



Invasive earthworms reduce chemical defense and increase herbivory and pathogen infection in native trees

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Abstract

1. Recent research shows that earthworms can alter defense traits of plants against herbivores and pathogens by affecting soil biochemistry. Yet, the effects of invasive earthworms on defense traits of native plants from previously earthworm-free ecosystems as well as the consequences for multitrophic interactions are virtually unknown.
2. Here we use a combination of an observational study and a complementary experimental study to investigate the effects of invasive earthworms on leaf defense traits, herbivore damage and pathogen infection in two poplar tree species (*Populus balsamifera* and *Populus tremuloides*) native to North American boreal forests.
3. Our observational study showed that earthworm invasion was associated with enhanced leaf herbivory (by leaf-chewing insects) in saplings of both tree species. However, we only detected significant shifts in the concentration of chemical defense compounds in response to earthworm invasion for *P. balsamifera*. Specifically, leaf phenolic concentrations, including salicinoids and catechin, were lower in *P. balsamifera* from earthworm-invaded sites.
4. Our experimental study confirmed an earthworm-induced reduction in leaf defense levels in *P. balsamifera* for one of the defense compounds, tremulacin. The experimental study additionally showed that invasive earthworms reduced leaf dry matter content, potentially increasing leaf palatability, and enhanced susceptibility of trees to infection by a fungal pathogen, but not to aphid infestation, in the same tree species.
5. *Synthesis.* Our results show that invasive earthworms can decrease the concentrations of some chemical defense compounds in *P. balsamifera*, which could make them susceptible to leaf-chewing insects. Such potential impacts of invasive earthworms are likely to have implications for tree survival and competition, native tree biodiversity and ecosystem functioning.

KEYWORDS

belowground invasion, boreal forests, invasion ecology, multi-trophic interactions, physical defense, plant-herbivore interactions, secondary metabolites

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1 | INTRODUCTION

Exotic ecosystem engineers can dramatically alter ecological features of recipient ecosystems if they become invasive (Cameron et al., 2016; Cuddington & Hastings, 2004). These ecological features may range from changes in the community structure of native species to changes in ecosystem productivity and the carbon balance within ecosystems. Invasive earthworms represent some of the most frequently investigated invasive ecosystem engineers, particularly in North American forest habitats, where European earthworms were introduced during the European settlement (Bohlen et al., 2004; Hendrix et al., 2008). Invasive earthworms alter the physico-chemical properties of soils mainly by reducing the soil organic layer in forests that usually degrades the habitat quality for native microorganisms (Ferlian et al., 2018), animals (Ferlian et al., 2018; Frelich et al., 2019) and plants (Craven et al., 2017). Despite the known negative effects of invasive earthworms on native biodiversity in forest ecosystems, we still know little about how traits of native species shift owing to earthworm invasion (Eisenhauer et al., 2019).

The spatial distribution and cycling of nutrients in soils are often altered by invasive earthworms (Ferlian et al., 2020), which can impact the growth of plants including trees (Donaldson et al., 2006; Jiang et al., 2018). For instance, soil nutrient availability can relax the trade-off between investments in growth and defense in tree species, which mainly relates to fitness costs for trees associated with defense against herbivores at the cost of their growth (Donaldson et al., 2006; Hahn & Maron, 2016). In general, N-availability in the mineral soil layer increases in earthworm-invaded sites (Ferlian et al., 2020). This mineral layer may represent the main layer of soil, where young trees extract nutrients from. As more nutrients become available, trees might invest more in growth and less in defense as proposed by the carbon-nutrient balance hypothesis (Bryant et al., 1983; Hamilton et al., 2001). While this hypothesis has been refuted as a general hypothesis for predicting defense investment in response to variation in environmental stoichiometry (Hamilton et al., 2001; Koricheva, 2002), it has been successful in cases predicting a reduction in carbon-based secondary metabolites, such as phenolic compounds in woody plants, as soil nutrients increase or become more accessible (Koricheva et al., 1998). Currently, it is unknown whether invasive earthworms can also alter carbon-based secondary metabolite production in trees, as reported for native earthworms (Xiao et al., 2018).

Here, we investigated the effects of invasive earthworms on a variety of defense traits against above-ground herbivores, mainly the (carbon-based) secondary metabolites of two common native tree species of North American boreal forests: *Populus balsamifera* L. (Balsam poplar) and *Populus tremuloides* Michx. (Trembling aspen). Both poplar species suffer from several kinds of insect and mammalian herbivores (Clausen et al., 1992; Lindroth & St. Clair, 2013; Roininen et al., 1997; Stevens & Lindroth, 2005). *Populus tremuloides* is widely distributed across Northern America, ranging from Canada to higher altitudes in Central Mexico. Its chemical defenses have been well studied and the species is known to produce a wide range of chemical defenses against a variety of herbivores (Lindroth & St. Clair, 2013; Philippe &

Bohlmann, 2007). *Populus balsamifera* has a more restricted northern distribution and is considered as the northernmost North American hardwood tree species (Peterson & Peterson, 1992). The survival of both tree species in their early development is often determined by the degree of damage caused by herbivores, as well as by fungal pathogens (Philippe & Bohlmann, 2007).

Poplars exert both chemical defenses, such as phenolic compounds and defense proteins, and physical defenses in the form of protective anatomical structures (Constabel & Major, 2005; Philippe & Bohlmann, 2007). We hypothesize that the presence of earthworms will reduce plant defenses in both tree species (i.e. prioritizing growth over defense) owing to commonly observed greater soil nutrient availability in earthworm-invaded soils. We studied salicinoids and flavonoids as chemical defense, two major groups of phenolics in poplar trees with anti-herbivore and anti-pathogen properties (Boeckler et al., 2011; Philippe & Bohlmann, 2007; Ullah et al., 2017). We also investigated physical defense in poplar trees by measuring leaf dry matter content, which is often related to leaf palatability to insects. Higher leaf dry matter content lowers leaf palatability and a decrease in leaf palatability reduces tree's susceptibility to leaf damage by leaf-chewing insects (Descobes et al., 2017; Elger & Willby, 2003; Schädler et al., 2003). Our research first involved an observational study in a boreal forest, part of which is invaded by earthworms, where we tested how leaf damage and defense in the saplings of two poplar species relate to earthworm invasion. Second, we performed an Ecotron experiment to examine how earthworm activities may alter the defense in the seedlings of the same two poplar species.

