EVALUATING ABOVE- AND BELOWGROUND EFFECTS OF NATIVE AND NON-NATIVE EARTHWORMS IN GRASSLANDS

By

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EVALUATING ABOVE- AND BELOWGROUND EFFECTS OF NATIVE AND NON-NATIVE EARTHWORMS IN GRASSLANDS

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Abstract: Previous research has shown non-native earthworms can alter ecosystem function and structure by altering plant communities, soil dynamics, and soil microbial functional groups, including disruption of arbuscular mycorrhizal (AM) fungi, in North America. Compared to previously earthworm-free regions, few studies have investigated effects of non-native earthworms in regions where native earthworms are currently present, with even fewer on earthworm invasions in tallgrass prairies. We conducted complimentary field and growth-chamber mesocosm studies to assess potential effects of non-native earthworms on plants and plant-microbial interactions in tallgrass prairies in north-central Oklahoma. In our field study we assessed earthworm abundance and species composition, plant species composition and biomass, total soil microbial biomass, and relative abundance of soil microbial functional groups including intra- and extra-radical AM fungal abundance. In our mesocosm study we assessed the individual and combined effects of native and non-native earthworms on *Andropogon gerardii* production and soil microbial communities. At our field site we found both native (*Diplocardia* spp.) and non-native (*Aporrectodea trapezoides*) earthworms, with natives dominating earthworm abundances. Neither native nor non-native earthworm abundances were significantly associated with percent vegetation cover, vegetation biomass and richness, or soil microbial communities. However, greater numbers of total earthworms (native or native and non-native combined) were inversely related to native vegetation cover, including *Aristida oligantha*, and positively related to non-native vegetation cover, including *Bothriochloa* spp. Our mesocosm study found no relationship between native nor non-native earthworms and above- or belowground biomass or flowering, or the microbial community, including AM fungi. However, native earthworms alone influenced plant root structure differently than when native and non-native earthworms were both included, suggesting cross-species interactions. Potential mechanisms are unknown, requiring further research assessing additional variables, including nutrient availability. Our research suggests total abundance of earthworms, but not specific species groups, may influence vegetation composition in tallgrass prairies by reducing native and promoting non-native plant cover. However, because the vegetation community is linked to total earthworm abundances, widespread ecosystem effects may increase as non-native earthworm invasion continues. Thus, continued studies of earthworm effects in tallgrass prairies is crucial to monitor further alterations of this endangered ecosystem.

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CHAPTER I

EVALUATING EFFECTS OF NATIVE AND NON-NATIVE EARTHWORMS ON MICROBIAL AND PLANT COMMUNITIES IN A NORTH AMERICAN GRASSLAND

ABSTRACT

Previous research has shown non-native earthworms can alter function and structure of ecosystems across North America. These changes include alterations to plant communities, soil dynamics, and soil microbial functional groups, including disruption of arbuscular mycorrhizal (AM) fungi. In tallgrass prairies of the southern Great Plains, both native and non-native earthworms occur together. Compared to previously earthworm-free regions, few studies have investigated effects of non-native earthworms in regions where native earthworms are currently present, with even fewer assess earthworm invasions in tallgrass prairies. We conducted a field study to assess potential effects of non-native earthworms on plants and plant-microbial interactions in tallgrass prairies in north-central Oklahoma. We assessed earthworm abundance and species composition, plant species composition and biomass, total soil microbial biomass, and relative abundance of soil microbial functional groups including intra- and extra-radical AM fungal abundance. Non-native and native earthworms were found in our study site, with 73 non-native (*Aporrectodea spp.*) and 451 native (*Diplocardia spp.*) individuals identified. Abundances of neither native nor non-native earthworms were significantly associated with percent vegetation

cover vegetation biomass, vegetation richness, or soil microbial composition or biomass. However, greater numbers of total earthworms (native and non-native combined) were inversely related to native vegetation cover and positively related to non-native vegetation cover. Our research suggests total abundance of earthworms, but not specific species groups, may influence vegetation composition in tallgrass prairies, potentially reducing cover of native plants and promoting abundance of non-native plants. Non-native earthworms (*A. trapezoides*) may have similar effects on tallgrass prairie ecosystems as native earthworms (*Diplocardia spp.*) because both occupy the same ecological niche (endogeic) and have similar feeding habits. However, as alterations in vegetation community link with earthworm abundances, widespread ecosystem effects may increase as non-native earthworm invasion continues. Thus, continued studies of earthworm effects in tallgrass prairies is crucial to monitor further alterations of this endangered ecosystem.

INTRODUCTION

Due to their role as ecosystem engineers, non-native earthworms cause substantial changes to ecosystems they invade, such as in forests throughout northern and eastern North America. Non-native earthworms in North America originate primarily from Europe and Asia and have spread beyond their native ranges through human dispersal (e.g., dumping of fishing bait) (Hendrix and Bohlen, 2002; Holdsworth et al. 2007b; Beauséjour et al., 2015). Most research investigating non-native earthworm impacts has focused on forests in previously glaciated regions, where ecosystems evolved devoid of any earthworms. In these ecosystems, earthworm invasions greatly alter soil nutrient cycling, the leaf litter layer and soil organic horizon, and soil microbial communities (Bohlen et al. 2004; Szlavecz et al. 2011; Welke and Parkinson, 2003; Bal et al. 2018;

Hale et al. 2006). Such changes to soil greatly impact structure and species composition of understory plant communities (Bohlen et al. 2004; Holdsworth et al. 2007a), growth, mortality, and species composition of trees (Hale et al. 2006; Larson et al. 2010; Drouin et al. 2016; Bal et al. 2018), and populations of wildlife reliant on forest vegetation (Maerz et al. 2009; Loss and Blair 2011). Earthworm invasions also facilitate non-native plant invasions, likely due to earthworm dispersal of seeds and/or creation of environments favoring non-native plants (Nuzzo et al., 2009; Clause et al., 2015; Roth et al., 2015; Craven et al. 2017).

Despite widespread occurrence of non-native earthworms in North America, few studies have evaluated effects of invasions in regions with native earthworms already present. However, these studies illustrate that non-native earthworms can have adverse effects in systems with native earthworms. For example, in mid-Atlantic forests of the U.S. that contain native earthworms, addition of non-native earthworms increases leaf litter decomposition and alters soil microbial communities, soil respiration, and tree seedling growth (Szlavecz et al., 2011). Of studies evaluating non-native earthworm effects where native species are present, only a few have focused on non-forested ecosystems, such as grasslands. For example, in grasslands of California, USA, non-native earthworms were found to reduce belowground plant biomass and root-associated microbial communities (Winsome et al. 2006). In tallgrass prairies of Kansas, USA, non-native species *Aporrectodea caliginosa* and *Octolasion cyaneum* reduced soil turnover and nutrient mineralization (James 1991), and the non-native *A. turgida* decreased plant root growth more than native earthworm species (*Diplocardia spp*.) (James and Seastedt 1986). However, in some cases, non-native earthworms may have similar, or even less-adverse,

effects than natives, as evidenced by a study showing that native earthworms caused greater reductions in plant growth and N acquisition compared to non-native species (Callaham et al. 2001). Due to the limited number of studies and varied conclusions, more research is needed to evaluate non-native earthworm effects in areas with native earthworms, especially in non-forested ecosystems.

