant Orders (**Figure 9**), which could impose metabolomic variation among them based solely on genetic distance . Additionally, they possess some distinct chemical or morphological features that could affect their metabolomes. *Lindera benzoin* is notably chemically defended , with very aromatic foliage, *R. multiflora* is defended with large prickles, and *E. alatus*produces tough, corky protrusions from its branches. In contrast,*N. sylvatica* possesses none of these features. Given this diversity, it was not surprising that the four species' metabolomes were quite distinct, with only 1.1% of the 2,333 detected metabolite features shared among them, and just 23% of the putative metabolic pathways shared among all four species (**Figure 2A, D**).

The dendrogram based on all metabolites produced four distinct clusters for the four species, but the two nonindigenous species, *E. alatus* and *R. multiflora* , clustered together (**Figure 2C**). In a recent study in eastern US forests, demonstrated metabolomic divergence between a large group of indigenous and non-indigenous species in the same plant community, along with evidence for greater regional invasion frequency in nonindigenous species with greater chemical novelty. In our case, with just two indigenous and two nonindigenous species, it is not possible to confidently attribute the clustering to their indigenous status, although our result is consistent with Sedio et al's observation. Also, *E. alatus* and *R. multiflora* happen to be more closely related phylogenetically than are the two indigenous species we studied (**Figure 9**); so their greater metabolomic similarity could be due simply to genetic relatedness rather than some similar physiology related to being successful invasive species. We also note that the two indigenous species, *N. sylvatica* and *L. benzoin* , shared the largest number of putative metabolic pathways that the detected metabolites may be part of, possibly suggesting greater similarity in their physiological ecologies. While variation among the four species based on the metabolite features is quite clear, any prediction that divergence in these metabolites indicates differences in the pathways is rather speculative, since the connection from metabolites to pathways is based on the only knowledge available at this point, which is from the model plant *Arabidopsis thaliana* . We must be very cautious when applying such evidence to quite different species and contexts , such as woody species in a forest understory.

Even given the differences among the species enumerated above, we had predicted that their shared suburban forest understory environment, with its overabundance of deer, would lead to an across-the-board divergence of the plants' metabolomes in fenced versus unfenced plots. In support of this hypothesis, we did indeed observe a global divergence (**Figure 3A, 3B**). We suspect that the difference was caused mainly by protection from deer herbivory in the fencing treatment, which is widely considered the common effect of deer exclosure . However, preventing deer access also may alter other ecological variables that could affect plant stress and influence a plants' metabolome, i.e. trampling of plants; soil compaction and therefore more difficult root penetration that negatively affects the soil microbial community and/or access to soil water and nutrients; elimination of deer fecal deposition, thereby altering soil nutrients; and release from herbivory for the entire plant community, creating increased competition with other plants . An important aim of ongoing research in deer-related plant ecometabolomics will be to disentangle all of these possible effects of deer on plant metabolomes in natural communities.

The overall divergence in the metabolomes of fenced and unfenced plants is by no means the entire story; rather, there were notable distinctions among the species/fencing treatment combinations in metabolites that were the main drivers of the fenced/unfenced divergence (**Figure 3C**). For the top five of these metabolites, a significant difference between fenced and unfenced samples within a species appeared in just some: *N. sylvatica* for metabolites 1, 2, 4, and 5; *L. benzoin* for metabolites 2 and 3; *R. multiflora* and *E. alatus* for metabolite 5. It should be noted that these were conservative pairwise tests, and the means in Figure 3C suggest additional marked differences, i.e. metabolite 3 in *R. multiflora*and *N. sylvatica* and metabolites 2 and 5 in *E. alatus* . In any case, the species' responses to protection from deer were variable for these five metabolites, and there was variation in whether the five increased or decreased when exposed to deer. For metabolites 1, 2, and 4, the significant differences were due to greater production in plants exposed to deer, but for metabolites 3 and 5, plants protected from deer by fencing had greater amounts. These findings on the top five metabolites responsible for the global fenced/unfenced divergence therefore partially support

the idea that metabolites involved in plant defense responses would be downregulated in fenced plots. More detailed knowledge of these chemicals' roles in the defense physiology of woody, understory plants is needed to fully understand why some were upregulated and some downregulated.

The separate analyses on each species provides further insight on the responses of defense-related metabolites to deer fencing and also enables discussion of our second hypothesis, that the effect of fencing on a plant's metabolomic profile is stronger for plant species that are more preferred by deer. Support for this hypothesis would add credence to the idea that it is herbivory by deer, rather than other fencing-related impacts, that affects the metabolome. Out of the four species, *N. sylvatica* stood out as the one with a metabolomic profile most affected by protection from deer; only for this species did the fenced and unfenced samples clearly cluster into separate groups on both the PCA based on all of its detected metabolites and in the HCA based on its top 30 significantly different metabolites (**Figure 4** vs. **Figures 5, 6, and 7**). Some of these top metabolites are putatively involved in pathways that produce intermediate compounds that can be used to produce defense secondary metabolites, e.g. pentose phosphate pathway and cyanoamino acid metabolism , or are involved in indirect defenses, e.g. monoterpenoid biosynthesis . Although fencing did not separate the other three species into distinct treatment-based clusters on the PCA plot, a significant numbers of metabolite features exhibited significant changes in their accumulation based on fencing treatment (**Figures 5D, 6D, and 7D**).

Deer did, indeed, browse *N. sylvatica* frequently, but the other species also were commonly browsed (**Figure 1**). In the summer of 2018, the same season when the samples were taken, *N. sylvatica*and *E. alatus* were browsed the most and at very similar rates, but the differences among the four species was not statistically significant. The browse rates from all observations pooled across all observed individuals from 2012-2019 provides more comprehensive knowledge of deer preference for these four species in the site, and revealed that the two indigenous species, *N. sylvatica* and*L. benzoin* , were preferred over the two non-indigenous species,*E. alatus* and *R. multiflora* . Full support for our second hypothesis would therefore require that both *N. sylvatica* and*L. benzoin* exhibit more divergent metabolomic profiles between the fenced and unfenced plants than did the less-preferred nonindigenous species, yet such divergence was only clear in *N. sylvatica* .

There are several aspects of herbivory that our data did not address and which may be key to understanding the variation among species in their metabolomic responses to protection from deer; these are all worth further study. First, as in other studies, e.g. , we used the proportion of observed plants with presence of browse signs as our metric for deer browse. A more fine-grained metric that captures the intensity, rather than presence, of browsing on a species could be a better predictor of how metabolomes respond to overabundant deer.

Second, plants are affected by herbivory not just by whether they are attacked, but also by their tolerance of any herbivory that does occur , and the four species we studied may differ in tolerance. For example,*N. sylvatica* is the only tree of the four species we studied, and this plant architecture, with one central stem and terminal bud, could make its seedlings less tolerant of a deer browse event that removes that bud, compared to more branched shrub species with more meristems . We may then expect a lack of tolerance to be correlated with stronger chemical defenses against herbivory , although some recent evidence for this idea is equivocal or negative). In any case, variation in tolerance to deer herbivory may correlate to variation in a species' metabolomic profile.