2 | MATERIALS AND METHODS

2.1 | Observational study

In July 2017, we conducted an observational study in a North American boreal forest located in the Kananaskis Valley in the front range of the Canadian Rocky Mountains on a south-facing slope at about 1,410 m above sea level in southwest Alberta (51°02'09"N, 115°03'41"W). In the study forest, *Populus balsamifera* and *Populus tremuloides* are the two main co-occurring deciduous tree species (Eisenhauer et al., 2007; Straube et al., 2009). The study forest also contains a dense understorey consisting of herbs (e.g. *Aster conspicuus* Lindl., *Aster laevis* L., *Viola canadensis* L., *Epilobium angustifolium* L., *Delphinium glaucum* S. Wats.), roses (*Rosa acicularis* Lindl) and grasses (e.g. *Bromus inermis*, *Calamagrostis rubescens*, *Leymus innovates*; O. Ferlian et al., unpubl. data). The soil in this forest is orthic grey luvisol and usually remains frozen from November until March. The climate is characterized by long and cold winters with intermittent warm Chinook winds and short dry summers. The mean annual precipitation at the study site is 625 mm and the mean annual temperature in the organic layer is -4°C (Eisenhauer et al., 2007; Mitchell, 1974).

Previous studies in this boreal forest have shown areas heavily invaded by European earthworms (Eisenhauer et al., 2007; Straube et al., 2009) as evidenced by high densities of endogeic and anecic

earthworms. Endogeic earthworms live in non-permanent horizontal burrows in the upper organic and mineral soil layers, anecic earthworms live in vertical burrows in soils and feed on surface leaf litter. A third group, epigeic earthworms live in and feed on the litter layer (Bouche, 1977). Previous research has shown that endogeic and anecic earthworms often have the strongest ecosystem effects (Craven et al., 2017; Eisenhauer et al., 2007; Frelich et al., 2006).

We identified the current earthworm invasion fronts during our field investigation in the year 2017 following a transect walk guided by the previously known earthworm invasion fronts in this forest (Eisenhauer et al., 2007; Straube et al., 2009; Figures S1 and S2). A transect walk was undertaken in north-western direction from previously known heavily invaded earthworm sites in the study forest to locate the invasion front (Figure S1). The north-west direction was chosen as deciduous forests change to coniferous forests in this direction where earthworms cannot invade due to acidic soils, as they are susceptible to acidic soils (e.g. acidic soils can damage their cuticle). When signs of earthworm activities (e.g. middens, casts and reduced litter cover) were identified, earthworm extractions were performed using mustard solution. Mustard solution consisted of 100 g of dry mustard powder and 20 ml of vinegar, mixed thoroughly in 10 L of water in a canister. The solution was incubated overnight. After thorough mixing, half of the mustard solution (5 L) was added to peat (~20 cm deep) of a 50 cm × 50 cm plot, which was carefully checked for 15 min for earthworm emergence. We repeated this procedure with the rest of the mustard solution (5 L) for another 15 min. The dug-up soils from the peat were further hand-sorted to obtain earthworm individuals, which were both counted and identified to species level.

Previously known sites of high earthworm density still showed higher signs of earthworm activity, which was confirmed by earthworm density data (Figure S2). As we moved north-west from the earthworm-invaded sites, we observed that earthworm densities were getting lower (Figure S1). When mustard extraction and hand sorting did not result in any endogeic or anecic earthworm collection, we identified this forest patch as a site with negligible earthworm activity. From further north of the invasion fronts (where endogeic and anecic earthworms were absent), we selected the plots for (insect) herbivory assessment of tree saplings (Figure S1). High and low earthworm activity sites were part of the same deciduous boreal forest where both study trees were present.

We randomly selected 10 plots (1 m × 1 m) each, for high and low earthworm activity sites. The maximum and minimum distances

between plots in two sites were comparable. In the high activity earthworm site, the maximum distance between plots was 106.0 m, whereas in the low density earthworm site, the maximum distance was 111.9 m. The minimum distances between plots were 5.0 m and 4.5 m in high and low earthworm activity sites respectively. We used the mustard extraction method and hand sorting as explained above to estimate earthworm densities from 50 cm × 50 cm subplots from each main plot (Figure S2). Since we occasionally found epigeic earthworms in low earthworm activity sites, we refer to them as 'low density earthworm sites', whereas the high earthworm activity sites are referred to as 'high density earthworm sites'. The average density and biomass of earthworm species found at the two sites are provided in Table S1.

From half of the plots, two saplings from *P. balsamifera*, and from the other half of the plots, two saplings from *P. tremuloides* were selected for leaf damage and leaf defense chemistry assessment. We accordingly assessed 40 saplings in total in our study (5 plots × 2 saplings × 2 sites × 2 tree species; Table S2). Tree saplings were on average 2.57 (±0.11) m high (e.g. Figure 1a) and were selected within a 5 m radius of our core plot of 1 m × 1 m (Figure S3). The height of each tree sapling is provided in Table S2. We further took three samples using soil cores of 5 cm diameter and pooled them for the assessment of the total soil N content (Figure S4). Soil samples were taken from a depth of 10 cm depth from another 50 cm × 50 cm subplot of the main plots and were divided into the organic layer (5 cm upper soil layer) and mineral layer (5 cm lower soil layer). From both layers, about 3 mg of dry soil (dried at 40°C for several days) was analysed using the dry combustion method in an elemental analyzer (Vario EL cube; Elementar Analysensysteme GmbH).

Leaf damage by leaf-chewing insects was assessed for each tree sapling in July from the entire foliage of the trees, resulting in a total number of 26,793 evaluated leaves (an average of ~670 leaves per sapling). Each leaf was visually categorized into one of three leaf damage classes (Figure S5): (A) no damage (intact leaf, no tissue damage), (B) moderate damage (<50% leaf damage) and (C) severe damage (≥50% leaf damage). Such categorical assessment of leaf damage is common in leaf damage studies and provides an overview of the severity of insect herbivory on plants (e.g. Schuldt et al., 2010; Zverev et al., 2017). These three classes of leaves were then converted into three response variables for the leaf damage assessment per sapling: (a) Overall leaf damage % ($100 \times ((B + C)/(A + B + C))$), (b) Moderate leaf damage % ($100 \times (B/(A + B + C))$) and (c) Severe leaf damage % ($100 \times (C/(A + B + C))$).

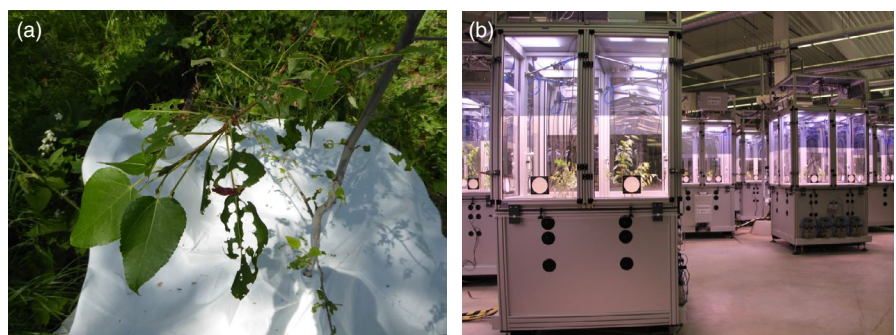


FIGURE 1 (a) A *Populus balsamifera* tree sapling in our observational study for the assessment of leaf damage in Alberta, Canada. (b) EcoUnits of the iDiv Ecotron indoor research facility, where we conducted our experiment (Bad Lauchstädt, Germany)

After the damage assessment, we pooled a fraction of the leaves (5 g) from each damage class (both young and old leaves were randomly mixed) and dried them in drying ovens for 72 hr at 28°C. The dried leaves were ground to fine powder with a ball mill at a vibration frequency of 25/s and stored at -20°C until chemical analyses.