Although non-native earthworms have been reported to have substantial effects on plant growth and community composition, the mechanisms driving these effects are not fully known. Changes in vegetation occur as a result of earthworm feeding and burrowing activities, which alter soil physical, chemical, and biological properties (Frelich et al. 2019), but few studies have evaluated whether vegetation changes are driven by earthworm-related effects on soil microbial communities, such as arbuscular mycorrhizal (AM) fungi (McLean et al. 2006; Paudel et al. 2016a). AM fungi play a crucial role in grassland ecosystems, as they form essential symbiotic relationships with plants, providing nutritional benefits (Miller et al. 2012) and disease and herbivory protection (Linderman, 2000; Graham, 2001; Kula et al. 2005). Limited previous research suggests non-native earthworms reduce extra- and intra-radical abundance of AM fungi (Lawrence et al., 2003; Welke and Parkinson, 2003; Dempsey et al., 2011; Dobson et al., 2019), but non-native earthworms have also been reported to increase AM fungal biomass and/or intra-radical abundance (Dempsey et al. 2013; Drouin et al. 2016). Alterations in AM fungal abundance by non-native earthworms may have cascading effects on plant communities, such as reduced abundance and cover of mycotrophic plant species and concomitant increases in less-mycotrophic plant species (Bohlen et al., 2004; Frelich et al., 2006; Holdsworth et al. 2007b), as well as dieback of tree species that are AM fungal-

dependent (Bal et al. 2018). However, few studies assessing earthworm-AM fungal interactions have examined non-forested ecosystems (Paudel et al. 2016a). Adverse effects of non-native earthworms on AM fungi in grasslands are expected to be especially important because the dominant plant species in these ecosystems are highly dependent on AM symbiosis (Wilson and Hartnett 1998, Miller et al., 2012).

To address these research gaps, we studied associations among native and non-native earthworms, soil microbial communities including AM fungi, and plants in a tallgrass prairie in the U.S. Great Plains. Our specific objectives were to evaluate independent and combined effects of native and non-native earthworms on: 1) intra- and extra-radical AM fungal abundance, and relative abundance of soil microbial functional groups (gram+ and gram- bacteria, saprophytic and AM fungi, and total microbial biomass), and 2) plant community composition and aboveground biomass production. Simultaneously investigating these above- and belowground ecosystem components can provide unique insight into the mechanisms by which earthworms affect plant communities, and therefore, the many crucial ecosystem services provided by grasslands (e.g., wildlife habitat, biodiversity, rangeland production, and carbon sequestration) (Hoekstra et al. 2005; Grant et al. 2009; Ratajczak et al. 2012).

METHODS

Study Area and Site Selection

Our study area was located southwest of Stillwater, Oklahoma, USA, at the Oklahoma State University Range Research Station (OSURRS) (36°03'52.6"N 97°13'52.1"W). OSURRS is at the western edge of the Cross Timbers ecoregion and

currently consists of tallgrass prairie and savanna vegetation types. The tallgrass prairie occurs on fine-textured clay and loamy soils and is dominated by native and non-native tallgrass graminoid species. Annual precipitation averages 93.2 cm, summer daily high temperature averages 34.4°C, and winter daily low temperature averages -1.1°C. Land management practices at OSURRS include light to moderate cattle grazing in tandem with a prescribed fire regime that includes dormant season (late winter/early spring) or growing season (late summer/early fall) burns approximately every three years to prevent woody encroachment and maintain grassland vegetation.

We selected four pastures at OSURRS that have similar grazing intensity (to control for effects of grazing on plant community composition), as well as similar fire return interval (~3 years) and time since fire (~2.5 years), since these fire-related factors may influence earthworm populations (James 1988). Using the Soil Survey Geographic Database (SSURGO) (Soil Survey Staff, NRCS), areas with similar soil types (loam, sandy loam, and loamy sand) (Loss et al. 2017) were selected within the four pastures to control for the effect of soil texture on earthworm abundance (Hendrix et al. 1992). Areas with high concentrations of woody vegetation were excluded. Under these constraints, one hundred random points were established using ArcMap (ESRI). We used these random points as locations for 1m x 1m plots in which we collected soil for microbial assessments, assessed plant community composition, and estimated native and non-native earthworm abundance. All sampling occurred in October 2019, a period during which precipitation occurred regularly and no frost occurred, conditions that are favorable for earthworm activity and detection (Loss et al. 2017).

Earthworm and Vegetation Sampling

Earthworm sampling was conducted by students in the Oklahoma State University Applied Ecology and Conservation lab course who were trained and directly supervised by authors of this paper (Y.M. and S.R.L.). Although students had no previous earthworm sampling experience, this approach to training, data collection, and supervision has been vetted by the authors and has resulted in past scientific publications (e.g. Loss et al. 2017). At each plot, earthworms were sampled from a single 33 cm x 33 cm subplot located 1m south of the plot center. We used the hand-sifting method to sample earthworms (Bouché & Gardner, 1984) because the mustard-solution extraction method is generally ineffective at this study site due to the slow rate of liquid infiltration into the relatively dense soil. In each subplot, we dug to approximately 30 cm depth, and excavated soil was sifted by hand and searched for earthworms. Collected earthworms were euthanized in 70% isopropyl alcohol and stored in 10% buffered formalin until identified by a taxonomic expert (S. James). Earthworms were identified to species when possible, but a small number could only be identified to genus or family.

We also estimated plant species richness and species composition within each 1 m x 1 m plot. Plant species composition was determined by estimating the percent cover of each plant species, total cover of all native and non-native plants, and total cover of all vegetation. Raw percentages of vegetation cover were converted to the following cover classes adopted from the Daubenmire scale (Mueller-Dombois and Ellenberg 1974): 0 to 5%, 6 to 25%, 26 to 50%, 51 to 75%, 76 to 95%, and 96 to 100%. Species richness was calculated for each plot as the total number of plant species observed, including graminoids, forbs, and woody plants. A 0.25 m x 0.25 m subplot was randomly located in

each plot; within this subplot, all aboveground plant material was clipped at the soil surface, plant samples were oven-dried to constant mass at 60℃ for 48 hours, and biomass was weighed to assess total aboveground production.

Evaluation of Soil Microbial Communities

Four randomly located, 2 cm soil cores (0-10 cm depth) were collected within each 1 m x 1 m plot. From among these 100 soil samples, we selected a subsample (n=31) that captured plots with all different observed combinations of native and non-native earthworms found during sampling (see Results), including plots with: native earthworms only, non-native earthworms only, natives and non-natives present, and no earthworms present.