Finally, our framework for investigating the effect of deer on the plants' metabolomes relied on comparing plants that had been protected from chronic deer herbivory for years (in fenced plots) versus those exposed to deer (in unfenced plots). Given the overall high browse rates on these species, it was reasonable to assume that at least some of the exposed plants would have experienced herbivory in the recent past, leading to metabolomic profiles different from protected plants. However, little is known about the timing of herbivory-induced metabolite production in long-lived woody plants. While there is evidence that woody plants maintain increased levels of some induced defense chemicals for months following herbivory , other studies in herbaceous plants showed that the metabolomic response to herbivory can be very rapid and then quickly wane . Similarly, the priming of the metabolome caused by an herbivory event, which readies the

plant or neighboring plants to rapidly defend against subsequent herbivory, may persist for months in woody plants, and even throughout the plant life cycle of herbaceous plants, or may last only days .

The four species included in this study exhibited variation in their metabolomes, but also in their ecological success in response to protection from deer. The connection between these responses is worth consideration. *Nyssa sylvatica* was the only species of the four that, in both measures of ecological success, percent cover and height, showed statistically significantly greater values in fenced vs. unfenced plots after 6.5 years (**Figure 8**). This suggests that *N. sylvatica* is particularly vulnerable to deer pressure in this forest. It was indeed browsed at one of the highest rates, suffering tissue loss, but it also was the one species that showed a clear metabolomic difference in fenced vs. unfenced plots, with increased production in deer-exposed plants of potentially costly secondary metabolites that are involved in defense-related pathways. The increased metabolite production did not appear to protect it from browse relative to the other three species, since it had among the higher browse rates in unfenced plots. Thus, we may expect this indigenous tree species to decline under severe, chronic deer herbivory, as in this suburban forest.

In contrast, one of the invasive species, *R. multiflora* , actually did better in the unfenced plots exposed to deer pressure, with a significantly greater mean height and a trend of increased cover. Although *R. multiflora* was browsed, it had among the lower browse rates and much less difference between metabolites in fenced vs. unfenced plants. This all suggests that *R. multiflora* is resistant and/or tolerant of deer browsing and, under the severe deer pressure in suburban forests, even may gain a competitive advantage over other, more susceptible species. Thus, we may expect this nonindigenous, invasive species to increase in the plant community.

It is important to note that species' responses to deer in a community are specific and likely cannot be generalized into, for example, indigenous vs. nonindigenous invasive species. Of the other two species, the indigenous *L. benzoin* did not show a strong difference among the metabolites in fenced vs. unfenced plants and exhibited only a weak positive effect on growth when protected from deer in the fences, but it had among the highest browse rates. The nonindigenous, invasive *E. alatus* was as highly browsed as the indigenous *N. sylvatica* in the summer of 2018, yet like the indigenous *L. benzoin* , had only a weak positive growth response to protection from deer, and did not have clear difference in its metabolites in fenced vs unfenced plants.

This study has set the stage for further research on how severe, chronic deer pressure affects the metabolomes of long-lived species in forests, with possible consequences for the ecology of communities that are now composed of a mix of indigenous and nonindigenous species, as in many suburban forests. While our research clearly indicates that protection from overabundant deer in suburban forests can influence a plants' metabolome, including affecting metabolites putatively involved in plant defense and stress pathways, it is only a first step. Needed next are ecometabolomic studies that include quantification of deer preference and herbivory intensity in various ways, measurements of tolerance to deer herbivory, documentation of the timing of metabolomic responses to deer herbivory in long-lived plants, and determination of the chemical identities of significant metabolite features.

References

Allevato, D.M., Kiyota, E., Mazzafera, P. & Nixon, K.C. (2019). Ecometabolomic analysis of wild populations of *Pilocarpus pennatifolius* (Rutaceae) using unimodal analyses. Frontiers in Plant Science, 10, 258. https://doi-org/10.3389/fpls.2019.00258.

Alverson, W.S., Waller, D.M. & Solheim, S.L. (1988). Forests too deer: Edge effects in northern Wisconsin. Conservation Biology, 2(4), 348-358. https://doi.org/10.1111/j.1523-1739.1988.tb00199.x.

Anderson, R.C. & Katz, A.J. (1993). Recovery of browse-sensitive tree species following release from white-tailed deer *Odocoileus virginianus* Zimmerman browsing pressure. Biological Conservation, 63(3), 203-208. https://doi.org/10.1016/0006-3207(93)90713-B.

Aronson, M.F., Handel, S.N., La Puma, I.P. & Clemants, S.E. (2015). Urbanization promotes non-native

woody species and diverse plant assemblages in the New York metropolitan region. Urban Ecosystems, 18(1), 31-45. https://doi.org/10.1007/s11252-014-0382-z.

Averill, K.M., Mortensen, D.A., Smithwick, E.A. & Post, E. (2016). Deer feeding selectivity for invasive plants. Biological Invasions, 18(5), 1247-1263. https://doi.org/10.1007/s10530-016-1063-z.

Baldwin, I.T. & Schultz, J.C. (2015). Rapid changes in tree leaf chemistry induced by damage: Evidence for communication between plants. Science, 221(4607), 277-279. https://doi.org/10.1126/science.221.4607.277.

Ballaré, C.L. (2014). Light regulation of plant defense. Annual Review of Plant Biology, 65(1), 335-363. https://doi.org/10.1146/annurev-arplant-050213-040145.

Batzli, G.O. & Dejaco, C.E. (2013). White-tailed deer (*Odocoileus virginianus*) facilitate the development of nonnative grasslands in central Illinois. American Midland Naturalist, 170(2), 323-334. https://doi.org/10.1674/0003-0031-170.2.323.

Berini, J.L., Brockman, S.A., Hegeman, A.D., Reich, P.B., Muthukrishnan, R., Montgomery, R.A. & Forester, J.D. (2018). Combinations of abiotic factors differentially alter production of plant secondary metabolites in five woody plant species in the boreal-temperate transition zone. Frontiers in Plant Science, 9, 1257. https://doi.org/10.3389/fpls.2018.01257.

Blossey, B., Curtis, P., Boulanger, J. & Dávalos, A. (2019). Red oak seedlings as indicators of deer browse pressure: Gauging the outcome of different white-tailed deer management approaches. Ecology and Evolution, 9(23), 13085-13103. https://doi.org/10.1002/ece3.5729.

Bressette, J.W., Beck, H. & Beauchamp, V.B. (2012). Beyond the browse line: Complex cascade effects mediated by white-tailed deer. Oikos, 121(11), 1749-1760. https://doi.org/10.1111/j.1600-0706.2011.20305.x.

Brown, M.J. & Parker, G.G. (1994). Canopy light transmittance in a chronosequence of mixed-species deciduous forests. Canadian Journal of Forest Research, 24(8), 1694-1703. https://doi.org/10.1139/x94-219.

