

## Article

# Ecological Impact of American Chestnut Hybrid Restoration on Invertebrate Communities Above- and Belowground

Jaq Reed <sup>1,†</sup>, Evan Hausler <sup>1,†</sup>, Abigail Levinson <sup>1</sup>, Jonathan Horton <sup>1</sup>, Denis S. Willett <sup>2</sup> and Camila C. Filgueiras <sup>1,\*</sup> 

<sup>1</sup> Department of Biology, UNC Asheville, Asheville, NC 28804, USA

<sup>2</sup> North Carolina Institute for Climate Studies, Department of Applied Ecology, NC State University, Raleigh, NC 27695, USA

\* Correspondence: camila@unca.edu

<sup>†</sup> These authors contributed equally to this work.

**Abstract:** The cross-hybridization of American chestnut (*Castanea dentata* (Marsh.) Borkh.) with Chinese chestnut (*Castanea mollissima* Bl.) is a promising strategy for restoring a blight-resistant strain of this keystone species to the Appalachian mountains. To assess the ecological impacts of hybridization on invertebrate communities, we conducted a study across chestnut plots with varying degrees of hybridization (75%, 94%, or 100% American chestnut). Our findings indicate American chestnut hybridization impacted invertebrate communities above- and belowground. Aboveground insect community composition, insect herbivory, gall infestation, and belowground invertebrate diversity were all altered. While some of these differences could be explained by different growth habits or environmental differences, stark differences in Asian chestnut gall wasp infestation (*Dryocosmus kuriphilus* Yasumatsu.) suggest a genetic component. These results suggest that chestnut hybridization, and particularly expanded restoration efforts using chestnut hybrids, could impact invertebrate communities above- and belowground in addition to pest dynamics. Understanding these effects is crucial for successful chestnut restoration and ecosystem management.



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**Keywords:** American chestnut; Chinese chestnut; entomopathogenic nematodes; population dynamics; soil arthropods

## 1. Introduction

Prior to the 20th century, American chestnuts (*Castanea dentata* (Marsh.) Borkh.) accounted for 25%–50% of the canopy within eastern hardwood forests of the United States and were once a vital keystone species providing food and habitat for wildlife [1]. These trees were well known for their strong, rot-resistant, fast-growing wood, which at the time was a valuable resource for timber production and construction industries [2]. The fungus *Cryphonectria parasitica* commonly known as chestnut blight was discovered in New York for the first time in 1904 and effectively eliminated approximately 4 billion trees by 1960 [3]. This catastrophe had wide-reaching economic and ecological effects. The sudden absence of the chestnut trees which previously made up a large portion of these forests fundamentally changed the dynamics of the biological community as other vegetation grew to fill in their niche. Knowledge of the specific ecological role that the American chestnut historically fulfilled is limited in comparison to other species as *C. parasitica* infection spread throughout the U.S. before a modern understanding of forest ecology was implemented [3].

In the 1980s, efforts to reintroduce the chestnut tree to American forests by breeding a blight-resistant hybrid began. Chinese chestnuts were selected for backcross breeding due to their naturally high resistance to *C. parasitica*, and Chinese–American hybrid trees have shown promising results in terms of survival during reintroduction trials [4]. Successful restoration of *C. dentata* hybrids to the tree’s historic range is projected to have wide-reaching beneficial effects on the surrounding ecological community. These impacts include

stabilizing food resources for foraging animals and increasing mammal presence responsible for the control of gypsy moth and lyme-carrying tick populations [1]. However, with the planting of these hybrids for nut farming and the return of the chestnut to American forests through conservation efforts, there comes a potential for unintended consequences. Studies have shown that this recent increase in chestnut tree farms has resulted in the reemergence of chestnut pests including the lesser chestnut weevil (*Curculio sayi*) [5] and the Asian Chestnut Gall Wasp (*Dryocosmus kuriphilus* Yasumatsu, Hymenoptera, Cynipidae) [6]. In addition to the limited amount of research conducted on these pests, there have been few studies thus far on the effect of the reintroduction of American–Chinese chestnut hybrids on invertebrate diversity above- and belowground.

One of the most well-known pests of chestnut trees is the Asian chestnut gall wasp [6,7]. Previous studies examining different plant hybridizations and the effects it has on parasite infestation have been largely variable [8]. The Asian chestnut gall wasp is considered one of the most successful invasive species [7]. They are a species of parasitic wasp that create galls, resulting in greatly reduced green biomass [9]. These parasitic wasps in high infestations damage the canopies, impacting chestnut production [10], as it leads to decreased flower and leaf production [9]. In heavy infestation, the Asian chestnut gall wasp damages the tree's survival and reproduction abilities, ultimately killing chestnut trees [10]. Understanding if American chestnut hybrids are impacted differently by this pest is paramount for reintroducing this species.