For these soil subsamples, roots were separated from soil for estimation of intra-radical AM fungal colonization (described in following section), and remaining soil was passed through a 6 mm diameter sieve to remove stones and coarse organic matter and assessed for soil microbial communities. Relative abundances of soil microbial functional groups (gram positive and gram negative bacteria, AM and saprophytic fungi) and total microbial biomass were assessed using signature fatty acids (Olsson et al. 1995). Phospholipid fatty acids (PLFAs) are constituents of biological membranes that can be used to estimate active biomass of bacteria and fungi as biovolume and cell surface area are well-correlated (Frostegard et al. 2011). Neutral lipid fatty acids (NLFAs) are basic storage products of many fungi and serve as the primary energy reserve in AM fungi (Sharma and Buyer, 2015). Soil samples were freeze-dried and finely ground with a mortar and pestle, and 5 g of soil was mixed with a phosphate buffer, methanol, and chloroform. The soil-solvent mixture was separated by centrifugation and then decanted

with 1:2 mix of chloroform and methanol. Phosphate buffer was added and left for phase separation to occur overnight, and then the chloroform layer containing the lipids was recovered and reduced by nitrogen flow at 50°C. Lipids were separated into neutral lipids, glycolipids, and phospholipids by solid-phase extraction by eluting with chloroform, acetone and methanol, respectively. Lipids were hydrolyzed and methylated. The methylated fatty acids were extracted with hexane and evaporated under nitrogen at 37°C.

The PLFA/NLFA analyses were performed using an Agilent 7890A gas chromatograph with an Agilent 5975C series mass selective detector. We utilized c:19 as an internal standard. Biomarkers used to select for the functional group of gram-positive bacteria consisted of i-15:0, a- 15:0, i-17:0, and i-16:0 (Liu et al., 2019). For gram-negative bacteria, selected biomarkers were 16:1ω7 (Liu et al., 2019), cy19:0, cy17:0ω9, 2-OH 14:0, 2-OH 16:0, 3-OH 14:0, and 18:1ω9 trans (Zhang et al., 2015). For AM fungal extra-radical hyphal (ERH) biomass, biomarkers consisted of 16:1ω5c, 20:1ω9, and 22:1ω13 for both PLFA and NLFA determination (Sakamoto et al. 2004). Biomarkers selected for saprophytic fungi were 18:2ω9,12 and 18:1ω9c (Bell et al., 2009). The abundances associated with these biomarkers were used to calculate a total nmol per gram of soil for each functional group. Total microbial biomass was calculated by adding abundances of all functional groups with the non-specific biomarkers, 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0 (Xiao et al., 2018). During PLFA analyses one sample failed to run, reducing the sample size to n=30.

Determination of Intra-radical AM Fungal Colonization

To quantify intra-radical AM fungal colonization (IRC, or percent AM fungal root colonization), live roots were extracted from soil samples collected for microbial community composition, washed, stained with trypan blue, and scored for AM fungal colonization using the magnified gridline intersect method (McGonigle et al., 1990). A digital microscope (HiroxKH 7700) was used to measure the percentages of root length colonized by hyphae, vesicles, coils, and arbuscules; these percentages were combined to determine total percent colonization.

Statistical Analysis

Earthworm effects on plants, microbial communities, and IRC, were investigated using generalized linear mixed models (GLMM), generalized linear models (GLM), and linear models (LM); for all response variables, we compared multiple candidate models using an information-theoretic approach (described in detail below). Vegetation response variables assessed (n=100) included: native plant species cover (%), non-native plant species cover (%), total vegetation cover (%), graminoid plant cover (%), forb plant cover (%), woody plant cover (%), percent cover of each of the 10 most abundant plant species (*Bothriochloa spp., Lespedeza cuneata, Panicum oligosanthes, Aristida oligantha, Schizachyrium scoparium, Panicum virgatum, Ambrosia psilostachya, Sorghastrum nutans, Bothriochloa laguroides, and Setaria spp*.), vegetation species richness, and aboveground plant biomass. AM fungi and other microbial response variables included: IRC (n=100), ERH (n=30), bacterial abundance (n=30), saprophytic fungal abundance (n=30), total fungal abundance (n=30), AM fungal spores (n=31), saprophytic fungal spores (n=31), and total fungal spores (n=31). Predictor variables evaluated in relation to all response variables included: (1) earthworm assemblage, a categorical variable

representing potential effects of the presence of native and non-native earthworms (categories included: only natives present, only non-natives present, natives and non-natives present, no earthworms present), (2) non-native earthworm density, a continuous variable representing a potential independent effect of earthworm invasion intensity, (3) native earthworm density, a continuous variable representing a potential independent effect of native earthworms to allow comparison to non-native earthworm effects, and (4) total earthworm density, a continuous variable representing combined effects of native and non-native earthworms. All earthworm density values were calculated per 1 m2 by multiplying earthworm counts from 0.33 x 0.33 m2 subplots by 9.18. Vegetation biomass estimates were calculated per 1 m2 by multiplying estimates from 0.25 m x 0.25 m subplots by 16. For vegetation percent cover analyses, cover classes were converted to midpoints to achieve a (0, 1) distribution.

To account for non-independence of sampling points within pastures, a random effect for pasture was used for most models described below. The only exceptions were models with response variables from PLFA and NLFA analyses; these variables did not require a random effect due to limited replication of these measurements within pastures. For analyses with response variables that were percentages (IRC and all plant cover response variables), we used GLMMs with a beta distribution due to the flexibility of this distribution and its suitability for percentage data. For plant species richness, we used a GLMM with a Poisson distribution because richness data were counts. For aboveground biomass, we used a GLMM with a Gamma distribution because biomass data were continuous and non-normally distributed. For analyses with AM fungal spores, saprophytic fungal spores, and total fungal spores as the response variables, we used

GLMs with a Gamma error distribution. The remaining response variables (ERH, bacterial biomass, saprophytic fungal biomass, and total fungal biomass) were all normally distributed, and thus, LMs with a Gaussian distribution were used.

For all response variables, we considered several single variable candidate models with each containing different predictor variables, and including a null (i.e., intercept-only) model with no predictors. Models were ranked using Akaike's Information Criterion corrected for small sample sizes (AICc; Burnham and Anderson, 2002). Model ranking was based on ΔAICc values with: ΔAICc = 0 indicating the most parsimonious model (i.e., the model that best achieves the tradeoff between simplicity and high explanatory power); ΔAICc = 0-2 indicating strong model support compared to other models in the candidate set, and ΔAICc > 2 indicating moderate-to-no model support. Thus, we considered models strongly supported if they had ΔAICc ≤ 2 and with ΔAICc at least 2 less than the null model (Burnham and Anderson, 2002). The 85% confidence intervals (CIs) for variables in supported models were checked to ensure they did not overlap with 0; 85% CIs were used in order to align with the selection criteria used by the AIC model selection framework so that variables supported by models with lower AIC are not discarded (Arnold 2010). Models with ΔAICc = 0-2 that were not 2 less than the null model, but had 85% CIs that did not overlap with 0, were considered moderately supported. For all strongly and moderately supported models, we evaluated effects of predictor variables using coefficient estimates.