Bruce, T.J.A. (2014). Variation in plant responsiveness to defence elicitors caused by genotype and environment. Frontiers in Plant Science, 5, 349. https://doi.org/10.3389/fpls.2014.00349.

Cash, V.W. & Fulbright, T.E. (2005). Nutrient enrichment, tannins, and thorns: Effects on browsing of shrub seedlings. The Journal of Wildlife Management, 69(2), 782-793. https://doi.org/10.2193/0022-541X(2005)069[0782:NETATE]2.0.CO;2.

Cipollini, D., Walters, D. & Voelckel, C. (2014). Costs of resistance in plants: From theory to evidence. Annual Plant Reviews (eds C. Voelckel & G. Jander), pp. 263-307. John Wiley & Sons, Ltd, Chichester, UK.

Cote, S.D., Rooney, T.P., Tremblay, J.P., Dussault, C. & Waller, D.M. (2004). Ecological impacts of deer overabundance. Annual Review of Ecology and Systematics, 35, 113-147. https://doi.org/10.1146/annurev.ecolsys.35.021103.105725.

Crandall, S.G., Gold, K.M., Jimenez-Gasco, M.d.M., Filgueiras, C.C. & Willett, D.S. (2020). A multi-omics approach to solving problems in plant disease ecology. PloS One, 15(9), e0237975. https://doi.org/10.1371/journal.pone.0237975.

Crawley, M.J. (1985). Reduction of oak fecundity by low-density herbivore populations. Nature, 314(6007), 163-164. https://doi.org/10.1038/314163a0.

Culbert, P.D., Radeloff, V.C., Flather, C.H., Kellndorfer, J.M., Rittenhouse, C.D. & Pidgeon, A.M. (2013). The influence of vertical and horizontal habitat structure on nationwide patterns of avian biodiversity. The Auk, 130(4), 656-665. https://doi.org/10.1525/auk.2013.13007.

Dodd, L.E., Lacki, M.J., Britzke, E.R., Buehler, D.A., Keyser, P.D., Larkin, J.L., Rodewald, A.D., Wigley, T.B., Wood, P.B. & Rieske, L.K. (2012). Forest structure affects trophic linkages: How silvi-

cultural disturbance impacts bats and their insect prey. Forest Ecology and Management, 267, 262-270. https://doi.org/10.1016/j.foreco.2011.12.016.

Dorion, S., Ouellet, J.C. & Rivoal, J. (2021). Glutathione metabolism in plants under stress: Beyond reactive oxygen species detoxification. Metabolites, 11(9), 641. https://doi.org/10.3390/metabo11090641.

Dubreuil-Maurizi, C. & Poinssot, B. (2012). Role of glutathione in plant signaling under biotic stress. Plant Signaling & Behavior, 7(2), 210-212. https://doi.org/10.4161/psb.18831.

Duncan, A.J., Hartley, S.E., Thurlow, M., Young, S. & Staines, B.W. (2001). Clonal variation in monoterpene concentrations in Sitka spruce (*Picea sitchensis*) saplings and its effect on their susceptibility to browsing damage by red deer (*Cervus elaphus*). Forest Ecology and Management, 148(1), 259-269. https://doi.org/10.1016/S0378-1127(00)00540-5.

Endara, M. & Coley, P.D. (2011). The resource availability hypothesis revisited: A meta-analysis. Functional Ecology, 25(2), 389-398. https://doi.org/10.1111/j.1365-435.2010.01803.x.

Endara, M., Weinhold, A., Cox, J.E., Wiggins, N.L., Coley, P.D. & Kursar, T.A. (2015). Divergent evolution in antiherbivore defences within species complexes at a single Amazonian site. Journal of Ecology, 103(5), 1107-1118. http://dx.doi.org/10.1111/1365-2745.12431.

Feeney, P. (1976). Plant apparency and chemical defense. Recent Advances in Phytochemistry, 10, 1-40. https://doi.org/10.1007/978-1-4684-2646-5_1.

Fineblum, W.L. & Rausher, M.D. (1995). Tradeoff between resistance and tolerance to herbivore damage in a morning glory. Nature, 377(6549), 517-520. https://doi.org/10.1038/377517a0.

Gargallo-Garriga, A., Sardans, J., Granda, V., Llusia, J., Peguero, G., Asensio, D., Ogaya, R., Urbina, I., Van Langenhove, L., Verryckt, L.T., Chave, J., Courtois, E.A., Stahl, C., Grau, O., Klem, K., Urban, O., Janssens, I.A. & Penuelas, J. (2020). Different "metabolomic niches" of the highly diverse tree species of the French Guiana rainforests. Scientific Reports, 10(1), 6937. https://doi.org/10.1038/s41598-020-63891-y.

Gomez, J.M. & Zamora, R. (2002). Thorns as induced mechanical defense in a long-lived shrub (*Hormathophylla spinosa* , Cruciferae). Ecology, 83(4), 885-890. https://doi-org.ezproxy.tcnj.edu/10.1890/0012-9658(2002)083[0885:TAIMDI]2.0.CO;2.

Habeck, C.W. & Schultz, A.K. (2015). Community-level impacts of white-tailed deer on understorey plants in North American forests: A meta-analysis. AoB PLANTS, 7, 10.1093/aobpla/plv119. https://doi.org/10.1093/aobpla/plv119.

Hansen, A.J., Knight, R.L., Marzluff, J.M., Powell, S., Brown, K., Gude, P. & Jones, K. (2005). Effects of exurban development on biodiversity: Patterns, mechanisms, and research needs. Ecological Applications, 15(6), 1893-1905. https://doi.org/10.1890/05-5221.

Haukioja, E. & Koricheva, J. (2000). Tolerance to herbivory in woody vs. herbaceous plants. Evolutionary Ecology, 14(4), 551-562. https://doi.org/10.1023/A:1011091606022.

Herms, D.A. & Mattson, W.J. (1992). The dilemma of plants: To grow or defend. Quarterly Review of Biology, 67(3), 283-335. https://doi.org/10.1086/417659.

Herron, P.M., Martine, C.T., Latimer, A.M. & Leicht-Young, S.A. (2007). Invasive plants and their ecological strategies: Prediction and explanation of woody plant invasion in New England. Diversity & Distributions, 13(5), 633-644. https://doi.org/10.1111/j.1472-4642.2007.00381.x.

Hilker, M., Schwachtje, J., Baier, M., Balazadeh, S., Baurle, I., Geiselhardt, S., Hincha, D.K., Kunze, R., Mueller-Roeber, B., Rillig, M.C., Rolff, J., Romeis, T., Schmulling, T., Steppuhn, A., van Dongen, J., Whitcomb, S.J., Wurst, S., Zuther, E. & Kopka, J. (2016). Priming and memory of stress responses in organisms lacking a nervous system. Biological Review, 91(4), 1118-1133. https://doi.org/10.1111/brv.12215.

Hill, E.M., Robinson, L.A., Abdul-Sada, A., Vanbergen, A.J., Hodge, A. & Hartley, S.E. (2018). Arbuscular mycorrhizal fungi and plant chemical defence: Effects of colonisation on aboveground and belowground metabolomes. Journal of Chemical Ecology, 44(2), 198-208. https://doi.org/10.1007/s10886-017-0921-1.