2.2 | Experimental study

In the same year, we carried out an indoor experiment with the same two tree species to investigate how earthworms could affect leaf defenses. This experimental study was carried out at the iDiv Ecotron indoor research facility in Germany (Eisenhauer & Türke, 2018; Figure 1b). We used 12 EcoUnits (Figure 1b, EcoUnit inner dimensions (length × width × height): 1.24 m × 1.24 m × 0.8 m) for this experiment, each of them filled with sterilized topsoil provided by Bauzentrum Farys GmbH, Laucha. For sterilization, the soil was perfused with approximately 100°C hot water steam for one hour. Such sterilization results in an excessive release of nutrients due to the death of soil organisms (Alphei & Scheu, 1993; Trevors, 1996), and was therefore thoroughly washed by tap water afterwards to reduce nutrient pulses (Jager et al., 1969). Each EcoUnit was filled with 1.23 m³ of this sterilized soil (soil pH = 7.05; soil C:N ratio = 9.30). To allow for interactions with a soil microbial community that mimics the ones encountered in native North American forests, the steam-sterilized soil was inoculated with soils (380 g per EcoUnit) from the earthworm-free parts of the Canadian field site. These soils were shipped to Germany in a cooling box and stored at 4°C until added to experimental units. The Canadian soil was first sieved (4 mm mesh size) and then mixed with 300 ml of tap water, homogenized and further sieved through 125 µm mesh size. We then added 100 ml of tap water to this 300 ml solution and distributed it homogeneously on the topsoil of the experimental units.

Poplar seedlings for the experiment were obtained from the tree nursery Pflanzenhandel Winkler Gbr (www.pflanzen-winkler.de) in Priestewitz, Germany. The nursery company had obtained these seedlings from their North American sources. Just before planting these tree seedlings into the EcoUnits, we thoroughly washed their roots with tap water to minimize any contamination that could be associated with potential earthworm presence in the sites where the saplings had been cultivated. All tree seedlings were planted into the EcoUnits in August 2017, with the height of *P. balsamifera* ranging from 34 to 56 cm and that of *P. tremuloides* ranging from 35 to 105 cm. Each EcoUnit received two seedlings per tree species planted diagonally.

At the beginning of the experiment, whenever a tree seedling died, we replaced it with a new one (within the same height range as the originally planted individuals), but we stopped replacing dead trees after the fifth week from the start of the experiment. To mimic natural conditions, we also planted EcoUnits with some common understorey vegetation (germinated in soils separately from poplar seedlings) resembling the Canadian field site after the fifth week of tree seedling growth. These understorey plants occur in both high

and low earthworm density sites of our study forest (Eisenhauer et al., 2007). The understorey vegetation community was composed of the two herb species *Aster laevis* [L.] (Common name: Smooth Aster) and *Achillea millefolium* [L.] (Common name: Common Yarrow) and the two grasses *Calamagrostis canadensis* [Michx.] (Common name: Bluejoint) and *Bromus ciliatus* [L.] (Common name: Fringed Brome). Both herbs and grasses were pre-grown in a greenhouse for about 4 weeks, before they were transplanted (ranging from ~2 cm to ~15 cm height depending on plant species) into the EcoUnits. Seeds of the grasses were obtained from Prairie Moon Nursery (MN, USA), whereas herb seeds were obtained from Wild About Flowers (Alberta, Canada). The herbaceous plants were planted in equal proportions at a distance of 10 cm but are not the focus of this study.

The earthworm invasion in our experiment was simulated by adding two species of earthworms: the anecic, large-bodied *Lumbricus terrestris* [L.] (commonly known as nightcrawler) and the endogeic *Aporrectodea rosea* [Savigny] (rosy-tipped worm). Both species of earthworms are invasive in northern North America (Addison, 2009), but only *L. terrestris* was present in our observational study site (Table S1), while another endogeic species was found in our study site (*Octolasion tyrtaeum*; Table S1). We added 50 individuals of *L. terrestris* and 15 individuals of *A. rosea* to half of the EcoUnits. These densities were in the range of earthworm densities found under field conditions in the study forest (Eisenhauer et al., 2007; Straube et al., 2009; Table S1) and maintained sufficient earthworm activities in our experimental soils (e.g. visually inspected through litter consumption and midden formation). We added anecic (*L. terrestris*) and endogeic (*A. rosea*) earthworms in our experiment to represent the two ecological groups of invasive earthworms that exert the strongest impact on ecosystems (Craven et al., 2017; Frelich et al., 2006). The earthworms were added thirteen days after planting the seedlings with a comparable total earthworm biomass across all EcoUnits. Furthermore, to account for the potential soil microbial contamination by earthworm excreta during their rearing, we added 4 g of the rearing soil to each of the earthworm-free EcoUnits. All individuals of *L. terrestris* were purchased from TZ-Terraristik Zentrum, Neunkirchen (www.terraristik-zentrum.de), Germany. We collected *A. rosea* individuals directly from the field, in the vicinity of Leipzig, Germany.

As the main food source for earthworms in the experiment, we added 50 g of fresh locally collected *Populus nigra* var. *italica* litter at three different timepoints (on the day of earthworm addition, and on the 23rd and 65th day after the earthworm addition, respectively) to all EcoUnits. All leaf material was cut into squares of 2 cm². As the experiment progressed, there were some unintended aphid infestations in some of the EcoUnits and also fungal infections on tree leaves (Figure S6). Both were visually assessed at the end of the experiment. Aphid infestation was expressed as percentage of leaf area covered by aphid-related materials including honeydew. Fungal infection was assessed as percentage of leaf area infected by the fungus. The aphids were identified as a species from the genus *Chaitophorus*, whereas the fungal species belonged to the genus *Melampsora*. Given the random distribution of EcoUnits with respect to the two earthworm treatments in the iDiv Ecotron hall, these

assessments provided the opportunity to study earthworm-induced effects on multitrophic interactions of seedlings with herbivores and with pathogens.