Understanding impacts of chestnut hybrid introduction extends to potential impacts on invertebrate diversity above- and belowground. Plants and soil biodiversity are inextricably linked. Subterranean animals aid in decomposition of organic matter and retention of moisture and nutrients within the soil necessary to plants' survival, and soil fauna depend on the resources and shelter provided by plant roots for their own livelihood [11,12]. These interactions keep the soil balanced and nutrient-rich, which results in a healthier ecosystem both above- and belowground. These organisms can also cause damage to plants by consuming and parasitizing their roots or leaving them susceptible to disease [12]. Biodiversity within soil communities is important and indicative of soil health, as invertebrates' interactions with each other and their surrounding environment are essential to maintaining ecological balance [11,12]. Soil biodiversity is positively correlated with plant diversity and nutrient retention and recycling [13]. By gaining an understanding of these interactions taking place in the soil surrounding American–Chinese chestnut hybrids, we can begin to examine how the reintroduction of these trees may be affecting our forests on both a micro and macro scale.

While morphologically similar, American–Chinese chestnut hybrids have slightly different maximum leaf size potential and curl compared to pure American chestnuts [14]. Understanding how these leaf differences could affect insect herbivory is crucial. Leaf size and shape have been seen to affect insect herbivores through the abundance and richness of different plant genotypes [15]. In terms of foliar chemistry, it was found that American chestnuts have higher concentrations of carbohydrates and lower tannin levels compared to hybrids, which means pure American chestnuts could be a higher-quality host for generalist herbivores but have a limited defense to insect herbivory [16]. Genetic differences between American chestnut and its hybrids could cause an effect on the dependent insect herbivore community [17] and also the enemies of those herbivores [18]. Following the theory of bottom-up interactions [19], impacts on insect herbivores would affect insects that prey on them. Genetically controlled plant traits have shown that hybridization can affect the distribution and abundance of associated insect species as well as impact insect diversity [20,21]. The insect diversity can be affected due to the accumulation of insects that would be more unique to each parental species [21]. With many introduced insects from the native region of the Chinese chestnut, this could pose a problem for reintroduction. For successful reintroduction of American chestnut trees through hybridization, we should understand the impact on insect diversity and the effects insects could have on them.

To evaluate the impact of chestnut hybrid restoration on invertebrate communities above- and belowground, a combination of trapping and monitoring assessments was used across three different hybrid types used in restoration efforts. Aboveground insect diversity was evaluated through trapping, assessments of herbivory, and assessments of galling. Belowground invertebrate diversity was evaluated using assessments of leaf litter and entomopathogenic nematode soil bio-indicators.

## 2. Materials and Methods

### 2.1. Experimental Design

Plots of chestnut hybrids were located in DuPont Recreational State Forest (DRSF) in Transylvania county of Western North Carolina. DRSF consists of 12,500 acres of coniferous, hardwood, and mixed temperate forest and was the site of experimental American chestnut tree planting during the late 2000's and mid 2010's. Treatment plots consisted of 100% American chestnuts (100% AC), 94% American chestnut hybrids with 6% Chinese chestnut (94% AC), and 75% American chestnut hybrids with 25% Chinese chestnut (75% AC) (Table 1).

**Table 1.** Plot information for American–Chinese chestnut hybridization in DuPont Recreational State Forest.

| Chestnut Hybrid | American | Chinese | Location               | Year Planted | Mean Height | Mean Diameter | Number of Trees (N) |
|-----------------|----------|---------|------------------------|--------------|-------------|---------------|---------------------|
| 100% AC         | 100%     | 0%      | 35.21939,<br>−82.59705 | 2013         | 35.89 cm    | 6.98 mm       | 46                  |
| 94% AC          | 94%      | 6%      | 35.21192,<br>−82.59662 | 2014         | 3.9 m       | 3.05 cm       | 62                  |
| 75% AC          | 75%      | 25%     | 35.21606,<br>−81.59480 | 2009         | 4.27 m      | 5.2 cm        | 30                  |

Plots with chestnut hybrids are integrated with natural vegetation, including common species such as oaks (*Quercus* spp.), pines (*Pinus* spp.), and sourwoods (*Oxydendrum* spp.). The 100% AC plot was located in a relatively flat densely forested area partially shaded by large surrounding trees. Trees planted within this site came from collections of chestnuts off of the Blue Ridge Parkway and were more spread out than those of other sites and had experienced a higher level of blight than the hybridized groups; the majority of the chestnut trees in this plot were small resprouts undergoing continual growth suppression as a result of *C. parasitica* infection. The 94% AC plot was located on a south facing slope with fewer large shading trees in comparison to the other two sites studied due to clearcutting of the area prior to chestnut planting. The 75% AC plot was located in a flat and densely wooded area. In all plots, competing vegetation, including large trees and shrubs, had been removed from the area directly surrounding chestnut stands. To evaluate above- and belowground impacts, samples for the assessments described below were collected during the summer of 2023 on May 25th, July 7th, July 20th, and August 10th.

### 2.2. Aboveground Insect Diversity

Four pyramid traps (Tedders Pyramid Trap, GL-5000-06, Great Lakes IPM, Vestaburg, MI, USA) were deployed across each of the three hybrid types (75% AC, 94% AC, and 100% AC) for a total of 12 traps. These pyramid traps are commonly used to collect and monitor insect abundance, particularly *Curculio* sp. Each trap was constructed with four 4-foot tall triangular pieces of upright black cardboard leading to a plastic trap cup at the top. These traps were evenly distributed throughout each hybrid type in the middle of each plot to avoid edge effects. The traps were placed on 15 May 2023, and collections continued until 29 August 2023.