Horsley, S.B., Stout, S.L. & deCalesta, D.S. (2003). White-tailed deer impact on the vegetation dynamics of a northern hardwood forest. Ecological Applications, 13(1), 98-118. https://doi.org/10.1890/1051-0761(2003)013[0098:WTDIOT]2.0.CO;2.

Huberty, M., Choi, Y.H., Heinen, R., Bezemer, T.M. & Chapman, S. (2020). Above-ground plant metabolomic responses to plant–soil feedbacks and herbivory. The Journal of Ecology, 108(4), 1703-1712. https://doi.org/10.1111/1365-2745.13394.

Hunter, J.C. & Mattice, J.A. (2002). The spread of woody exotics into the forests of a northeastern landscape, 1938-1999. Journal of the Torrey Botanical Society, 129(3), 220-227. https://doi.org/10.2307/3088772.

Ingersoll, C.M., Niesenbaum, R.A., Weigle, C.E. & Lehman, J.H. (2010). Total phenolics and individual phenolic acids vary with light environment in *Lindera benzoin* . Botany, 88(11), 1007. https://doi.org/10.1139/B10-072.

Ji, H., Du, B., Wen, J., Liu, C. & Ossipov, V. (2019). Differences in the relationship between metabolomic and ionomic traits of *Quercus variabilis* growing at contrasting geologic-phosphorus sites in subtropics. Plant and Soil, 439(1-2), 339-355. https://doi.org/10.1007/s11104-019-04020-1.

Jones, O.A.H., Maguire, M.L., Griffin, J.L., Dias, D.A., Spurgeon, D.J. & Svendsen, C. (2013). Metabolomics and its use in ecology. Austral Ecology, 38(6), 713-720. https://doi.org/10.1111/aec.12019.

Kain, M., Battaglia, L., Royo, A. & Carson, W.P. (2011). Over-browsing in Pennsylvania creates a depauperate forest dominated by an understory tree: Results from a 60-year-old deer exclosure. The Journal of the Torrey Botanical Society, 138(3), 322-326. https://doi.org/10.3159/TORREY-D-11-00018.1.

Kant, M.R. & Baldwin, I.T. (2007). The ecogenetics and ecogenomics of plant–herbivore interactions: Rapid progress on a slippery road. Current Opinion in Genetics & Development, 17(6), 519-524. https://doi.org/10.1016/j.gde.2007.09.002.

Karban, R. (2011). The ecology and evolution of induced resistance against herbivores. Functional Ecology, 25(2), 339-347. https://doi.org/10.1111/j.1365-2435.2010.01789.x.

Kuhl, C., Tautenhahn, R., Bottcher, C., Larson, T.R. & Neumann, S. (2012). CAMERA: An integrated strategy for compound spectra extraction and annotation of liquid chromatography/mass spectrometry data sets. Analytical Chemistry, 84(1), 283-289. https://doi.org/10.1021/ac202450g.

Leimu, R. & Koricheva, J. (2006). A meta-analysis of tradeoffs between plant tolerance and resistance to herbivores: Combining the evidence from ecological and agricultural studies. Oikos, 112(1), 1-9. https://doi.org/10.1111/j.0030-1299.2006.41023.x.

Lind, E.M., Myron, E.P., Giaccai, J. & Parker, J.D. (2012). White-tailed deer alter specialist and generalist insect herbivory through plant traits. Environmental Entomology, 41(6), 1409-1416. https://doi.org/10.1603/EN12094.

Lindroth, R.L., Donaldson, J.R., Stevens, M.T. & Gusse, A.C. (2007). Browse quality in quaking aspen (*Populus tremuloides*): Effects of genotype, nutrients, defoliation, and coppicing. Journal of Chemical Ecology, 33(5), 1049-1064. https://doi.org/10.1007/s10886-007-9281-6.

Maag, D., Erb, M. & Glauser, G. (2015). Metabolomics in plant–herbivore interactions: Challenges and applications. Entomologia Experimentalis Et Applicata, 157(1), 18-29. https://doi.org/10.1111/eea.12336.

Machingura, M., Salomon, E., Jez, J.M. & Ebbs, S.D. (2016). Theβ-cyanoalanine synthase pathway: Beyond cyanide detoxification. Plant, Cell and Environment, 39(10), 2329-2341. https://doi.org/10.1111/pce.12755.

Masse, A. & Côté, S.D. (2012). Linking habitat heterogeneity to space use by large herbivores at multiple scales: From habitat mosaics to forest canopy openings. Forest Ecology and Management, 285, 67-76. https://doi.org/10.1016/j.foreco.2012.07.039.

Mauch-Mani, B., Baccelli, I., Luna, E. & Flors, V. (2017). Defense priming: An adaptive part of induced resistance. Annual Review of Plant Biology, 68(1), 485-512. https://doi.org/10.1146/annurev-arplant-042916-041132.

Meijden, E.v.d., Wijn, M. & Verkaar, H.J. (1988). Defence and regrowth, alternative plant strategies in the struggle against herbivores. Oikos, 51(3), 355-363. https://doi.org/10.2307/3565318.

Morrison, J.A. & Brown, L. (2004). Effect of herbivore exclosure caging on the invasive plant *Alliaria petiolata* in three southeastern New York forests. Bartonia, 62, 25-43.

Nephali, L., Piater, L.A., Dubery, I.A., Patterson, V., Huyser, J., Burgess, K. & Tugizimana, F. (2020). Biostimulants for plant growth and mitigation of abiotic stresses: A metabolomics perspective. Metabolites, 10(12), 505. https://doi.org/10.3390/metabo10120505.

New Jersey Farm Bureau (2019). New Jersey white-tailed deer (*Odocoileus virginiana*) population density survey using sUAS infrared: New Jersey Farm Bureau - 2019 study, https://njfb.org/wp-content/uploads/2019/10/NJFB-SG-State-Report_10.2019.pdf.

Nosko, P. & Embury, K. (2018). Induction and persistence of allelochemicals in the foliage of balsam fir seedlings following simulated browsing. Plant Ecology, 219(6), 611-619. https://doi.org/10.1007/s11258-018-0821-7.

Ohse, B., Hammerbacher, A., Seele, C., Meldau, S., Reichelt, M., Ortmann, S., Wirth, C. & Koricheva, J. (2017). Salivary cues: Simulated roe deer browsing induces systemic changes in phytohormones and defence chemistry in wild-grown maple and beech saplings. Functional Ecology, 31(2), 340-349. https://doi.org/10.1111/1365-2435.12717.

Pais, A.L., Li, X. & (Jenny) Xiang, Q. (2018). Discovering variation of secondary metabolite diversity and its relationship with disease resistance in *Cornus florida* L. Ecology and Evolution, 8(11), 5619-5636. https://doi.org/10.1002/ece3.4090.