All analyses were conducted in R version 3.6.1 (R Core Team 2013) using the following packages: 'glmmTMB' (Brooks et al., 2017) for the GLMMs with the beta

distribution, and 'lme4' (Bates et al., 2013) for the GLMMs, GLMs, and LMs. For AIC model comparisons, the 'AICcmodavg' package (MJ Mazerolle, 2017) was used.

RESULTS

We collected a total of 524 earthworms across 91 of the 100 plots (i.e., no earthworms found at 9 plots) (Table 1). Of these, 451 earthworms were identified as native, all belonging to the genus *Diplocardia*, including *D. longiseta* (80), *D. singularis* (98), *D. kansensis* (21), and juveniles only identifiable to genus (252). Native earthworms were found at 85 of the 100 plots, and 45 of these had only native earthworms. A total of 68 non-native earthworms were collected, all in the genus *Aporrectodea*, including adults (29) and juveniles only identifiable to genus (39). Non-native earthworms were found at 42 of the 100 plots, and 5 of these plots contained only non-native earthworms; 37 plots contained both native and non-native earthworms (average counts of earthworms in these plots included: 5 natives and 2 non-natives). Of the 524 earthworms, 5 were only identifiable to family Lumbricidae, which consists of both North American and Eurasian species; thus, the native status of these earthworms could not be determined. Unidentified earthworms were found at 4 plots, and 3 of these plots had native earthworms as well, with 1 plot containing only the unidentified earthworm.

Vegetation sampling determined that the majority of plant species at our study site were native, and only six non-native species were observed, two of which were rare and only found in one plot each (Table 1). However, the most abundant plant species overall was a non-native, *Bothriochloa spp*., a warm-season grass commonly known as old world bluestem and hereafter referred to as *Bothriochloa*.

With the exception of the four below-described plant cover response variables, no models outperformed the null model for most response variables (i.e., either the null model was top-ranked, or other models ranked higher than the null, but they were not more than 2 ΔAIC units above it and/or had coefficients with 85% CI's that overlapped zero). The response variables with no strongly supported models included: plant species richness, % total vegetation cover; % cover of major plant growth forms (grasses, forbs, and woody plants), aboveground plant biomass, and all AM fungal and other microbial group response variables (bacterial and total microbial abundance; saprophytic, AM, and total fungal hyphal abundance (PLFA); saprophytic, AM, and total spore abundance (NLFA); and IRC). These results indicate that there was minimal support for any of these response variables being related to the earthworm variables we evaluated, including the categorical variable capturing different combinations of native and non-native earthworm presence, non-native earthworm density, native earthworm density, and total earthworm density.

For the analysis with percent cover of native vegetation as the response variable, two models were moderately supported (i.e., ΔAIC = 0-2; null model ΔAIC = 1.77, and 85% CI of the coefficient estimate not overlapping zero). The top model included native earthworm density, and the second most strongly supported model (ΔAIC = 0.27) included total earthworm density. Coefficients in these models indicated that both native and total earthworm density were inversely associated with percent cover of native vegetation (Figure 1). For the analysis with percent cover of non-native vegetation as the response variable, there were two strongly supported models. As with the native plant cover analysis, the top model and second-most strongly supported model (ΔAIC = 0.02)

included native earthworm density and total earthworm density, respectively. Coefficients in these models indicated that both total and native earthworm density were positively related to percent cover of non-native vegetation (Figure 2).

For analyses of percent cover of each of the ten most common plant species in our study area, only two plant species had models that were supported and outperformed the null model, *Bothriochloa* and *Aristida oligantha*, a native annual warm-season grass commonly known as prairie threeawn or oldfield threeawn. For each species, two models were supported, one each containing total earthworm density and native earthworm density. For *Bothriochloa*, total earthworm density was in the top model and native earthworm density was in the second-ranked model [ΔAIC = 0.07]; support for these models was moderate because ΔAIC = 1.25 for the null model and 85% CIs for both earthworm variables did not overlap 0. For *A. oligantha*, native earthworm density was in the top model and total earthworm density was in the second-ranked model [ΔAIC = 0.43]. Both total and native earthworm density had positive associations with *Bothriochloa* cover (Figure 3) and negative associations with *A. oligantha* cover (Figure 4).

DISCUSSION

Although we did not find significant effects of non-native earthworms by themselves, our study suggests that numbers of total earthworms, and of native earthworms, influence plant communities in tallgrass prairies of the North American Great Plains. Specifically, native and total earthworm abundance had positive relationships with non-native plant cover and inverse relationships with native plant

cover. Past research has found that non-native earthworms greatly change plant communities by increasing non-native plant cover and reducing native plant cover (Hale et al., 2008; Nuzzo et al., 2009; Gibson et al., 2013; Drouin et al., 2016; Paudel et al., 2016b; Craven et al., 2017). However, our study suggests that such effects can occur with increasing numbers of earthworms regardless of their native status. This pattern may have arisen because both the native and non-native earthworm taxa in our study area fill the same ecological niche (i.e. the non-native *A. trapezoides* and native *Diplocardia spp*. are endogeic, meaning they inhabit and forage within mineral soil layers). Native and total earthworm abundances were also negatively related to cover of the native plant *Aristida oligantha* and positively related to cover of *Bothriochloa*, a highly invasive European species common in Great Plains grasslands. Our investigation of possible mechanisms underlying observed vegetation changes illustrated no significant effects of either native or non-native earthworms on the soil microbial community, including AM fungal intra- or extra-radical abundance.

Earthworm Associations Native and Non-native Plants

Many past studies have shown that non-native earthworms can facilitate non-native plant invasions into forests and grasslands, especially those that were historically devoid of any earthworms (Clause et al., 2015; Roth et al., 2015; Paudel et al. 2016b; Frelich et al., 2019). Our study, which assessed effects of native and non-native earthworms in sites where they co-occur, suggests that high numbers of total earthworms and of native earthworms, but not of non-native earthworms per se, may facilitate invasions of non-native plants (e.g. *Bothriochloa*) into grassland ecosystems. Because *Bothriochloa* has competitive superiority over native grasses, it may readily benefit from

changes that can be caused by both native and non-native earthworms, such as increased nutrient availability (Schmidt et al., 2008). Szlavecz et al. (2011) reported that non-native earthworms altered microbial communities and reduced seedling growth in an ecosystem with native earthworms present. However, unlike our study in which native earthworms were much more abundant than non-natives, non-natives were more common in that study. The relative dominance of native and non-native earthworms could influence the nature of effects on plant communities, or alternatively, total numbers of earthworms present, regardless of native status, could be most important in some systems. We speculate that the latter explanation may apply in our study area because both the native and non-native taxa we documented fill the same ecological niche (i.e., *A. trapezoides* and *Diplocardia spp*. are endogeic, meaning they forage in and inhabit mineral soil layers). Thus, increasing numbers of earthworms, regardless of native status, could lead to stronger ecosystem effects due to increasing numbers of individuals with foraging and movement behaviors that affect the soil and plants in similar ways.