Air temperatures in EcoUnits were set to 20°C during the day and 15°C at night. The day-night cycle was set to 16 hr/8 hr with night lasting from 9 p.m. to 5 a.m. Light conditions were set to 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation (PAR) using light of a wavelength between 400 and 700 nm. At the bottom of the EcoUnits, soil temperature was set to 18°C in order to mimic a near-natural soil temperature gradient. This feature was accomplished by a capillary system at the base of the experimental units which contain a circulating cooling liquid (ethylene-glycol-water mixture). Irrigation to EcoUnit soils was initially set high at 2.4 L per EcoUnit every six hours to facilitate the initial growth of the tree species. This corresponds to 1.8 L on the soil per EcoUnit as there were some water losses during irrigation which equals 1.17 L per square metre as each EcoUnit has a surface of approximately 1.54 m. This amount (1.8 L/EcoUnit) is twice as much as the average precipitation in June at the Canadian field site (<http://climate.weather.gc.ca>). In the following two weeks, the water additions were halved to 1.2 L per EcoUnit every 6 hr which corresponded to 0.8 L on the soil and 0.52 L per square metre which enabled us to maintain a soil moisture of $\leq 40\%$.

The experiment ran for 16 weeks (113 days) from the day of tree seedling addition. All leaves (21,099 in total) were collected from all the seedlings and assessed for aphid infestations and fungal infections (only in infected and non-infected categories). After these assessments, a random fraction of total leaves was pooled (5 g) per tree seedling and subsequently dried at 28°C for 72 hr for the analysis of chemical defense compounds and for leaf dry matter content.

2.3 | Measurement of tree defense

The dried leaves from the experimental study (both infected and non-infected leaves were mixed) were also finely ground with a 5 mm diameter metal ball at a vibration frequency of 25/s for two min in a ball mill (Retsch MM400). Ground leaf material was then stored at -20°C until further processing.

Five salicinoids (salicin, salicortin, salicortin-6-benzoate, tremulacin and homalosid D), one flavonol glycoside (rutin) and one flavan-3-ol (catechin) were determined using high performance liquid chromatography (HPLC, Agilent 1100 Series) after their extraction from the dried and ground leaf samples (Boeckler et al., 2013). For their extraction, a 10-mg portion of each sample was placed into the wells of a 96 well plate (Micronic) and 1 ml MeOH containing 0.8 mg/ml phenyl- β -glucopyranoside (Sigma Aldrich) was added. The mixtures were extracted by agitation for 30 s on a paint shaker (Scandex) and shaken for another 30 min at 200 rpm on a second shaker (IKA Labortechnik). After centrifuging at 3,200 rpm for 5 min, 200 μl of the supernatants were transferred into a new 96 well plate (Nunc). Extracts were diluted 1:1 with milli-Q water and 10 μl of each extract was injected into an HPLC (Agilent 1100 series)

accommodated with a chromatographic column (EC 250 \times 4.6 mm NUCLEODUR Sphinx RP, 5 μm , Macherey Nagel) connected to a precolumn (C18, 5 μm , 4 mm \times 3 mm, Phenomenex). The temperature of the column oven was set to 25°C. The mobile phase consisted of two solvents, solvent A (Milli-Q water) and solvent B (acetonitrile), from which solvent B was used in a gradient mode with time/concentration (min/%) of: 0:00/0; 19:00/52; 10:10/100; 21:00/100; 21:10/14; 26:00/14). The flow rate was set to 1ml/min. The signal was detected using a photodiode array detector. The compounds' concentrations (mg/g based on leaf dry weight) were then calculated on the basis of the peak areas at 200 nm (salicin, salicortin, salicortin-6-benzoate, homaloside D, tremulacin and catechin), and 330 nm (rutin).

We determined the physical defense of trees by estimating leaf dry matter content (LDMC). This was only done in the experimental study. We randomly sampled 20 leaves per seedling (including infected and non-infected leaves) per EcoUnit for the LDMC estimation. LDMC was calculated by dividing the oven-dried mass of a leaf in milligrams by its water-saturated fresh mass in grams (Wilson et al., 1999). Relative growth rates of tree seedlings were also estimated by periodically measuring their height during the experiment (Figure S7).

2.4 | Statistical analyses

For the observational study, we used linear mixed-effects models to analyse how leaf damage in the two tree species was affected by earthworm invasion (earthworms high or low density sites) as the fixed factor and plots as the random intercept, as we assessed two individual tree saplings per plot. Thus, using plots as random intercept accounts for the pseudo-replication at the plot level. We used Gaussian error terms for analysing the effect of earthworm invasion on the three leaf damage (%) classes in tree saplings. The models were run separately for the two poplar species.

We used non-metric multidimensional scaling (NMDS), based on Bray-Curtis dissimilarities, to visualize differences in seven chemical defense compounds in earthworm-invaded and non-invaded sites. We ran permutational multivariate analysis of variance (PERMANOVA) to test whether earthworms affected the composition of chemical defense concentrations in the two saplings. We performed the same analysis for data from the experimental study.

The experimental study had a nested design with two tree individuals per species in each EcoUnit. We accordingly used linear mixed-effects models with the EcoUnit as the random intercept to test differences between the two earthworm treatments for each measured response variable separately for both poplar species. For all response variables (i.e. chemical compounds, leaf dry matter content, fungal infection, and aphid infestation), we used the same model structure with Gaussian error terms. For the experimental data, we were only able to run models with *P. balsamifera*, because most of the *P. tremuloides* seedlings died during the experiment. For

instance, out of 21,099 total leaves in the experimental seedlings, 20,057 were the leaves of *P. balsamifera*. The proportion of leaf area covered by unintended fungal pathogen infection and aphid infestation was also analysed using mixed-effects models. For the analysis of the leaf infestation by aphids, we used generalized mixed-effects models with negative binomial error terms to account for overdispersion and zero-inflation in the data. Finally, we explored the association between chemical defense compounds and leaf dry matter content (as a measurement of a physical defense trait) to test how tree seedlings change their levels of chemical and physical defenses in the presence of earthworms using an analysis of covariance (ANCOVA) test.

All statistical analyses and figures were made using the R statistical software (R Core Team, 2018). Mixed-effects models were run with the LME4 package (Bates et al., 2015). Type II Wald Chi-square tests for these models were obtained using the CAR package (Fox & Weisberg, 2011). We made PERMANOVA tests with the VEGAN package (Oksanen et al., 2019). The conditional R^2 (combination of fixed and random effects) was obtained from the MuMIn package (Barton, 2018). Model diagnostics for linearity model assumptions were done in the DHARMA package (Hartig, 2017). The zero-inflation test for leaf infestation caused by aphids was also done using the DHARMA package (Hartig, 2017). Figures were made in the GGPLOT2 package (Wickham, 2016).