Peebles-Spencer, J.R., Haffey, C.M. & Gorchov, D.L. (2018). Browse by white-tailed deer decreases cover and growth of the invasive shrub,*Lonicera maackii* . The American Midland Naturalist, 179(1), 68-77. https://doi.org/10.1674/0003-0031-179.1.68.

Peters, K., Worrich, A., Weinhold, A., Alka, O., Balcke, G., Birkemeyer, C., Bruelheide, H., Calf, O.W., Dietz, S., Duhrkop, K., Gaquerel, E., Heinig, U., Kucklich, M., Macel, M., Muller, C., Poeschl, Y., Pohnert, G., Ristok, C., Rodriguez, V.M., Ruttkies, C. et al. (2018). Current challenges in plant eco-metabolomics. International Journal of Molecular Sciences, 19(5), 1385. https://doi.org/10.3390/ijms19051385.

Pierson, T.G. & deCalesta, D.S. (2015). Methodology for estimating deer browsing impact. Human-Wildlife Interactions, 9(1), 67. https://doi.org/10.26077/rbw0-bn49.

Quinn, A.C., Williams, D.M. & Porter, W.F. (2013). Landscape structure influences space use by white-tailed deer. Journal of Mammalogy, 94(2), 398-407. https://doi.org/10.1644/11-MAMM-A-221.1.

R Core Team (2021). R: A language and environment for statistical computing, https://www.R-project.org/.

Relva, M.A., Nunez, M.A. & Simberloff, D. (2010). Introduced deer reduce native plant cover and facilitate invasion of non-native tree species: Evidence for invasional meltdown. Biological Invasions, 12(2), 303-311. https://doi.org/10.1007/s10530-009-9623-0.

Rivas-Ubach, A., Sardans, J., Hodar, J.A., Garcia-Porta, J., Guenther, A., Paša-Tolić, L., Oravec, M., Urban, O. & Peñuelas, J. (2017). Close and distant: Contrasting the metabolism of two closely related subspecies

of Scots pine under the effects of folivory and summer drought. Ecology and Evolution, 7(21), 8976-8988. https://doi.org/10.1002/ece3.3343.

Rooney, T.P. (2009). High white-tailed deer densities benefit graminoids and contribute to biotic homogenization of forest ground-layer vegetation. Plant Ecology, 202(1), 103-111. https://doi.org/10.1007/s11258-008-9489-8.

Rooney, T.P. & Waller, D.M. (2003). Direct and indirect effects of white-tailed deer in forest ecosystems. Forest Ecology and Management, 181(1–2), 165-176. https://doi.org/10.1016/S0378-1127(03)00130-0.

Russell, F.L., Zippin, D.B. & Fowler, N.L. (2001). Effects of white-tailed deer (*Odocoileus virginianus*) on plants, plant populations and communities: A review. American Midland Naturalist, 146(1), 1-26. https://doi.org/10.1674/0003-0031(2001)146[0001:EOWTDO]2.0.CO;2.

Scholes, D.R. & Paige, K.N. (2015). Transcriptomics of plant responses to apical damage reveals no negative correlation between tolerance and defense. Plant Ecology, 216(8), 1177-1190. https://doi.org/10.1007/s11258-015-0500-x.

Schuman, M.C. & Baldwin, I.T. (2016). The layers of plant responses to insect herbivores. Annual Review of Entomology, 61(1), 373-394. https://doi.org/10.1146/annurev-ento-010715-023851.

Schwachtje, J., Fischer, A., Erban, A. & Kopka, J. (2018). Primed primary metabolism in systemic leaves: A functional systems analysis. Scientific Reports, 8(1), 216. https://doi.org/10.1038/s41598-017-18397-5.

Sedio, B.E. (2017). Recent breakthroughs in metabolomics promise to reveal the cryptic chemical traits that mediate plant community composition, character evolution and lineage diversification. The New Phytologist, 214(3), 952-958. https://doi.org/10.1111/nph.14438.

Sedio, B.E., Parker, J.D., McMahon, S.M. & Wright, S.J. (2018). Comparative foliar metabolomics of a tropical and a temperate forest community. Ecology, 99(12), 2647-2653. https://doi.org/10.1002/ecy.2533.

Sedio, B.E., Rojas Echeverri, J.C., Boya P, C.A. & Wright, S.J. (2017). Sources of variation in foliar secondary chemistry in a tropical forest tree community. Ecology, 98(3), 616-623. https://doi.org/10.1111/nph.1443810.1002/ecy.1689.

Sedio, B.E., Devaney, J.L., Pullen, J., Parker, G.G., Wright, S.J. & Parker, J.D. (2020). Chemical novelty facilitates herbivore resistance and biological invasions in some introduced plant species. Ecology and Evolution, 10(16), 8770-8792. https://doi.org/10.1111/nph.1443810.1002/ece3.6575.

Sedio, B.E., Durant Archibold, A., Rojas Echeverri, J.C., Debyser, C., Boya P, C.A. & Wright, S.J. (2019). A comparison of inducible, ontogenetic, and interspecific sources of variation in the foliar metabolome in tropical trees. PeerJ, 7, e7536. https://doi.org/10.7717/peerj.7536.

Shimazaki, A. & Miyashita, T. (2002). Deer browsing reduces leaf damage by herbivorous insects through an induced response of the host plant. Ecological Research, 17(5), 527-533. https://doi.org/10.1046/j.1440-1703.2002.00510.x.

Singh, B. & Sharma, R.A. (2014). Plant terpenes: Defense responses, phylogenetic analysis, regulation and clinical applications. 3 Biotech, 5(2), 129-151. https://doi.org/10.1007/s13205-014-0220-2.

Smith, C.A., Want, E.J., O'Maille, G., Abagyan, R. & Siuzdak, G. (2006). XCMS: Processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Analytical Chemistry, 78(3), 779-787. https://doi.org/10.1021/ac051437y.

Snoeren, T.A.L., Kappers, I.F., Broekgaarden, C., Mumm, R., Dicke, M. & Bouwmeester, H.J. (2010). Natural variation in herbivore-induced volatiles in *Arabidopsis thaliana* . Journal of Experimental Botany, 61(11), 3041-3056. https://doi.org/10.1093/jxb/erq127.

Stephan, J.G., Pourazari, F., Tattersdill, K., Kobayashi, T., Nishizawa, K. & De Long, J.R. (2017). Long-term deer exclosure alters soil properties, plant traits, understory plant community and insect herbivory, but not the functional relationships among them. Oecologia, 184(3), 685-699. https://doi.org/10.1007/s00442-017-3895-3.

Stork, W., Diezel, C., Halitschke, R., Galis, I. & Baldwin, I.T. (2009). An ecological analysis of the herbivory-elicited JA burst and its metabolism: Plant memory processes and predictions of the moving target model. PloS One, 4(3), e4697. https://doi.org/10.1371/journal.pone.0004697.

Stowe, K.A., Marquis, R.J., Hochwender, C.G. & Simms, E.L. (2000). The evolutionary ecology of tolerance to consumer damage. Annual Review of Ecology and Systematics, 31(1), 565-595. https://doi.org/10.1146/annurev.ecolsys.31.1.565.