As evidenced by the relatively low proportion of non-native earthworms, the earthworm invasion in our study area may be relatively recent (Loss et al. 2017). If non-native populations increase in the future, and native earthworm abundances remain stable, then overall increases in earthworm numbers could lead to intensified effects on plant communities. Alternatively, non-native earthworms can outcompete natives without increasing overall earthworm abundances (Hendrix et al. 2006), and this could also result in a greater magnitude of effects by non-natives. However, a study by Kalisz and Dotson (1989) indicates that *Diplocardia spp*. are resistant to ecosystem disturbances and non-native earthworm invasions by *A. trapezoides*, especially when consistent prescribed

burning was applied, presumably because *Diplocardia spp*. are better able to tolerate effects of fire (Callaham et al., 2003; James, 1988). Due to the potential for future increases in proportional abundance of non-native earthworms and/or total earthworm abundance, continued research is needed to evaluate how native and non-native earthworms interact to affect grassland ecosystems.

The associations we documented between earthworm abundances and native and non-native plant cover could have broader ecosystem implications related to wildlife populations and communities, and to grazing and livestock production in tallgrass prairies. Previous research indicates that cattle production on pastures dominated by *Bothriochloa* are less profitable compared to native pastures, suggesting potential financial implications of earthworm-related increases in non-native plant cover (Coleman et al., 2001; Phillips & Coleman, 1995). *Bothriochloa* has also been found to change invertebrate communities by shifting dominant functional groups and reducing overall diversity and biomass (Hickman et al., 2006; McIntyre & Thompson, 2003; Mitchell & Litt, 2016). Effects on invertebrates may have cascading implications for insectivorous wildlife, such as many grassland bird species including quail and songbirds (Kuvlesky et al., 2002; Wiens & Rotenberry, 1979), and increased *Bothriochloa* cover can decrease bird species richness and total abundance (Fulbright et al., 2019; George et al., 2013; Hickman et al., 2006). Decreasing cover of native plant species, including *A. oligantha*, may also impact wildlife in tallgrass prairies. For example, this plant species is a known source of food and cover for the uncommon and declining Smith's Longspur (*Calcarius pictus*), a species that has a geographically restricted wintering range centered in grasslands of the southern Great Plains including our study area (Holimon et al., 2012).

Past research in forests of northern and eastern North America indicates that invasive earthworm-caused changes to plant communities cause declines in wildlife populations (Loss & Blair, 2011; Maerz et al., 2009; Ziemba et al., 2016). However, research evaluating wildlife responses to non-native earthworm invasions, and to varying numbers of native earthworms, has never been conducted in grassland ecosystems. Such research is needed to increase understanding of potential links between earthworms and wildlife populations and communities in non-forested ecosystems that have both native and non-native earthworms present.

Earthworms and Microbial Communities

To investigate mechanisms underlying earthworm associations with plant cover, we assessed relationships between native and non-native earthworm abundance and soil microbial functional groups, including AM fungal colonization root extra-radical abundance. Previous research indicates earthworms can reduce abundance of AM fungi, which can lead to decreased abundance and cover of dominant, highly mycotrophic plant species with increases in less-mycotrophic species (Frelich et al., 2006; Hale et al., 2006). However, we found no significant associations between earthworm numbers and any of the microbial community variables we assessed. These results suggest that the observed earthworm-associated alterations in plant cover were not driven by shifts in microbial communities, including AM fungi. Our findings were unexpected, because in addition to the above-described relationships with mycotrophic and non-mycotrophic plant species, several studies have suggested that non-native earthworm-caused alterations to plant communities may arise primarily from changes in soil microbial communities (Hale et al., 2006; Paudel et al., 2016a; Laushman et al., 2018). For example, in north-temperate

forests, earthworms contribute to canopy tree dieback and reduced cover and regeneration of seedlings for sugar maple (Acer saccharum), a mycorrhizal-dependent tree species (Holdsworth et al., 2007a; Drouin et al., 2016; Bal et al., 2018); such effects may arise at least in part through reduced AM fungi colonization of tree roots (Lawrence et al. 2003). As with the above explanation for our plant cover results, the lack of significant findings related to soil microbes may relate to the earthworms occupying a similar ecological niche. Notably, other studies that evaluated effects of endogeic earthworm groups also found no effects on AM fungal root colonization, including for the non-native earthworms *A. caliginosa* (Wurst et al. 2004) and *A. trapezoides* (Pattinson et al. 1997). Thus, behaviors characteristic of endogeic earthworms may result in few impacts on soil microbes, at least in grassland ecosystems where native and non-native earthworms are both present. Further research is needed to evaluate how effects of earthworms on plants, AM fungi, and other microbes, are influenced by the ecological niches of both the invading earthworms and those that are already present.

Given that we documented no relationships between earthworms and microbes, including AM fungi, the mechanisms underlying significant relationships between earthworm abundance and plant cover in our study site are unclear. However, additional factors that we did not examine and that have previously been found to mediate earthworm effects on plants include earthworm consumption and dispersal of seeds, and effects on seedling emergence and soil nutrients, structure, moisture, and organic matter (Scheu & Parkinson, 1994; Clause et al., 2015; Hale et al., 2008; Roth et al., 2015; Welke & Parkinson, 2003). Additionally, earthworms and plant cover may not be causally related; instead, the statistically significant relationships we documented could simply be

correlations that arise from shared environmental preferences or modes of introduction (Gabbard & Fowler, 2007; Paudel et al., 2016b). For example, both non-native earthworms and *Bothriochloa* tend to be present near roads (Gabbard & Fowler, 2007; Loss et al., 2017) and both thrive in medium-textured, loamy soils (Berg, 1993; Lowe & Butt, 2005). A common mode of road-based dispersal and similar environmental preferences could thus result in a positive, but not causal, relationship between earthworms and plants.

Conclusions

While there has been substantial and increased attention given to researching and managing ecosystem-level effects of non-native earthworms in areas with no native earthworms present, such as in glaciated north-temperate forests of North America (Frelich et al. 2019), relatively little research has evaluated such effects of earthworm invasions in regions and ecosystems where native earthworms are already present. Our study revealed novel relationships between native and non-native plant cover and native and total earthworm abundance in tallgrass prairies of the North American Great Plains. Specifically, cover of native plant species decreased and cover of non-native plants increased with increasing earthworm numbers. However, the mechanisms underlying these patterns are uncertain, as we found no evidence that soil microbial communities, including AM fungi, were influenced by earthworms and therefore likely did not drive plant community changes. However, earthworm effects have the potential to evolve, and new adverse effects of non-native earthworms could emerge if earthworm community composition and total abundance change in the future with addition of greater numbers and/or new species of non-native earthworms to grassland ecosystems. A controlled study

in which earthworm communities are experimentally manipulated, and earthworm abundance and/or density are controlled and accounted for, could elucidate mechanisms underlying the independent and combined effects of native and non-native earthworms on plant growth. Further field monitoring and research into the effects of dynamic earthworm invasions and native earthworm populations are also needed to evaluate potential changing effects on plant communities, wildlife, biodiversity, rangeland productivity, and the other ecosystem functions and services provided by grassland ecosystems.