Each trap was staked to the ground within a meter of an adjacent chestnut tree. The trap catch was collected roughly every ten days and traps were cleaned in between collections. All traps were collected on the same day and arthropods were identified to taxonomic order once frozen [22]. Arthropods that were too mutilated in the traps to identify were discarded. Traps

that had fallen between collections were excluded from the data and redeployed immediately. These traps are designed to mimic tree trunks and capture arthropods climbing skywards.

Aboveground arthropod diversity was evaluated using comparisons of abundances, diversity indices, and canonical correspondance analysis to compare differences in aboveground arthropod communities between chestnut hybrids. Seasonal abundance was totaled by order and trap over the season and evaluated with a one-way Analysis of Variance (ANOVA). Differences of Shannon and Simpson diversity indices were also evaluated using ANOVA [23,24]. A permutational analysis of variance was used to evaluate differences in Jaccard similarity scores across chestnut types. Canonical correspondence analysis was used to evaluate the differences in community composition between chestnut hybrid types. Post hoc comparisons across chestnut types were conducted using the Tukey method.

### 2.3. Insect Herbivory

To evaluate the impact of herbivory on chestnut hybrids, ten leaves were collected from different trees from each hybrid type every month from June 2023 until August 2023. Each collection was based on a stratified random sample of trees and leaves were selected through blind collection. All the leaves were collected within 3 m in height off the ground.

After the collection, leaves were placed in the freezer at  $-18^{\circ}\text{C}$  for 24 h to preserve and flatten. To capture images without shadows, we constructed an examination box, consisting of a cardboard box with a light source inside and a paper-covered top. Another box without a bottom surface was placed on top, creating an enclosed space. Photos were taken using an iPhone 13 with dual 12MP cameras through a hole cut in the top box. Each leaf was placed in between the boxes on the illuminated surface.

Photos were processed using the Bioleaf Application [25] with the setting of no reflection to find the percent of herbivory (leaf area eaten) for each leaf. Leaf area eaten in this case is calculated as the percentage of the leaf missing due to insect herbivory. If a leaf's edge was defoliated, the missing portion was estimated based on the opposite side of the leaf. Only herbivory attributed to insect herbivory was evaluated, while herbivory likely caused by mammals or other natural elements was excluded. Insect herbivory was determined based on patterns of known insect herbivory patterns which cause more rippled sections of damage compared to mammal herbivory or tears that are larger with straighter cuts. Leaves with gall infestation were not collected for herbivory assessments.

Insect herbivory was evaluated across chestnut types using a hurdle model to independently model the likelihood of herbivory (presence/absence, probability of herbivory being present) and the severity of herbivory (percent herbivory, the amount of leaf area eaten). A binomial model was used for herbivory likelihood and a Poisson model used for herbivory severity. Insect herbivory was converted to an index of herbivory damage by taking the ceiling of percent leaf area eaten for use in the hurdle model. Post hoc comparisons of insect herbivory were conducted using the Tukey method.

### 2.4. Asian Chestnut Gall Wasp

To evaluate the impact of Asian Chestnut Gall Wasp infestation on chestnut hybrids, a gall sampling method was adapted from previous work [26]. Ten trees were selected from each hybrid type through stratified random sampling. Those ten trees were then monitored for galls formed by the Asian chestnut gall wasp. Gall counts were recorded every ten days from 15 May 2023 to 29 August 2023. Only current-year gall growth was considered, excluding any old gall growth from previous years.

Gall counts on a per tree basis were separated into four categories (extended from [26]) of tree infestation: None (0 Galls), Low (1–5 Galls), Moderate (6–40 Galls), Severe (>41 Galls). Presence of galls by chestnut type was modeled using logistic regression. Post hoc comparisons of probability of infestation were conducted using Dunnett's test. To compare the differences in infestation levels and the proportions of the presence of galls among the treatments, a Fisher test was conducted.

## 2.5. Soil Arthropods

To evaluate soil arthropod diversity, leaf litter samples were acquired during field collections from May to August 2023. Samples were taken by hand from sites corresponding to pyramid traps described above. The same locations surrounding the traps were used during each sampling and were distinguished using marking flags. The leaf litter samples were stored in one gallon ziplock bags before being processed in the lab.

Leaf litter samples were transported to the NEMA Lab at the University of North Carolina Asheville where they were mixed by hand and added to Berlese funnels in an amount which completely filled the 8.5" × 8.5" funnel. The samples were heated at 40 °C for 48 h, while the majority of the organisms were driven into a 50 mL conical centrifuge tube collecting container containing approximately 25 mL of 70% ethanol where they were preserved until identification [27]. To quantify and sort arthropods to taxonomic class, organisms in samples were identified visually via external morphology using a dissecting microscope and pipetted into glass vials [28].