Strauss, S.Y. & Agrawal, A.A. (1999). The ecology and evolution of plant tolerance to herbivory. Elsevier Ltd, Oxford.

Takada, M., Asada, M. & Miyashita, T. (2001). Regional differences in the morphology of a shrub *Damnacanthus indicus* : An induced resistance to deer herbivory? Ecological Research, 16(4), 809-813. https://doi.org/10.1046/j.1440-1703.2001.00436.x.

Takada, M., Asada, M. & Miyashita, T. (2003). Can spines deter deer browsing?: A field experiment using a shrub *Damnacanthus indicus* . Journal of Forest Research, 8(4), 321-323. https://doi.org/10.1007/s10310-003-0043-1.

Tugizimana, F., Piater, L. & Dubery, I. (2013). Plant metabolomics: A new frontier in phytochemical analysis. South African Journal of Science, 109(5-6), 1-11. https://doi.org/10.1590/sajs.2013/20120005.

Umair, M., Sun, N., Du, H., Yuan, J., Arshad Mehmood Abbasi, Wen, J., Yu, W., Zhou, J. & Liu, C. (2019). Differential metabolic responses of shrubs and grasses to water additions in arid karst region, southwestern China. Scientific Reports, 9, 1-21. https://doi.org/10.1038/s41598-019-46083-1.

Urbanek, R.E. & Nielsen, C.K. (2013). Influence of landscape factors on density of suburban white-tailed deer. Landscape and Urban Planning, 114(0), 28-36. https://doi.org/10.1016/j.landurbplan.2013.02.006.

Vourc'h, G., Martin, J., Duncan, P., Escarre, J. & Clausen, T.P. (2001). Defensive adaptations of *Thuja plicata* to ungulate browsing: A comparative study between mainland and island populations. Oecologia, 126(1), 84-93. https://doi.org/10.1007/s004420000491.

Vourc'h, G., Vila, B., Gillon, D., Escarre, J., Guibal, F., Fritz, H., Clausen, T.P. & Martin, J. (2002). Disentangling the causes of damage variation by deer browsing on young *Thuja plicata* . Oikos, 98, 271-283. https://doi.org/10.1034/j.1600-0706.2002.980209.x.

War, A.R., Paulraj, M.G., Ahmad, T., Buhroo, A.A., Hussain, B., Ignacimuthu, S. & Sharma, H.C. (2012). Mechanisms of plant defense against insect herbivores. Plant Signaling & Behavior, 7(10), 1306-1320. https://doi.org/10.4161/psb.21663.

Ward, J.S., Williams, S.C. & Linske, M.A. (2018). Influence of invasive shrubs and deer browsing on regeneration in temperate deciduous forests. Canadian Journal of Forest Research, 48(1), 58-67. https://doi.org/10.1139/cjfr-2017-0208.

Wiggins, N.L., Forrister, D.L., Endara, M., Coley, P.D. & Kursar, T.A. (2016). Quantitative and qualitative shifts in defensive metabolites define chemical defense investment during leaf development in*Inga* , a genus of tropical trees. Ecology and Evolution, 6(2), 478-492. https://doi.org/10.1002/ece3.1896.

Xiong, Y., DeFraia, C., Williams, D., Zhang, X. & Mou, Z. (2009). Characterization of Arabidopsis 6-phosphogluconolactonase T-DNA insertion mutants reveals an essential role for the oxidative section of the plastidic pentose phosphate pathway in plant growth and development. Plant and Cell Physiology, 50(7), 1277-1291. https://doi.org/10.1093/pcp/pcp070.

Yates, E.D., Levia, D.F.J. & Williams, C.L. (2004). Recruitment of three non-native invasive plants into a fragmented forest in southern Illinois. Forest Ecology and Management, 190, 119-130. https://doi.org/10.1016/j.foreco.2003.11.008.

Zeier, J. (2013). New insights into the regulation of plant immunity by amino acid metabolic pathways. Plant, Cell and Environment, 36(12), 2085-2103. https://doi.org/10.1111/pce.12122.

Appendix Table 1. Metabolite features that are shared by two or more species.

Species	**Number of metabolites**	**Metabolite features**
E. alatus, L. benzoin, N. sylvatica and *R. multiflora*	27	113.0594; 127.0752; 99.9244; 105.9344; 1
E. alatus, N. sylvatica and *R. multiflora*	14	89.0229; 162.0478; 149.0597; 188.9322; 1
E. alatus, L. benzoin and *R. multiflora*	10	317.9508; 181.0708; 160.841; 163.0601; 3
E. alatus, L. benzoin and *N. sylvatica*	28	646.8527; 190.9276; 454.289; 297.1524; 3
L. benzoin, N. sylvatica and *R. multiflora*	20	258.9156; 180.8986; 78.9575; 171.0653; 1
E.alatus and *R.multiflora*	27	121.9427; 489.0854; 198.9355; 600.5289;
E. alatus and N. sylvatica	28	148.0321; 192.928; 448.0965; 510.8784; 2
E. alatus and *L. benzoin*	61	138.0548; 228.1602; 222.9189; 100.9322;
N. sylvatica and *R. multiflora*	28	118.9228; 107.0237; 384.9351; 72.0156; 1
L. benzoin and *R. multiflora*	25	153.0182; 128.034; 125.0595; 173.9252; 1
L. benzoin and *N. sylvatica*	27	187.0968; 208.0536; 164.9262; 588.8942;

Appendix Table 2 . Unique and shared putative metabolic pathways that are identified by the GSEA pathway analysis of the MS Peaks to Pathways module of metaboanalyst. Asterisks denote metabolic pathways involved in defense.

Species	**Total number of metabolites**	**Predicted metabolic pathway**
E. alatus, *L. benzoin*, *N. sylvatica* and *R. multiflora*	14	Amino sugar and nucleotide sugar metabolism Starch and sucrose metabolism Flavonoid biosynthesis* Flavone and flavonol biosynthesis* Phenylpropanoid biosynthesis* Purine metabolism Galactose metabolism Ubiquinone and other terpenoid-quinone biosynthesis Glucosinolate biosynthesis* Ascorbate and aldarate metabolism Cysteine and methionine metabolism Pentose phosphate pathway Riboflavin metabolism Steroid biosynthesis
E. alatus, *L. benzoin* and *R. multiflora*	2	Pantothenate and CoA biosynthesis Glutathione metabolism
E. alatus, *L. benzoin* and *N. sylvatica*	3	Glyoxylate and dicarboxylate metabolism Cyanoamino acid metabolism Arginine and proline metabolism