3 | RESULTS

3.1 | Observational study

Saplings from the high invasive earthworm density site had significantly higher leaf damage than trees from the low invasive earthworm density site. The proportion of overall leaf damage was 28% and 46% higher in the high density earthworm site in *P. balsamifera* (χ^2 -value = 7.94, p -value = 0.02, Figure 2a) and *P. tremuloides* (χ^2 -value = 4.40, p -value = 0.03, Figure 2a) respectively. In *P. balsamifera*, the proportion of leaves in the moderate leaf damage class was 31% higher in the high density earthworm site than in the low density site (χ^2 -value = 8.17, p -value < 0.01, Figure 2b), whereas the proportion of leaves in the severe leaf damage class did not differ between the high and low earthworm density sites (χ^2 -value = 0.10, p -value = 0.75, Figure 2c). In *P. tremuloides*, neither moderate (χ^2 -value = 2.43, p -value = 0.11) nor severe leaf damage (χ^2 -value = 3.35, p -value = 0.06) differed between the high and low earthworm density sites, but were marginally higher in the high earthworm density site (Figure 2b,c).

We found that the total concentration of defense compounds (sum of seven compounds) was lower in the high earthworm density site in *P. balsamifera* saplings and not in *P. tremuloides* (Table 1). In *P. balsamifera*, we found a significant decrease in

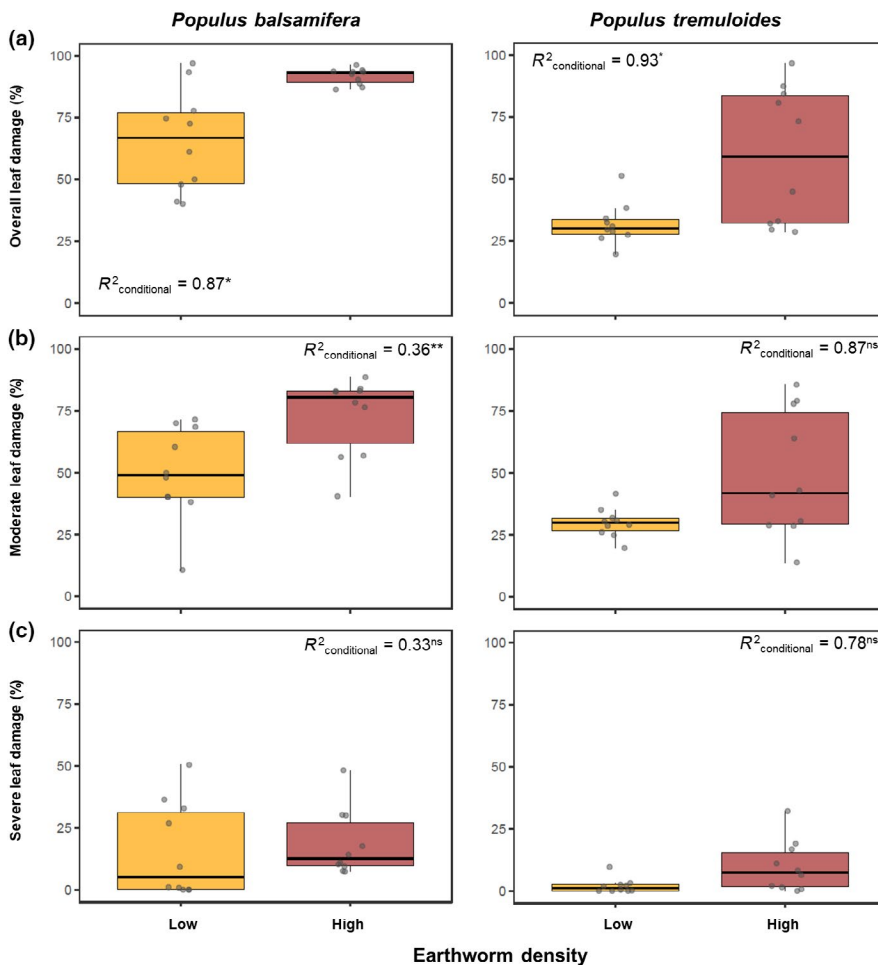


FIGURE 2 Leaf damage by insect herbivores on two Poplar tree species in the low and high earthworm density sites from the observational study. (a) Overall leaf damage in saplings of two tree species; (b) moderate leaf damage (<50% damage) and (c) severe (more than 50% damage) leaf damage. Conditional R^2 are the sum of R^2 from fixed effects (earthworms) and random effects (plots). ns: p -value > 0.05, * p -value < 0.05, ** p -value < 0.01

TABLE 1 Response of secondary metabolites (concentration in mg/g) in our observational and experimental study to earthworms and their statistical significance. The bold numbers indicate statistical significance (p -value < 0.05). Conditional R^2 are the sum of R^2 from fixed effects (earthworms) and random effects (plots in observational study, EcoUnits in experimental study). n.d.: not detected. The grey area in the table indicates that *P. tremulooides* were excluded from the experiment given that only few individuals survived

| Compound family | Defense compounds | <i>Populus balsamifera</i> | | | | <i>Populus tremulooides</i> | | | | | | |
|--------------------|-----------------------|---|--|-----------------|---------------------|---|--|-----------------|---------------------|-------|------|-------|
| | | Low density earthworm site (M \pm SE) | High density earthworm site (M \pm SE) | χ^2 -value | R^2 (conditional) | Low density earthworm site (M \pm SE) | High density earthworm site (M \pm SE) | χ^2 -value | R^2 (conditional) | | | |
| | | | | p -value | | | | p -value | | | | |
| Total | | 94.10 \pm 8.76 | 21.80 \pm 7.31 | 40.17 | <0.001 | 0.67 | 0.67 | 105 \pm 9.32 | 106 \pm 11.50 | <0.01 | 0.95 | <0.01 |
| Salicinoids | Salicin | 14.6 \pm 1.60 | 1.43 \pm 0.59 | 59.92 | <0.001 | 0.75 | 0.75 | 14.8 \pm 1.22 | 13.5 \pm 2.35 | 0.21 | 0.64 | 0.69 |
| Salicinoids | Salicortin | 66.5 \pm 5.83 | 15.8 \pm 6.29 | 29.86 | <0.001 | 0.69 | 0.69 | 53.3 \pm 6.16 | 56 \pm 6.82 | 0.01 | 0.89 | 0.37 |
| Salicinoids | Salicortin-6-Benzoate | 0.24 \pm 0.21 | <0.01 \pm <0.01 | 1.21 | 0.27 | 0.05 | 0.05 | 1.67 \pm 0.28 | 1.15 \pm 0.20 | 2.26 | 0.13 | 0.10 |
| Salicinoids | Tremulacin | n.d. | | | | | | 27 \pm 3.78 | 28.5 \pm 3.01 | 0.10 | 0.75 | <0.01 |
| Salicinoids | Homaloid D | 7.78 \pm 0.75 | 0.88 \pm 0.59 | 48.47 | <0.001 | 0.74 | 0.74 | 6.96 \pm 0.96 | 5.13 \pm 0.93 | 1.85 | 0.17 | 0.08 |
| Flavonol glycoside | Rutin | 0.26 \pm 0.10 | 0.29 \pm 0.11 | 0.03 | 0.84 | <0.01 | <0.01 | 0.25 \pm 0.09 | 0.35 \pm 0.11 | 0.26 | 0.60 | 0.32 |
| Tannin | Catechin | 0.89 \pm 0.17 | 3.37 \pm 0.26 | 48.84 | <0.001 | 0.82 | 0.82 | 0.69 \pm 0.11 | 0.86 \pm 0.11 | 0.75 | 0.38 | 0.18 |