TABLES

Table 1. Total number of individual earthworms and their mean density (±SE) collected in 2019 at the Oklahoma State University Range Research Station near Stillwater, Oklahoma, USA.

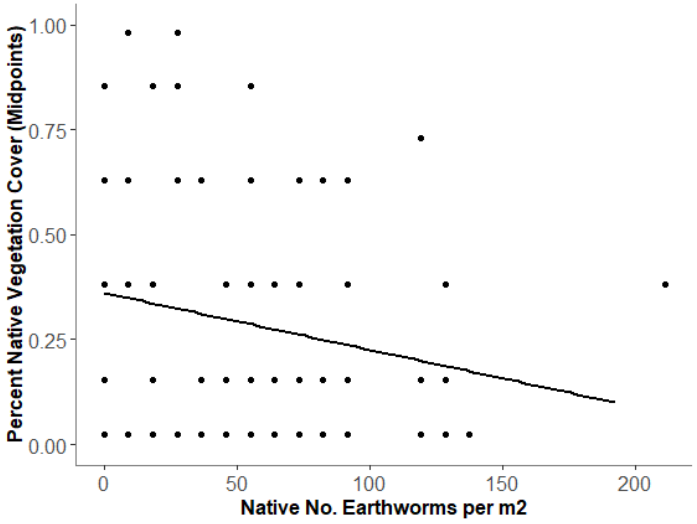
Earthworm Species/Taxa Sampled	Origin	# Individuals	Density (individuals m^{-2})
Diplocardia spp.	**Native**	**451**	**4.51 (0.40)**
Diplocardia longiseta	Native	80	0.8 (0.14)
Diplocardia singularis/caroliniana	Native	98	0.98 (0.17)
Diplocardia kansensis	Native	21	0.21 (0.06)
Juveniles	Native	252	2.52 (0.27)
Aporrectodea spp.	**Eurasian**	**68**	**0.68 (0.10)**
Aporrectodea adults	Eurasian	29	0.29 (0.07)
Juveniles	Eurasian	39	0.39 (0.07)
Lumbricidae (excl. *Aporrectodea*)	Native/Eurasian	5	0.05 (0.03)

Table 2. For plant species found at 3 or more plots (out of 100 total sampled) at the Oklahoma State University Range Research Station near Stillwater, Oklahoma, USA, number of plots with the species and average percent cover (%) across all plots (standard error in parentheses).

Plant Species	Number of plots with species	Percent Cover (%)
Grasses		
Bothriochloa spp.	79	45.72 (3.44)
Panicum oligosanthes	67	4.16 (0.54)
Aristida oligantha	15	3.85 (1.08)
Schizachyrium scoparium	41	3.28 (0.61)
Panicum virgatum	25	2.33 (0.65)
Setaria spp.	23	3.02 (0.87)
Sorghastrum nutans	43	1.82 (0.33)
*Bothriochloa laguroides**	14	1.21 (0.48)
Andropogon virginicus	9	3.38 (0.43)
Digitaria sanguinalis	5	0.96 (0.43)
Andropogon ternarius	9	0.77 (0.37)
Digitaria cognata	4	0.55 (0.37)
Paspalum floridanum	9	0.41 (0.22)
Panicum anceps	4	2.63 (0.13)
Eragrostis pectinacea	5	0.08 (0.04)
Andropogon gerardii	3	0.07 (0.05)
Forbs		
Lespedeza cuneata	68	17.08 (1.93)
Symphyotrichum ericoides	12	0.73 (0.25)
Gutierrezia sarothrae	12	0.84 (0.29)
Ambrosia psilostachya	28	2.33 (0.56)
Artemisia ludoviciana	5	0.47 (0.26)
Solidago rigida	5	0.4 (0.21)
Helianthus mollis	4	0.4 (0.23)
Chrysopsis pilosa	5	0.18 (0.11)
Liatris punctata	3	0.12 (0.10)
Acalypha virginica	3	0.07 (0.05)
Chamaecrista fasciculata	3	0.05 (0.03)
Euphorbia dentata	4	0.04 (0.02)
Woody		
Ceanothus cuneatus	5	0.23 (0.15)
Rhus copallinum	3	0.21 (0.13)

FIGURES

(a)



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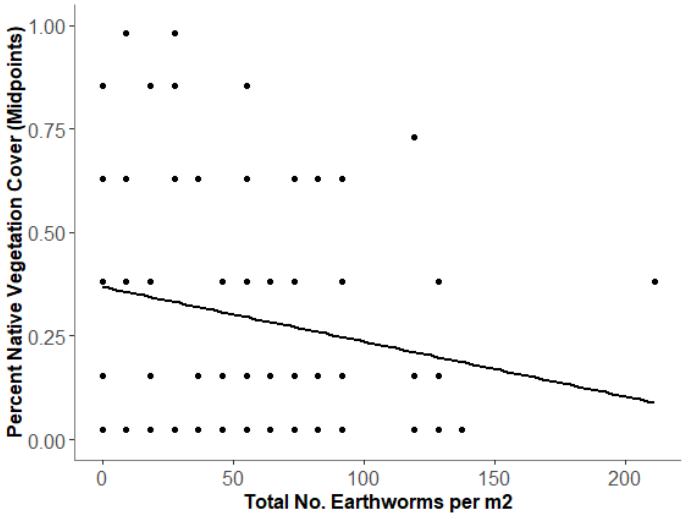
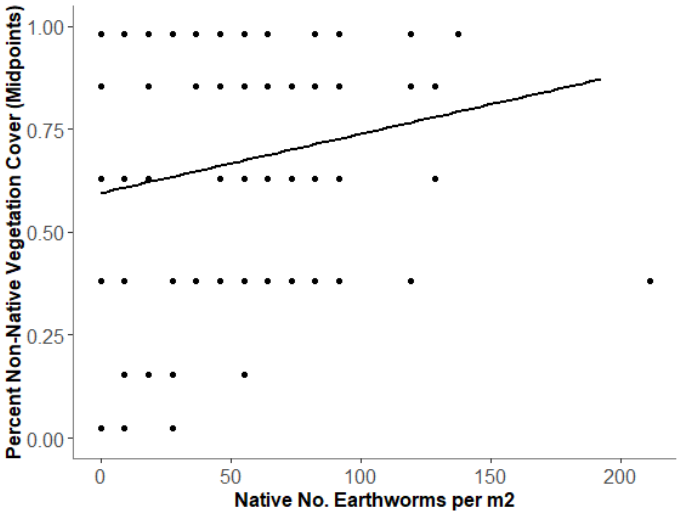


Figure 1. Relationship between percent total cover (%) of native vegetation (midpoints from range of estimates described in text; e.g., 0-5% cover is plotted as 2.5%) and native (a) and total (b) earthworm density (earthworms per m2) at the Oklahoma State University Range Research Station near Stillwater, Oklahoma, USA.