Species	**Total number of metabolites**	**Predicted metabolic pathway**
E. alatus, *N. sylvatica* and *R. multiflora*	3	Tyrosine metabolism Arginine biosynthesis Stilbenoid, diarylheptanoid and gingerol biosynthesis
L. benzoin, *N. sylvatica* and *R. multiflora*	4	Glycerolipid metabolism Pentose and glucuronate interconversions Carbon fixation in photosynthetic organisms Terpenoid backbone biosynthesis*
E. alatus and *L. benzoin*	4	Porphyrin and chlorophyll metabolism Citrate cycle (TCA cycle) Carotenoid biosynthesis beta-Alanine metabolism
E. alatus and *R. multiflora*	1	alpha-Linolenic acid metabolism*
E. alatus and *N. sylvatica*	2	Phenylalanine, tyrosine and tryptophan biosynthesis* Isoquinoline alkaloid biosynthesis
L. benzoin and *R. multiflora*	2	Zeatin biosynthesis* Sulfur metabolism
L. benzoin and *N. sylvatica*	9	Inositol phosphate metabolism N-Glycan biosynthesis Phosphonate and phosphinate metabolism Fructose and mannose metabolism Pyrimidine metabolism Glycolysis / Gluconeogenesis Folate biosynthesis Phosphatidylinositol signaling system Glycine, serine and threonine metabolism
N. sylvatica and *R. multiflora*	1	Lysine biosynthesis
E. alatus	4	Alanine, aspartate and glutamate metabolism Glycerophospholipid metabolism Histidine metabolism Fatty acid biosynthesis
L. benzoin	4	Vitamin B6 metabolism Caffeine metabolism Diterpenoid biosynthesis* Anthocyanin biosynthesis
R. multiflora	3	Nicotinate and nicotinamide metabolism Glycosylphosphatidylinositol (GPI)-anchor biosynthesis Thiamine metabolism

Species	Total number of metabolites	Predicted metabolic pathway
N. sylvatica	5	Selenocompound metabolism Valine, leucine and isoleucine degradation Tryptophan metabolism* Sesquiterpenoid and triterpenoid biosynthesis* Monoterpenoid biosynthesis*

Appendix Table 3 . Metabolic pathways identified based on the metabolite features identified for *N. sylvatica* using the GSEA pathway analysis of the MS Peaks to Pathways module of metaboanalyst. Asterisks denote metabolic pathways involved in defense.

Metabolic pathway	Pathway Total	Hits	P_val	P_adj	NES	EC Hits
Pentose phosphate pathway	5	5	0.0119	0.402	-1.73	EC0004;EC0002
Starch and sucrose metabolism	3	3	0.02941	0.402	1.664	EC0002;EC0009;EC0006
Pyrimidine metabolism	1	1	0.05455	0.402	1.378	EC0002
Cyanoamino acid metabolism	1	1	0.05455	0.402	1.378	EC0002
Riboflavin metabolism	1	1	0.05455	0.402	1.414	EC0002
Monoterpenoid biosynthesis*	1	1	0.05882	0.402	-1.481	EC0002
Arginine biosynthesis	1	1	0.1273	0.7455	1.308	EC00025;EC00041
Folate biosynthesis	1	1	0.1765	0.8039	-1.367	EC0005;EC00020;EC000
Lysine biosynthesis	1	1	0.2545	0.8039	1.237	EC00012
Glycine, serine and threonine metabolism	1	1	0.2549	0.8039	-1.253	EC0003;EC00013;EC000
Glyoxylate and dicarboxylate metabolism	1	1	0.2549	0.8039	-1.253	EC00010
Selenocompound metabolism	1	1	0.2941	0.8039	-1.215	EC0004
Purine metabolism	3	3	0.3529	0.8039	1.033	EC00021;EC0004
Terpenoid backbone biosynthesis*	3	3	0.3676	0.8039	-1.125	EC00036
Phenylpropanoid biosynthesis*	8	8	0.4	0.8039	1.054	EC00014

Appendix Table 4 . Metabolic pathways identified based on the metabolite features identified for *L. benzoin* using the GSEA pathway analysis of the MS Peaks to Pathways module of metaboanalyst. Asterisks denote metabolic pathways involved in defense.

Metabolic pathway	Pathway Total	Hits	P_val	P_adj	NES	EC Hits
Glyoxylate and dicarboxylate metabolism	2	2	0.01639	0.5854	-1.498	EC0006;EC0002
Pentose phosphate pathway	3	3	0.02857	0.5854	-1.502	EC0005
Glucosinolate biosynthesis	4	4	0.0625	0.5854	-1.509	EC0002;EC0009;EC0
Vitamin B6 metabolism	1	1	0.06818	0.5854	-1.327	EC0002
Purine metabolism	1	1	0.08333	0.5854	1.314	EC0002
Ascorbate and aldarate metabolism	3	3	0.09375	0.5854	1.478	EC00020;EC0002;EC0
Pantothenate and CoA biosynthesis	2	2	0.09756	0.5854	1.448	EC0002;EC00012;EC0
Pyrimidine metabolism	1	1	0.1167	0.6125	1.286	EC00047
Citrate cycle (TCA cycle)	1	1	0.1591	0.6682	-1.266	EC00036
Glycine, serine and threonine metabolism	1	1	0.1591	0.6682	-1.297	EC0001
Steroid biosynthesis	1	1	0.2045	0.7344	-1.236	EC00037
Flavone and flavonol biosynthesis*	4	4	0.225	0.7344	-1.215	EC00010
Glycolysis / Gluconeogenesis	2	2	0.2623	0.7344	-1.154	EC0006
Glycerolipid metabolism	2	2	0.2623	0.7344	-1.154	EC00026;EC00017;EC

Carbon fixation in photosynthetic organisms	2	2	0.2623	0.7344	-1.154	EC00022

Appendix Table 5. Top 15 metabolic pathways identified based on the metabolite features of *R. multiflora* using the GSEA pathway analysis of the MS Peaks to Pathways module of metaboanalyst. Asterisks denote metabolic pathways involved in defense.

Metabolite pathway	**Pathway Total**	**Hits**	**P_val**	**P_adj**	**NES**	**EC Hits**
Pentose phosphate pathway	1	1	0.04348	0.5068	-1.348	EC0008
Carbon fixation in photosynthetic organisms	1	1	0.04348	0.5068	-1.378	EC0001
Glucosinolate biosynthesis*	3	3	0.06757	0.5068	-1.502	EC0004;EC0001
Pentose and glucuronate interconversions	1	1	0.125	0.5068	1.306	EC0001;EC00016
Glycerolipid metabolism	1	1	0.125	0.5068	1.306	EC00046
Nicotinate and nicotinamide metabolism	1	1	0.125	0.5068	1.277	EC00039;EC00027;EC
Zeatin biosynthesis*	1	1	0.125	0.5068	1.306	EC00012
Ascorbate and aldarate metabolism	2	2	0.1351	0.5068	1.49	EC00011;EC00013;EC
Glutathione metabolism	1	1	0.1739	0.5797	-1.256	EC00019
Arginine biosynthesis	1	1	0.1964	0.5893	1.219	EC00014
Steroid biosynthesis	1	1	0.2174	0.5929	-1.225	EC00026
Galactose metabolism	2	2	0.2424	0.6061	-1.167	EC0005
Lysine biosynthesis	1	1	0.3036	0.7005	1.161	EC0004;EC00015;EC
Sulfur metabolism	2	2	0.4394	0.8696	-1.037	EC00030;EC0001;EC
Thiamine metabolism	1	1	0.4821	0.8696	1.045	EC0001

Appendix Table 6. Top 15 metabolic pathways identified based on the metabolite features identified for *E. alatus* using the GSEA pathway analysis of the MS Peaks to Pathways module of metaboanalyst. Asterisks denote metabolic pathways involved in defense.