Belowground arthropod diversity was evaluated using comparisons of abundances, diversity indices, and canonical correspondence analysis to compare differences in belowground insect communities between chestnut hybrids. Seasonal abundance was totaled by taxonomic class and trap over the season then evaluated with a one-way Analysis of Variance (ANOVA). Differences of Shannon and Simpson diversity indices were also evaluated using ANOVA [23,24]. Canonical correspondence analysis was used to evaluate the differences in community composition between chestnut hybrids. Post hoc comparisons across chestnut types were conducted using the Tukey method (Diversity Indices) and the Dunnett Method (Community Composition Index).

## 2.6. Entomopathogenic Nematodes

Entomopathogenic nematodes (EPNs) are soil-dwelling roundworms which are obligate endoparasites of insect hosts. EPNs require a single insect host to complete their life cycle and are therefore monoxenous with indirect transmission involving infective juveniles (IJs) passing through the environment. Because of their dependence on insect hosts and their requirements for healthy soil environments, EPNS are ideal bioindicators of healthy soils. Large EPN populations reflect healthy populations of their host insects [29]. In addition, prior to infection, free-living IJs reside within soil pore space and are susceptible to changes in temperature and moisture, as well as presence of toxic chemicals [30]. Any of these factors which negatively impact nematode populations can result in changes to the stability of the larger ecological community.

Soil samples were collected using soil probes to determine the association of entomopathogenic nematode (EPN) presence, a bioindicator, with chestnut hybrid types [31]. Samples were extracted from two marked sites in proximity to each of four pyramid traps located in the experimental plots described above, resulting in a total of eight soil samples collected per plot per collection date. Each sample consisted of the top 15 cm of three 3/4" diameter soil cores from the same site which were stored in 16 oz containers until lab analysis.

EPNs were extracted from soil samples using sentinel *Galleria mellonella* larvae [32]. Water was added to each sample to increase moisture content along with 10 *G. mellonella* to be used as an insect host for any EPN infective juveniles present in the soil. The samples were allowed to rest for five days at room temperature without disturbance. *G. mellonella* were then removed from soil, externally sterilized using 10% bleach solution, separated according to color in order to prevent bacterial and fungal cross contamination, and added to white traps. The filter paper within the white traps was moistened with deionized water using a syringe and checked for fungal and bacterial growth every 1–2 days until EPNs began to emerge. Moisture levels were maintained within traps to facilitate EPN movement into water [33].

Entomopathogenic nematode presence or absence was evaluated using Fisher's exact test and logistic regression both within and across the observation season.