| Experimental study | | | | | | | | | |
|--------------------|-------------------------|-------------------------------|--------------------------------|-----------------|------------|---------------------|-----------------|------------|---------------------|
| Compound family | Defense compounds | Earthworm absent (M \pm SE) | Earthworm present (M \pm SE) | χ^2 -value | p -value | R^2 (conditional) | χ^2 -value | p -value | R^2 (conditional) |
| Total | | 216 \pm 4.61 | 208 \pm 7.68 | 0.65 | 0.41 | 0.01 | 0.65 | 0.41 | 0.01 |
| Salicinoids | Salicin | 8.05 \pm 0.78 | 11.4 \pm 2.07 | 1.85 | 0.17 | 0.28 | 1.85 | 0.17 | 0.28 |
| Salicinoids | Salicortin | 117 \pm 3.33 | 110 \pm 6.58 | 0.61 | 0.43 | 0.35 | 0.61 | 0.43 | 0.35 |
| Salicinoids | Salicortin-6-Benzoate | 10.40 \pm 0.42 | 10.40 \pm 0.41 | 0.01 | 0.89 | 0.61 | 0.01 | 0.89 | 0.61 |
| Salicinoids | Tremulacin (log-scaled) | 59.70 \pm 1.72 | 53.8 \pm 2.49 | 4.27 | 0.03 | 0.16 | 4.27 | 0.03 | 0.16 |
| Salicinoids | Homaloid D | 19.70 \pm 1.85 | 21.70 \pm 0.69 | 0.94 | 0.32 | 0.04 | 0.94 | 0.32 | 0.04 |
| Flavonol glycoside | Rutin | 0.34 \pm 1.44 | 0.24 \pm 0.10 | 0.33 | 0.56 | 0.01 | 0.33 | 0.56 | 0.01 |
| Tannin | Catechin (log-scaled) | 0.56 \pm 0.09 | 0.31 \pm 0.05 | 3.32 | 0.06 | 0.61 | 3.32 | 0.06 | 0.61 |

Abbreviations: M, mean; SE, standard error.

salicin, salicortin and homalosid D concentrations, whereas the concentration of the flavanol catechin was significantly higher in the high density earthworm site (Table 1). Likewise, our multivariate analyses revealed that the composition of these defense compounds was significantly different between tree saplings from the high and low density earthworm sites in *P. balsamifera*, but not in *P. tremuloides* (Figure 3). We also found a significant interaction between the effects of earthworm density and soil layers in driving the total soil N variations in the field (χ^2 -value = 24.50, p -value < 0.001, Figure S3). The differences between organic and mineral soil layers disappeared at the high earthworm density site (Figure S4).

3.2 | Experimental study

In contrast to the field study, our multivariate analysis did not reveal any significant shift in chemical defense composition in *P. balsamifera* seedlings in response to earthworm presence (Figure 4a). When chemical compounds were individually analysed (Table 1), we only found a significant decrease in response to earthworms for the salicinoid tremulacin (χ^2 -value = 4.27, p -value = 0.03, Figure 4b) that had undetectable levels in the observational study (Table 1) but was the second dominant salicinoid in the experimental trees (Table 1). The total concentration of defense compounds in *P. balsamifera* did not vary significantly between earthworm treatments

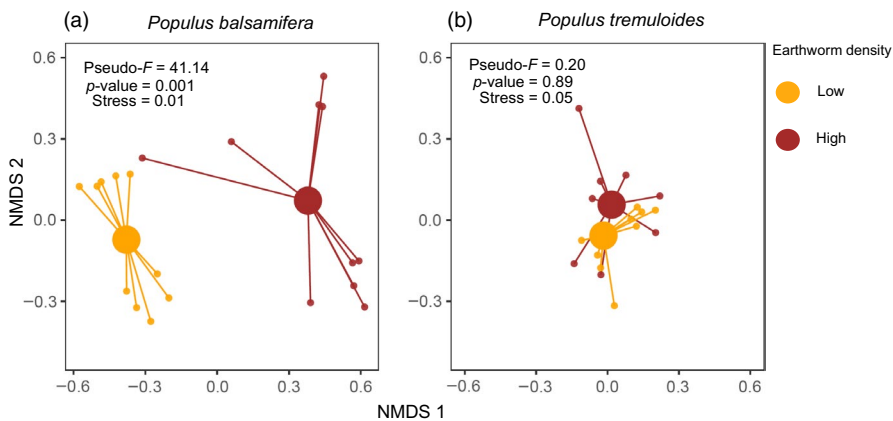


FIGURE 3 Non-metric dimensional scaling (NMDS) of defense compounds detected in the leaves of (a) *Populus balsamifera* and (b) *Populus tremuloides* in the observational study. The chemical-specific responses are provided in Table S2