Metabolic pathway	**Pathway Total**	**Hits**	**P_val**	**P_adj**	**NES**	**EC Hits**
Glutathione metabolism	1	1	0.02041	0.4286	-1.358	EC0004;EC0008
Pentose phosphate pathway	1	1	0.04082	0.4286	-1.268	EC0005
Alanine, aspartate and glutamate metabolism	1	1	0.04082	0.4286	-1.298	EC00021;EC00010;E
Ascorbate and aldarate metabolism	2	2	0.07042	0.4286	-1.376	EC0008;EC00014
Porphyrin and chlorophyll metabolism	1	1	0.07407	0.4286	1.294	EC00020
Isoquinoline alkaloid biosynthesis	2	2	0.09859	0.4286	-1.315	EC00047
Glucosinolate biosynthesis	2	2	0.09859	0.4286	-1.312	EC00018;EC00040
Tyrosine metabolism	3	3	0.1039	0.4286	-1.279	EC00013
Steroid biosynthesis	1	1	0.1224	0.449	-1.238	EC00030;EC00011;E
Starch and sucrose metabolism	2	2	0.1613	0.5233	1.277	EC0006
Flavone and flavonol biosynthesis*	6	6	0.1744	0.5233	-1.316	EC00024
Arginine biosynthesis	1	1	0.2222	0.5577	1.182	EC00037
Glycerophospholipid metabolism	1	1	0.2222	0.5577	1.21	EC00030
Citrate cycle (TCA cycle)	2	2	0.2535	0.5577	-1.17	EC00017;EC00018;E
Glyoxylate and dicarboxylate metabolism	2	2	0.2535	0.5577	-1.17	EC00018

Figure Captions

Figure 1. Proportion of sampled unfenced plants with deer browse signs in the understory of a central New Jersey suburban forest, Herrontown Woods Preserve. LIBE, *Lindera benzoin* ; NYSY, *Nyssa sylvatica* ; EUAL, *Euonymus alatus* ; ROMU, *Rosa multiflora*Number of sampled plants from right to left: LIBE,

30, 476; NYSY, 29, 308; EUAL, 80, 743; ROMU, 55, 492. G-tests for heterogeneity indicated a significant difference among species only for the pooled 2012-2019 data; different letters indicate species that were significantly different (P< 0.02) in pairwise tests.

Figure 2. Comparison of the metabolite profiles of the tree species in the fenced and unfenced plots. A) The number of common and unique metabolite features identified for the four woody tree species is depicted in the Venn diagram. B) Principal component analysis (PCA) depicts the clustering of all the samples based on species. C) Dendrogram displays the relationship of the samples based on genotype and presence or absence of fences. D) Common and unique metabolic pathways predicted based on the differentially accumulating metabolites.

Figure 3. Identification of metabolites that accumulate differentially in the four species following the treatment gradient. A) Partial least squares discriminant analysis (PLS-DA) separates the samples into eight distinct groups corresponding to species and containment in fence or unfenced plots. B) Important features that contributed to the PLS-DA based separation of the samples are depicted with their pattern of accumulation shown by the color code. C) Normalized concentrations (mean+ SE) of the top five metabolite features identified by PLS-DA. Different letters indicate statistically significant differences (ANOVA and Tukey's HSD).

Figure 4. Untargeted metabolomic analysis of *N. sylvatica* samples from fenced and open plots. A) Principal component analysis (PCA) grouped the samples into two clusters that correspond with the fencing treatment. B) PLS-DA clustering identified two distinct groups based on treatment. C) The top 15 metabolite features that contributed to the separation of the samples into two clusters on the PLS-DA plot and their relative abundances is shown. D) Hierarchical cluster analysis (HCA) computed based on the top 30 significantly different metabolite features groups the samples into two groups.

Figure 5. Metabolomic comparison of fenced and unfenced *L. benzoin* samples. A) PCA plot demonstrates the metabolite-based relationship of the fenced and unfenced *L. benzoin* samples. B) PLS-DA plot clusters *L. benzoin* samples into two groups based on significant features. C) The top 15 metabolite features that are responsible for the PLS-DA grouping of the samples and their relative abundances in fenced and unfenced plants is displayed. D) Pearson Correlation and HCA identified the top 25 metabolites that varied significantly among the samples and clustered the sampled into groups based on these features.

Figure 6. Untargeted metabolomic analysis of *R. multiflora* under fenced and unfenced plots. A) PCA plot displays the similarities and differences of *R. multiflora* samples based on the identified metabolite features. B) PLS-DA identifies two clusters that correspond to treatments. C) Important features that differentiate the samples into fenced and unfenced groups. D) HCA displays the top 25 significantly different metabolites and their relative abundances among the *R. multiflora* samples.

Figure 7. Comparative metabolomic analysis of *E. alatus* samples from fenced and open plots. A) PCA indicates no treatment-based clustering of the samples. B) PLS-DA clusters the *E. alatus*samples into two treatment-related groups. C) Fifteen of the top most important features were identified by PLS-DA analysis and their relative abundances in fenced and unfenced plots are shown. D) HCA depicts the relative concentrations of the top 25 metabolite features that differed among the fenced and unfenced samples significantly.

Figure 8. Means + 95% CL of percent cover and height of four woody and semi-woody species in the understory of a central New Jersey suburban forest, Herrontown Woods Preserve, in fall 2019. Fenced plots had excluded deer since spring 2013. LIBE, *Lindera benzoin* ; NYSY,*Nyssa sylvatica* ; EUAL, *Euonymus alatus* ; ROMU, *Rosa multiflora* . Top, N fenced = 18, N not fenced = 20. Bottom, N from left to right: 23, 41, 14, 5, 65, 18, 11, 17. * P<0.05, **P<0.01, ****P<0.0001, from one-tailed t-tests between fenced and no-fenced values for each species.

Figure 9. Phylogenetic relatedness of the four species included in the study. Species Order classifications are from the Integrated Taxonomic Information System (http://www.itis.gov), and the Order-level tree is from

the Angiosperm Phylogeny Website (http://www.mobot.org/MOBOT/research/APweb/).

DATA ACCESSIBILITY STATEMENT

Data used for this paper will be available from the Dryad Digital Repository.

COMPETING INTERESTS STATEMENT

There are no competing interests.

AUTHORS' CONTRIBUTIONS

Morrison and Woldemariam conceived of and designed the study. Morrison was responsible for all field work and field data analysis; Woldemariam was responsible for all laboratory work and analysis of all metabolomic data. Morrison outlined the manuscript, was lead author on the Abstract, Introduction, the field data Methods and Results, and the Discussion. Woldemariam was lead author on the metabolomics Methods and Results. Both authors edited the entire manuscript and gave final approval for publication.

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