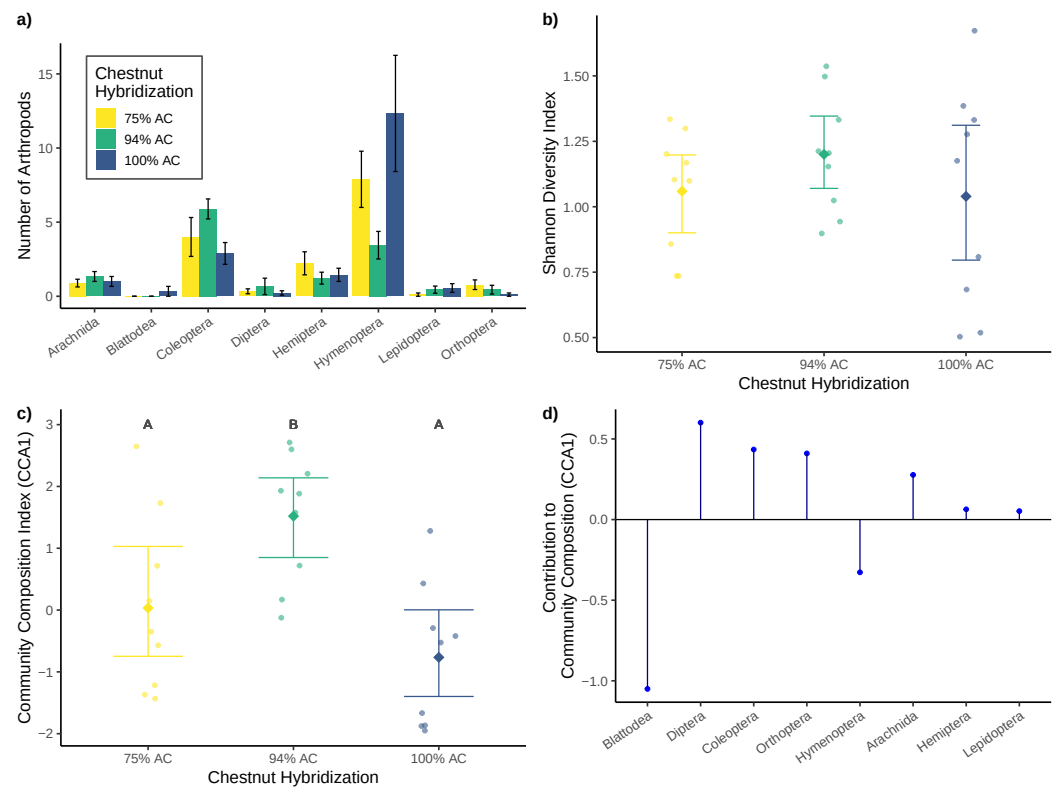
## 2.7. Statistical Analysis

All data were collected into tabular CSV files then analyzed using the R Statistical Programming Language (version 4.3.3) and the RStudio IDE (version 2024.04.1+748) [34,35]. The following packages facilitated analysis: *tidyverse* for data cleaning [36], *here* for file management [37], *pscl* for hurdle models [38,39], *emmeans* for post hoc comparisons [40], *cowplot* for figure arrangement [41], *vegan* for canonical correspondence analysis [42], *car* for regression statistics [43], *janitor* for cleaning [44]. Full datasets and code used to produce this analysis are available upon request.

## 3. Results

### 3.1. Aboveground Insect Diversity

A total of eight orders of invertebrates were collected from pyramid traps over the collection period: Arachnida, Blattodea, Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, and Orthoptera (Figure 1a). Of these eight orders, Hymenoptera was the most abundant in every treatment followed by Coleoptera. There were no significant differences ( $p > 0.05$ ) in order abundance across chestnut type. Shannon (Figure 1b) and Simpson diversity indices varied within chestnut type, but were not significantly different ( $p > 0.05$ ) across chestnut types as evaluated by ANOVA.

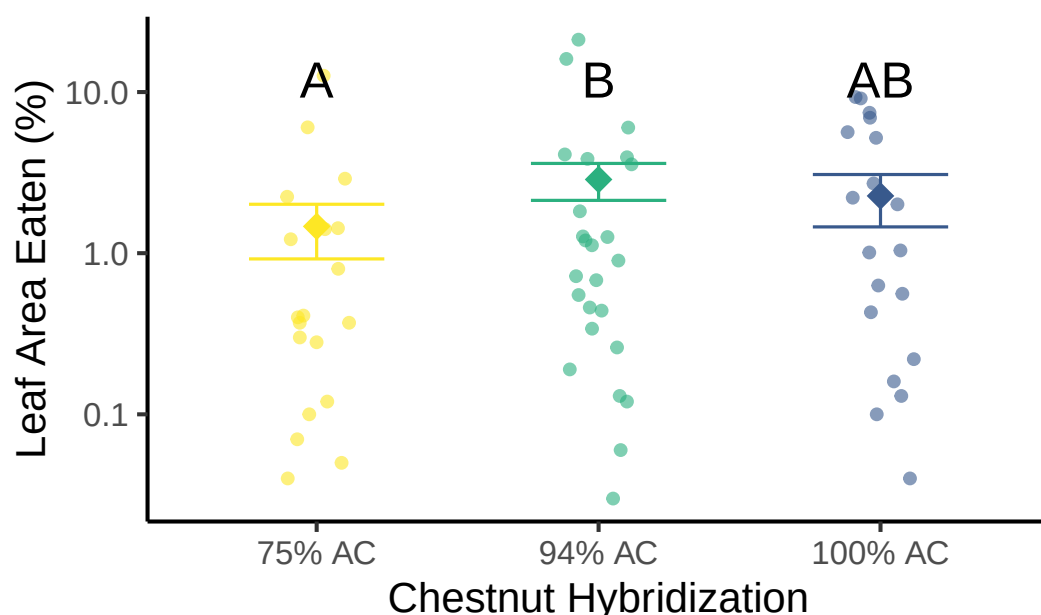


**Figure 1.** Aboveground insect diversity across chestnut hybrid types. **(a)** Total seasonal abundance of arthropods collected in pyramid traps by order. Bar height and error bars denote mean and standard error, respectively. **(b)** Shannon diversity indices by chestnut hybrid types. Points denote index values per site. Diamond points and error bars denote mean and 95% confidence intervals, respectively. **(c)** Community Composition Index (first axis from canonical correspondence analysis) explaining 84% of the constrained variance. Points denote site values. Diamond points and error bars denote mean and 95% confidence intervals, respectively. Letters denote significant differences between chestnut hybrid types at  $p < 0.05$ . **(d)** Relative contribution to community composition index in (c). Absolute values of bar height denote weights assigned to order by the canonical correspondence analysis.

Chestnut type significantly explained approximately 13% of the observed differences in Jaccard similarity indices ( $F = 1.84$ ,  $df = 2$ ,  $p = 0.03$ ). Investigating this further showed that the canonical correspondence analysis model relating chestnut type to community composition significantly ( $F = 2.4$ ,  $df = 2$ ,  $p = 0.015$ ) explained 16.6% of observed variance in community composition. Chestnut type was the significant factor ( $F = 2.4$ ,  $df = 2$ ,  $p = 0.01$ ) driving differences in community composition that were resolved significantly ( $F = 4$ ,  $df = 1$ ,  $p = 0.02$ ) by the first canonical axis explaining 84% of the constrained variation. The 94% American Chestnut hybrids had significantly different ( $p < 0.05$ ) community composition compared with other chestnut hybrid types (Figure 1c). These differences in community composition were driven by the relative contributions of order abundance (Figure 1d).