(a)



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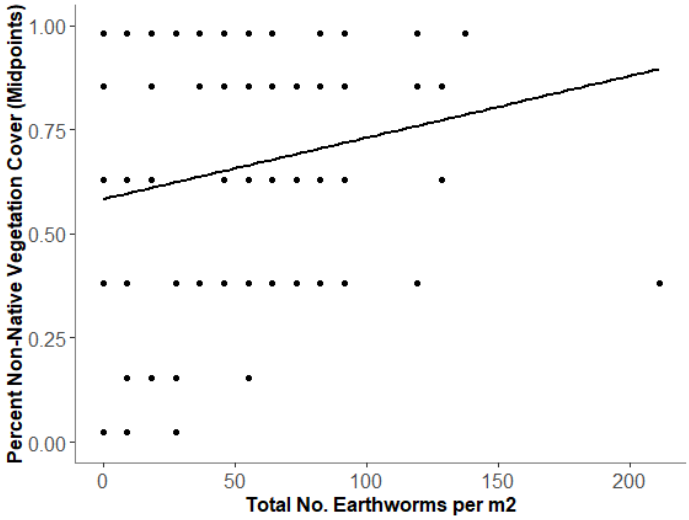
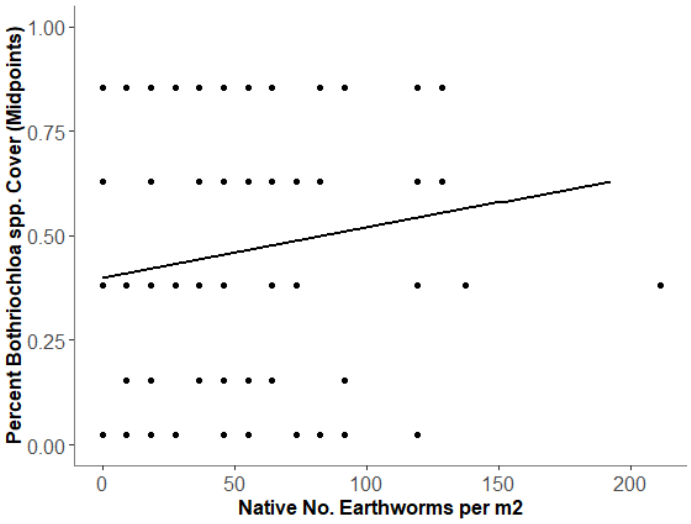


Figure 2. Relationship between percent total cover (%) of non-native vegetation (midpoints from range of estimates described in text; e.g., 0-5% cover is plotted as 2.5%) and native (a) and total (b) earthworm density (earthworms per m2) at the Oklahoma State University Range Research Station near Stillwater, Oklahoma, USA.

(a)



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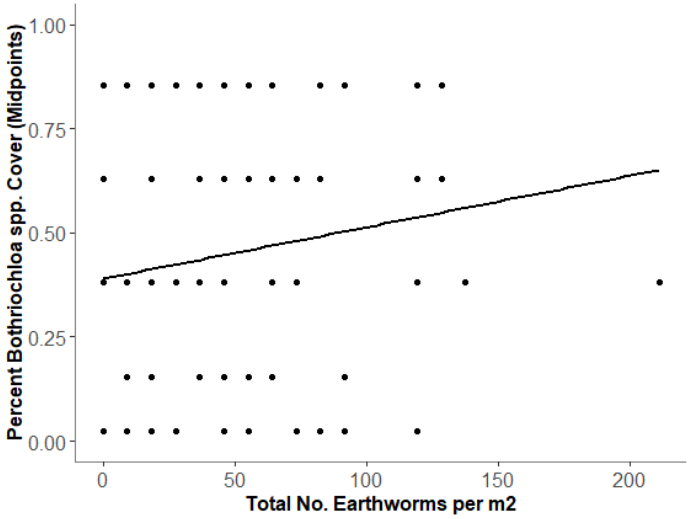
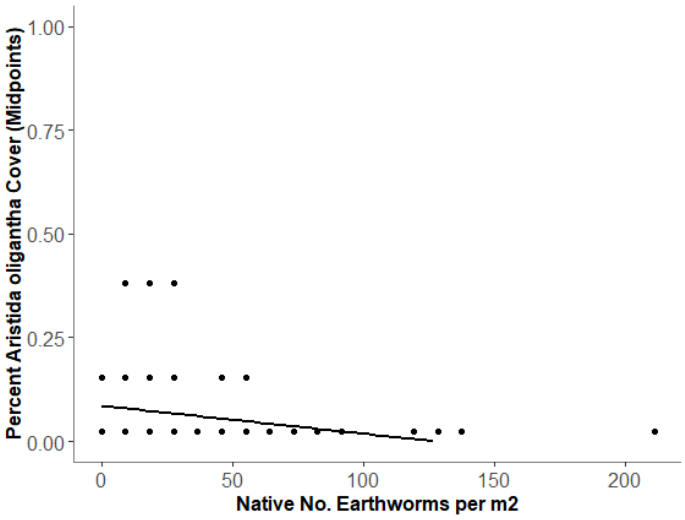


Figure 3. Relationship between percent cover (%) of *Bothriochloa* (midpoints from range of estimates described in text; e.g., 0-5% cover is plotted as 2.5%) and native (a) and total (b) earthworm density (earthworms per m2) at the Oklahoma State University Range Research Station near Stillwater, Oklahoma, USA.

(a)



(b)

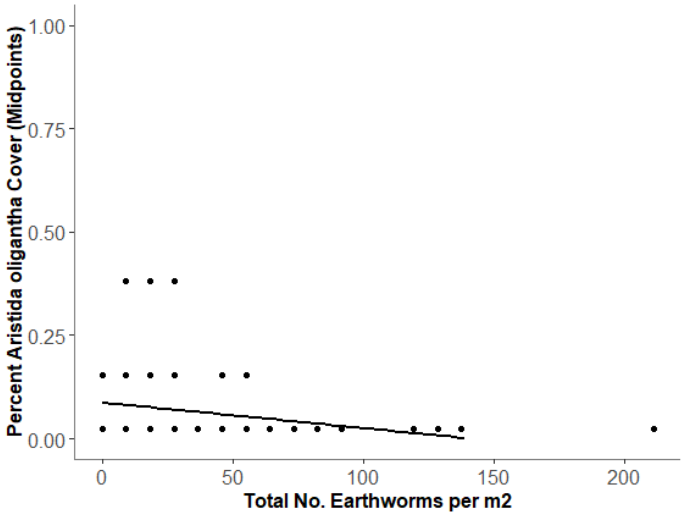


Figure 4. Relationship between percent cover (%) of *Aristida oligantha* (midpoints from range of estimates described in text; e.g., 0-5% cover is plotted as 2.5%) and native (a) and total (b) earthworm density (earthworms per m2) at the Oklahoma State University Range Research Station near Stillwater, Oklahoma, USA.