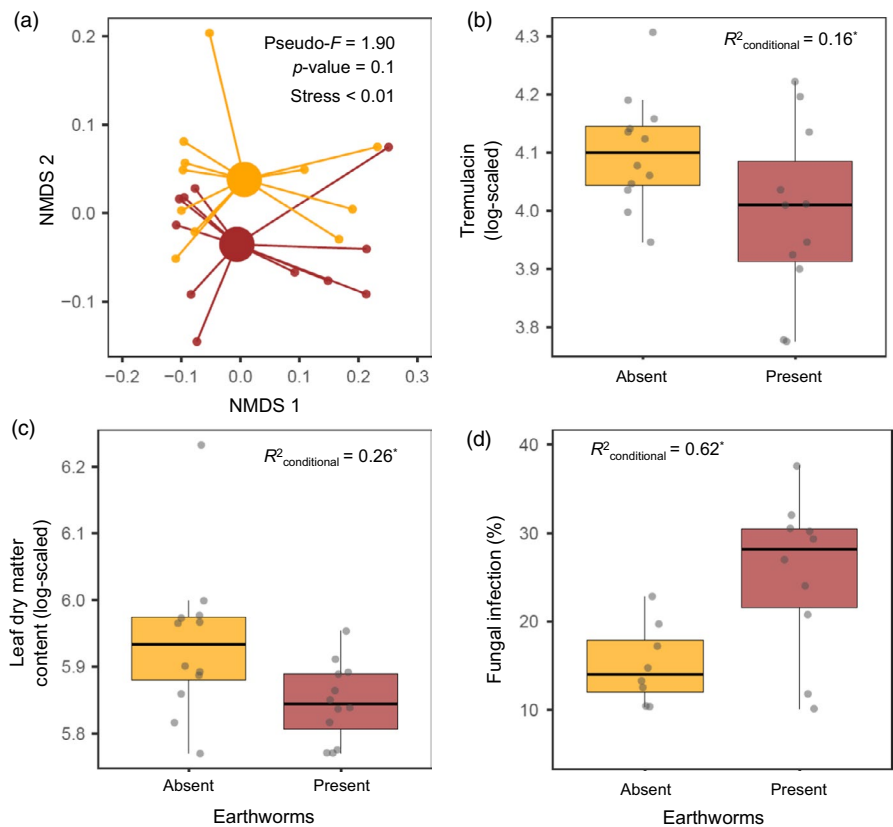


FIGURE 4 Response of *Populus balsamifera* to earthworm treatments in the experimental study. (a) Non-metric dimensional scaling (NMDS) of defense compounds detected in the leaves of *P. balsamifera*. (b) Tremulacin (mg/g) concentration in the leaves of *P. balsamifera* in earthworm treatments. (c) Leaf dry matter content (LDMC, mg/g) of *P. balsamifera* leaves in earthworm treatments. (d) Fungal infections of *P. balsamifera* leaves in earthworm treatments. Conditional R^2 are the sum of R^2 from fixed effects (earthworms) and random effects (experimental sub-units). * p -value < 0.05

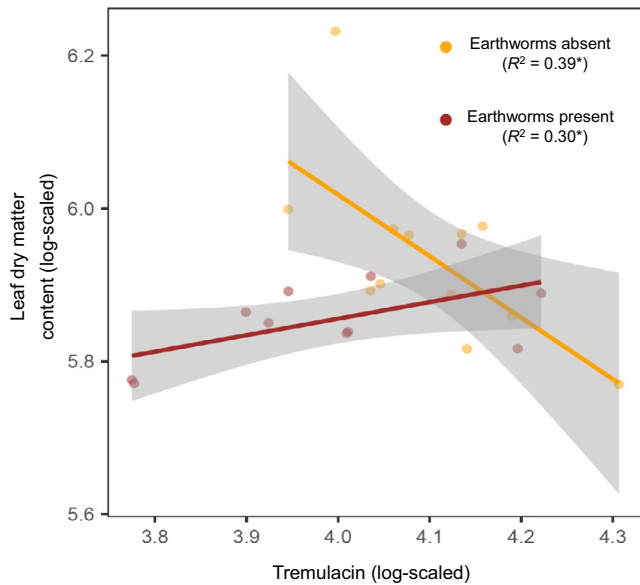


FIGURE 5 Relationship between the physical (defense) trait leaf dry matter content (as a measure of leaf palatability) and the chemical (defense) trait tremulacin in the presence and absence of earthworms. * p -value < 0.05

(Table 1). Notably, leaf dry matter content (a proxy of leaf palatability) also significantly decreased in the presence of earthworms (χ^2 -value = 5.11, p -value = 0.02, Figure 4c). Moreover, we found that leaf dry matter content negatively correlated with tremulacin in the absence of earthworms, but this relationship turned into a positive one in the presence of earthworms (Figure 5). Earthworm treatments significantly increased fungal infections on *P. balsamifera* leaves (χ^2 -value = 4.74, p -value = 0.02, Figure 4d), whereas they had no significant effect on leaf infestation by aphids (χ^2 -value = 0.01, p -value = 0.91, Figure S8).

4 | DISCUSSION

Several meta-analyses and synthesis work have pointed out that invasive earthworms are detrimental to native biodiversity and can alter the functions of an ecosystem with no recent history of earthworms (Craven et al., 2017; Ferlian et al., 2018, 2020; Frelich et al., 2019; Hendrix et al., 2008). Our results indicate that saplings of two common deciduous trees (*P. balsamifera* and *P. tremuloides*) in North American boreal forests may become susceptible to insect herbivory at sites where the density of invasive earthworms is higher (Figure 2). More specifically, our results demonstrate that earthworm densities are associated with differences in leaf secondary metabolites involved in herbivore defense. Contrary to our expectation, our results could only confirm chemical defense reduction in *P. balsamifera*, not in *P. tremuloides*. We are still far from understanding the underlying factors behind invasive earthworm-induced shifts in chemical defenses in native trees. However, our experimental study indicated that earthworms could potentially relax a trade-off in *P. balsamifera*'s resource allocation between a

physical (LDMC) and a chemical defense (tremulacin). Clearly, our observational study cannot unequivocally demonstrate a causal relationship between earthworm density and tree metabolic profile, i.e. despite the uniformity of the forest stand prior to earthworm invasion, unmeasured environmental factors may have affected both earthworm density and tree metabolic profile. Yet, our results suggest that invasive earthworms can potentially make *P. balsamifera* more susceptible to some natural enemies like leaf-chewing insects (observational study) and fungal pathogens (experimental study). We discuss the results of our observational and experimental study in light of how these results may impact the earthworm-invaded ecosystems.

Insect herbivory is widespread in both *P. balsamifera* and *P. tremuloides* despite differences in their growth and defense strategies (Peterson & Peterson, 1992; Philippe & Bohlmann, 2007). Invasive earthworms increase soil nutrient availability in mineral soil layers (Ferlian et al., 2020), at least in the initial years after invasion (Eisenhauer et al., 2019). In general, particular forms of soil N, such as soil nitrate, increase in earthworm-invaded mineral soil layers (Ferlian et al., 2020). Accordingly, we expected a decrease in secondary metabolites in native trees as predicted by the carbon-nutrient balance hypothesis (Bryant et al., 1983). Our results indeed show a strong reduction in the concentration of the phenylpropanoid-derived salicinoids in response to higher density of invasive earthworms, and a concomitant sharp increase in the levels of leaf herbivory in *P. balsamifera*. These results are in agreement with a previous study in which a higher nutrient availability indeed decreased the concentration of secondary metabolites, although in another poplar tree species (*Populus nigra*; Glynn et al., 2003). Given that we found no differences in total N in mineral soil layers between the high and low density earthworm sites (Figure S3), we can not confirm that differences in secondary metabolites were indeed due to soil N. Nevertheless, we also cannot rule out the contribution of other forms of N to increase in mineral soil layers with higher earthworm densities, as shown by a recent meta-analysis (Ferlian et al., 2020). Compared to *P. balsamifera*, *P. tremuloides* showed a similar increase in overall leaf herbivory at the high density earthworm site, but it did not show a similar decrease in leaf salicinoids, indicating that the increased herbivory was likely mediated by other traits.