### 3.2. Insect Herbivory

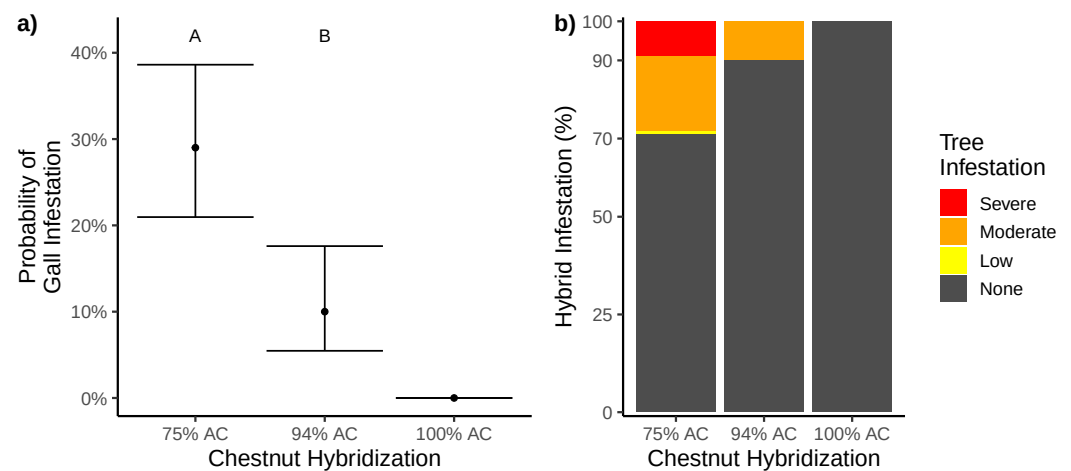
Insect herbivory as measured by percentage of leaf area eaten was explained by chestnut hybrid type. Probability of herbivory (from the zero hurdle model) was marginally significant ( $p < 0.1$ ). Severity of herbivory was significantly explained by chestnut hybrid type ( $p < 0.05$ ). The 94% American Chestnut hybrids had  $1.4\% \pm 0.46\%$  more insect herbivory ( $t = 3.1$ ,  $df = 84$ ,  $p = 0.01$ ) than 75% American Chestnut hybrids (Figure 2).



**Figure 2.** Insect herbivory as measured by percent leaf area eaten on chestnut tree leaves. Leaf area eaten is the percent of missing leaf area due to insect herbivory. Points denote observed values. Diamond points and error bars denote mean and 95% confidence intervals, respectively. Letters denote significant differences between chestnut hybrid types at  $p < 0.05$ .

### 3.3. Asian Chestnut Gall Wasp

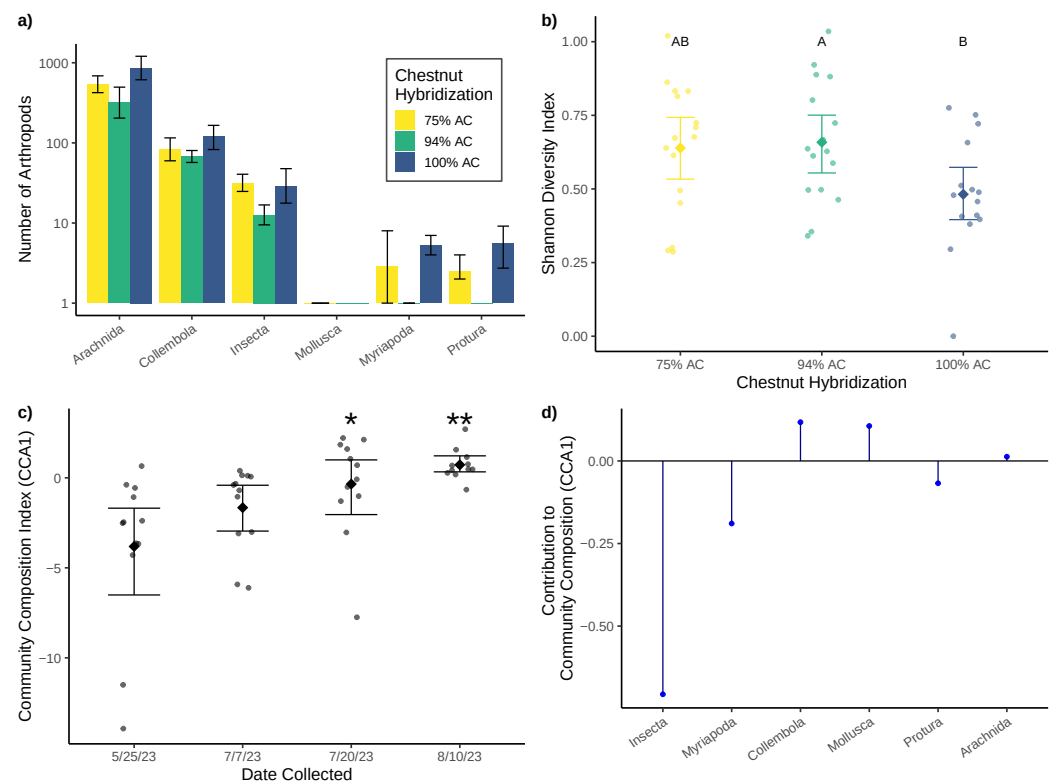
Chestnut type significantly explained ( $\chi^2 = 46.4$ ,  $df = 2$ ,  $p < 0.001$ ) the probability of infestation by the Asian Chestnut Gall Wasp. The 75% American chestnut hybrids are 3.7 times more likely ( $z = 3.3$ ,  $p = 0.002$ ) to have galls than 94% American chestnut hybrids (Figure 3a). Severity of infestation was also significantly different ( $p < 0.001$ ) across chestnut hybrid types (Figure 3b). The 75% American chestnut hybrids were the only hybrid type with severe levels of gall infestation on individual trees and had the highest proportion (29%) of infested trees. The 100% American chestnut hybrids did not have any galls.



**Figure 3.** Asian Chestnut Gall Wasp infestation. (a) Probability of infestation by Asian Chestnut Gall Wasp. Points and error bars denote mean probabilities and 95% confidence intervals, respectively. Letters denote significant differences at  $p < 0.05$ . (b) Percent infestation across stands by Asian Chestnut Gall Wasp on American Chestnut Hybrids. Severity of infestation was assessed on a per tree basis.

### 3.4. Soil Arthropods

Five arthropod classes were identified during our sampling, including Arachnida, Collembola, Insecta, Myriapoda, and Protura, along with three total molluscs (Figure 4a). There were minor significant differences ( $p < 0.05$ ) between chestnut hybrids for Arachnida and Protura, with 100% AC having higher numbers than 94% AC.



**Figure 4.** Soil arthropod diversity. (a) Total seasonal abundance of arthropods collected from leaf litter samples by taxonomic class. Bar height and error bars denote mean and 95% confidence intervals, respectively. (b) Shannon diversity indices by chestnut hybrid types. Points denote index values per

site. Diamond points and error bars denote mean and 95% confidence intervals, respectively. Letters denote significant differences between chestnut hybrid types at  $p < 0.05$ . (c) Community Composition Index (first axis from canonical correspondence analysis) explaining 63% of the constrained variance. Points denote site values. Diamond points and error bars denote mean and 95% confidence intervals, respectively. Single asterisk denotes significance at  $p < 0.05$  compared to first collection date. Double asterisk denotes significance at  $p < 0.01$  compared to first collection date. (d) Relative contribution to community composition index in (c). Absolute values of bar height denote weights assigned to order by the canonical correspondence analysis.

Chestnut hybrid type significantly ( $F = 3.4$ ,  $df = 2$ ,  $p = 0.04$ ) explained 14% of the observed variance in Shannon diversity index values. The 94% American chestnut hybrids had significantly more ( $0.18 \pm 0.07$ , Mean  $\pm$  SE;  $t = 2.4$ ,  $df = 45$ ,  $p = 0.0495$ ) diversity than 100% American chestnuts (Figure 4b). Analysis of diversity using Simpson indices produced similar results.

A canonical correspondence analysis model relating chestnut type and collection date to community composition significantly ( $F = 2.6$ ,  $df = 5$ ,  $p = 0.006$ ) explained 24% of observed variance in community composition. Collection date, but not chestnut hybrid type, was the significant factor ( $F = 3.6$ ,  $df = 3$ ,  $p = 0.001$ ) driving differences in community composition that were resolved significantly ( $F = 8.2$ ,  $df = 1$ ,  $p = 0.012$ ) by the first canonical axis explaining 63% of the constrained variation. Collection dates later in the season had significantly higher ( $p < 0.05$ ) community composition index scores compared with the first collection (Figure 4c). These differences in community composition were driven by the relative contributions of class abundance (Figure 4d).

### 3.5. Entomopathogenic Nematodes

Entomopathogenic nematodes were found across all chestnut hybrid types and no significant difference ( $p > 0.05$ ) was found between chestnut types within or across the season.

## 4. Discussion

American chestnut hybridization impacts invertebrate communities above- and belowground. Chestnut hybridization changed community composition of aboveground insects, as monitored through pyramid traps, insect herbivory, and galling. The 94% American chestnut hybrid insect communities were significantly different than other hybrid types, with Diptera and Coleoptera having the largest positive influence on these differences (Figure 1). In addition, 94% American chestnut hybrids had higher levels of insect herbivory as measured by leaf area eaten (Figure 2). Chinese chestnut hybrids also had much higher probabilities of infestation from the Asian Chestnut Gall Wasp and higher levels of infestation (Figure 3). The 100% American chestnuts had no incidence of galling, while both the 94% and 75% American chestnut hybrids had moderate levels of Asian Chestnut Gall Wasp infestation (Figure 3b). The 75% American chestnut hybrids had nearly 10% of Severe infestation (Figure 3b).