Populus balsamifera is a relatively slow-growing tree species in comparison to *P. tremuloides*, and it is considered to have a higher nutrient demand and a longer life span (Peterson & Peterson, 1992). Earlier studies have shown that *P. tremuloides*, as a pioneer species, typically prospers during early forest succession, whereas *P. balsamifera* becomes dominant in the longer run in North American deciduous boreal forests (Peterson & Peterson, 1992). Given the differences in their growth rates, it is likely that their strategies to overcome herbivore pressure also vary. For instance, slow-growing trees like *P. balsamifera* may invest more into defense in their early growth stage relative to faster-growing *P. tremuloides* (Karban, 2011). Such differences between slow- and fast-growing trees often relate to differences in adaptive strategies to cope with limited resource availability in the soil (Endara & Coley, 2011; Stamp, 2003). Within the

framework of the carbon-nutrient balance hypothesis, it is therefore tempting to speculate that in the fast-growing *P. tremuloides*, investment in salicinoids was less dependent on soil nutrient availability than for the slow-growing *P. balsamifera*. Hence, *P. tremuloides* may have been less responsive to the presence of earthworms and potential shifts in soil nutrient availability in terms of carbon-based secondary metabolites than *P. balsamifera*. However, since we lack more detailed insight in the demand side of carbon and nutrients in the two species, this remains a speculation.

Our experimental manipulation of earthworms did not support the patterns observed in the observational study in terms of the field-observed decline in several secondary metabolites in *P. balsamifera* leaves in response to earthworm invasion. Indeed, the genetic makeup of field saplings and experimental seedlings was different, which makes their comparison difficult. Nonetheless, mismatches in secondary metabolite responses between the observational and the experimental study could relate to some other key differences between the two studies. For instance, despite unintended herbivory (by aphids) and infection by fungal pathogens in our experimental units, the herbivory damage on *P. balsamifera* saplings in the field was much higher. The variation in the degree of herbivory damage can strongly regulate the variability in defense concentrations of poplar leaves (Constabel & Lindroth, 2010). The difference in growth stage of field saplings and experimental seedlings could also have played an important role in detectability of secondary metabolites and their responsiveness to earthworms (Neilson et al., 2013). For instance, total concentration of secondary metabolites was higher in experimental seedlings than in observational saplings in our study (Table 1). Total concentration of secondary metabolites may also have been affected by our drying method (oven drying of leaves instead of freeze-drying). Another factor that could affect variable earthworm effects on secondary metabolites is the use of one litter type from a different poplar tree as a food source for earthworms in our experiment, in contrast to diverse litter types present in the field. This difference is likely to affect nutrient quality in the soil and in turn, nutrient turnover with subsequent effects for the tree's allocation to secondary metabolites.

Despite various differences between observational and experimental study conditions, we still found a decline in tremulacin in *P. balsamifera* in earthworm treatments. While tremulacin was not even detected in the field, its reduction in the presence of earthworms indicates the potential of *P. balsamifera* to shift its defense strategy in the presence of earthworms (Figure 4b). Indeed, reduction of tremulacin in poplar trees increases their susceptibility to leaf-chewing insects (Boeckler et al., 2011; Clausen et al., 1989). Susceptibility to leaf-chewing insects may further exacerbate when leaf palatability increases as also observed in our experiment through a decrease in leaf dry matter content in *P. balsamifera* leaves (Elger & Willby, 2003; Schuldt et al., 2012). Moreover, shifts in a potential trade-off between physical (leaf dry matter content) and chemical defense (tremulacin) in *P. balsamifera* seedlings further points towards the possibility of earthworm-induced changes in tree strategies (Figure 5). Such trade-offs between physical and chemical

defense in trees have been reported (Eichenberg et al., 2015). Our findings indicate that earthworms can mitigate such a trade-off in *P. balsamifera* seedlings, which can have implications for their susceptibility to different kinds of herbivores (Züst & Agrawal, 2017). However, this shift in the trade-off occurred in the absence of leaf-chewing insects, as we did not experimentally manipulate insect herbivory in our experiment and studies have shown that shifts in trade-offs are often specific to plant enemy types (Biere et al., 2004; Hahn & Maron, 2016; Huang et al., 2010; Koricheva et al., 2004). Nevertheless, our results provide some first empirical indication for how physical and chemical defense strategies may be altered owing to earthworm invasion in a deciduous boreal tree species. Our results encourage future studies to examine whether earthworm-induced changes in the trade-off between a physical and chemical defense in *P. balsamifera* are sustained in the presence of leaf-chewing herbivores, as well as how such interactions are shaped by environmental contexts that could further determine earthworm effects (Van Groenigen et al., 2014).

Results from our observational study suggest that invasive earthworms can, in part, make North American poplar trees (*P. balsamifera* and *P. tremuloides*) more susceptible to leaf-chewing insect herbivores. While we cannot rule out that this increased susceptibility was partly due to earthworm-induced changes in leaf primary nutrient status, we speculate that these are to some extent driven by reductions in the concentration of secondary metabolites in *P. balsamifera* but not so in *P. tremuloides*. Indeed, the experiment did not support the patterns of chemical defense from the observational study, which is not that surprising given the various differences in biotic and abiotic conditions between the two studies. However, experimental addition of earthworms still decreased one of the secondary metabolites and enhanced fungal infection in *P. balsamifera*. Notably, our results are in contrast to the previously observed effects of native earthworms on herbaceous plants in their natural ranges, where native earthworms are often associated with enhanced concentrations of secondary metabolites (Xiao et al., 2018). We do acknowledge that our leaf damage assessments lack temporal assessments to account for seasonal variations in insect herbivory pressure on our study tree species. Furthermore, our leaf damage categories also do not capture all the variations in leaf damage. Greater temporal resolution of leaf damage assessment (with more resolved damage classes), insect herbivore assessments and tree growth measurements in field conditions will provide a better understanding of how earthworm-induced changes in resource allocation strategies in deciduous trees impact native forest functioning. This will further help understand the opposing effects of native earthworms on secondary metabolite production versus ecosystems where earthworms are invasive.

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AUTHORS' CONTRIBUTIONS

M.P.T. and N.E. conceived the study; T.K., M.P.T., S.B.U., O.F., U.P., L.T. and M.T. performed the study; M.P.T. and T.K. analysed the data; M.P.T. wrote the manuscript with substantial inputs from A.B., N.E. and S.B.U. All authors contributed to revisions.

PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/1365-2745.13504>.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository <https://doi.org/10.5061/dryad.g1jwstqpb> (Thakur et al., 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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