Similarly, Chestnut hybridization impacted invertebrate communities belowground. Diversity indices were significantly higher in 94% American chestnut hybrids compared with 100% American chestnuts (Figure 4b). Belowground differences in community composition were driven by changes in community over time, not by differences in chestnut hybridization (Figure 4c). Bio-indicator analysis of entomopathogenic nematodes did not show differences between hybrid types because entomopathogenic nematodes were present across all chestnut hybrid types. This is a good bioindicator of healthy soils across chestnut hybrid types.

The observed impacts of chestnut hybridization on invertebrate communities above- and belowground could be attributed to a wide range of factors. These differences could result from different locations and growth habits. While these trees were all relatively colocated, there were differences in site placement, planting date, and orientation that could not be overcome by experimental design. Tree age and growth pattern could also

play an outsize role, given that 100% American chestnuts were mostly sprouts from blight-affected trees and were markedly smaller than other hybrid types (Table 1).

The 94% American chestnut trees were larger, healthier, and made up a greater percentage of the vegetative cover than 100% American chestnuts, which were almost entirely sprouts experiencing continual growth suppression due to *C. parasitica* infection. Multiple studies have found that arthropod abundance and diversity were positively correlated with greater plant cover and diversity [45–47]. Plant community heterogeneity is also known to promote soil fertility and decomposition rates as well as create more complex habitats to support a greater number and diversity of belowground organisms [47].

It is also important to note the terrain differences between chestnut stands. Both the 75% American chestnut hybrids and 100% American chestnut trees were located in relatively flat areas surrounded on all sides by the Eastern hardwood forest present in DuPont Recreational State Forest. By contrast, the 94% American chestnut hybrids were planted on a south facing slope which had been clearcut prior to chestnut planting, unlike the other two experimental plots. Temperatures were noticeably hotter when performing field collections on this plot in comparison to the other two, likely due to its orientation in combination with a lack of canopy cover. It has been shown that a greater number and diversity of shade-producing trees are associated with higher soil arthropod abundance and richness [46]. However, a study on the effect of tree thinning on soil organisms in coniferous stands found contrasting evidence where plots with intense thinning had the highest richness and abundance of arthropod communities and that biodiversity was not affected across treatment groups [48]. High soil surface temperatures and exposure to UV radiation from sunlight are also connected with decreased biodiversity of subterranean arthropods [49,50].

The 100% American chestnut plot was also characterized by the presence of several large ant colonies in the area, which were not observed in the other two plots studied. Previous studies have shown that ant activity alters the surrounding soil by creating pore space and increasing the amount of several key nutrients, including sodium [51,52]. An increase in plant and microbe biomass and richness has also been noted surrounding ant colonies, which in turn likely alters the community composition of larger belowground organisms [51,52]. The greater number of ants within the 100% American chestnut plot contributed to the larger abundance of insects sampled, and it is also possible that ant activity altered the community dynamics of other arthropods in the area.

The role of genetics in these interactions is beginning to be established in clear cases, like chestnut resistance to galling wasps [53], where clear genetic signals can be mapped to environmental and community outcomes.

While these characteristics could drive some of the observed differences, if these characteristics were solely responsible, we would have expected to see larger impacts across chestnut types akin to the stark differences we observed in galling. Infestation levels by the Asian Chestnut Gall Wasp were markedly different between chestnut hybrid types, with no observed infestation in 100% American chestnuts. This lack of infestation could result from the 100% American chestnuts being entirely sprouts. The 94% and 75% American chestnut hybrid trees were more similar in size; differences in probability of infestation and infestation levels could point to a genetic component where higher levels of Chinese chestnut genetic makeup contribute to higher levels of galling infestation.

In comparison with the clear differences in gall infestation, the other observed differences in community composition were much more subtle; there were not large taxonomic shifts in communities. Given the close proximity of these locations, this suggests that enough mixing could have occurred to obviate differences in site placement. If this were the case, the observed differences in community composition could suggest that the 94% American chestnut hybrids could be different from the other hybrid types. Community composition aboveground, insect herbivory, and diversity indices belowground were all significantly different for 94% American chestnut hybrids.

## 5. Conclusions

A comparison of invertebrate communities above- and belowground across chestnut hybrid types shows that there are differences in aboveground community composition, herbivory, galling, and belowground diversity. While some of these differences may be attributed to growth characteristics of the different chestnut hybrid types or the nature of their planting, a genetic component may be at play, particularly for infestation with Asian Chestnut Gall Wasp. While the long-term impacts of these differences in invertebrate communities remain to be seen, the continued efforts at expanding the reintroduction of American Chestnut hybrids across the eastern seaboard of the United States suggest that these impacts will become more widespread. A judicious management approach that considers these impacts, particularly in terms of the Asian Chestnut Gall Wasp and other potential pests, could be beneficial in maintaining a successful reintroduction strategy. Reintroducing chestnut hybrids as keystone species in forest ecosystems will engender a host of abiotic and biotic impacts. Considering these impacts above- and belowground will assist in orienting reintroduction priorities and strategies.

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## Abbreviations

The following abbreviations are used in this manuscript:

EPN    Entomopathogenic nematode  
AC    American chestnut

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