CHAPTER II

EXPERIMENTAL EVALUATION OF NATIVE AND NON-NATIVE EARTHWORMS ON SOIL MICROBIAL COMMUNITIES AND A DOMINANT WARM-SEASON GRASS IN TALLGRASS PRAIRIE

ABSTRACT

Past research shows that earthworms can affect plant growth either directly or indirectly through alteration of root structure and plant biomass, nutrient availability, soil moisture, and relative abundance of microbial functional groups including arbuscular mycorrhizal (AM) fungi (via disruption of intra- and inter-radical hyphae). Although much research has addressed the widespread impacts of non-native earthworms on plant communities in historically earthworm-free regions, few studies have compared effects of native and non-native earthworms on plant growth in ecosystems with native earthworms, such as tallgrass prairies. We conducted a mesocosm study to experimentally assess the individual and combined effects of native and non-native earthworms on big bluestem (*Andropogon gerardii)*, a dominant plant species in North American tallgrass prairies. Each mesocosm included a single plant and was subjected to one of the following earthworm treatments for 16 weeks: no earthworms (control), natives only, non-

natives only, and both natives and non-natives. Among all treatments, we compared above- and belowground plant biomass, root structure, relative abundance of soil microbial functional groups, and intra- and inter-radical AM fungal abundance. Our results show that neither native nor non-native earthworms individually affected aboveground plant biomass, relative abundance of soil microbial functional groups, or intra- and inter-radical AM fungal abundance. However, the combined native and non-native treatment had a significantly greater fine:coarse root ratio and lower average root diameter compared to native earthworms only, and no combination of native and non-native earthworms was significantly different from plants grown without earthworms. We found no significant effects of earthworms on belowground biomass or specific root length. Our findings suggest that while earthworms do not affect total plant growth or root growth, they do affect root structure and, therefore, will likely affect the ability of plants to acquire nutrients and water. More research is needed to further evaluate the separate and combined effects of native and non-native earthworms on plant growth and soil microbial composition in tallgrass prairies, as non-native earthworm abundances are projected to increase in these ecosystems.

INTRODUCTION

Non-native earthworms have been shown to have adverse ecological effects in a variety of ecosystems across the globe, including North America (Bohlen & Hendrix, 2002; Hendrix P.F., 2006). Earthworm invasions substantially affect abiotic conditions (e.g., soil temperature, texture, and structure), populations and communities of plants and animals, and ecosystem-level processes such as decomposition, nutrient cycling, and primary productivity (Frelich et al., 2019). Earthworm-caused changes to the soil and leaf litter layer can greatly influence plant community composition through species-specific

impacts on seedling emergence, and plant growth and mortality (Eisenhauer et al., 2010; Bal et al., 2018). Earthworm invasions often decrease overall plant diversity and facilitate invasions by non-native plant species, which can impact fauna including invertebrates, birds, and salamanders, as well as ecosystem functioning and services (Maerz et al., 2009; Nuzzo et al., 2009; Loss and Blair, 2011; Clause et al., 2015; Craven et al., 2017; Dobson et al., 2017; Duncan & Whitfeld, 2017).

Understanding the mechanistic processes driving non-native earthworm effects on plant growth, community composition, and subsequent ecosystem processes and productivity, is crucial for predicting and managing effects of non-native earthworm invasions. Earthworm-caused changes in plant growth and productivity, which are often evidenced by changes in aboveground, belowground, or total plant biomass (Milleret et al., 2009; Jana et al., 2010; Cameron et al., 2014; Coulis et al., 2014; Van Groenigen et al., 2014), can be driven by biological, chemical, and physical mechanisms. Such mechanisms include root abrasion and seed consumption caused by earthworm feeding and burrowing activities, changes in soil nutrients arising from earthworm-caused changes in soil structure, plant production of growth-influencing substances in response to earthworms and other stressors, and changing populations and species composition of soil microbes including bacteria and fungi (Canellas et al., 2002; Brown et al., 2004; Dempsey et al., 2011; Cameron et al., 2014; Paudel et al., 2016). The relative importance of these different mechanisms is likely to vary among different plant species and in relation to the species of invading earthworms and the ecological niches they fill (i.e., epigeic, endogeic, or anecic earthworms, groupings that respectively correspond to surface-dwelling, topsoil-dwelling, and deep-burrowing life histories). Notably, while

important advances have been made to document mechanisms for non-native earthworm impacts on plants, limited research has been conducted in controlled laboratory or greenhouse settings (Hale et al., 2008; Eisenhauer et al., 2010; Clause et al., 2015; Blume-Werry et al., 2020). Such controlled research is needed to allow manipulation of plant and earthworm communities, and thus isolation and causal inferences about the mechanistic factors underlying invasive earthworm effects on ecosystems.

One particularly understudied but potentially important mechanism by which earthworms affect plant growth is through their effects on soil microbial communities, particularly arbuscular mycorrhizal (AM) fungi. AM fungi form symbiotic and often obligate relationships with most terrestrial plant species by increasing nutrient and water acquisition, which expands the range of plant-accessible soil and allows plants to acquire resources needed for survival and growth (Cavagnaro et al., 2015; Bowles et al., 2018). By impacting AM fungi, and by extension the plant-fungal symbiotic relationship, earthworms can have large negative influences on plant growth of AM-dependent species. Overall, impacts on plants can be either positive or negative, arising from earthworm-driven processes like dispersal and consumption of spores and hyphae and changes in overall AM fungal abundances and colonization (Lawrence et al., 2003; Gormsen et al., 2004; Butenschoen et al., 2007; Drouin et al., 2016; Bal et al., 2018). Earthworm activity can also impact the greater microbial community by altering relative abundances of bacteria or bacteria:fungal ratio (Tiunov & Scheu, 1999; Dempsey et al. 2011; Stromberger et al. 2012; Heděnec et al. 2019). Despite the apparent context-dependence of non-native earthworm effects on AM fungi, earthworm-caused changes to AM fungal and microbial communities may be a major factor driving reductions in plant

diversity and increased invasions of non-native plant species, as well as by shifting dominance from highly mycorrhizal-dependent to less dependent plant species (Bohlen et al., 2004; Holdsworth et al., 2007; Paudel et al., 2016). In addition to influencing bacterial and fungal abundances, earthworm-induced increases in mineralization rates and excretion of bacteria-rich casts can also directly affect plant growth (Lachnicht et al. 2002; Jana et al., 2010; Cameron et al. 2014; Huang et al. 2016). However, additional research including controlled experimental studies is needed to improve causal inferences determining the role of microbes, particularly AM fungi, in mediating earthworm effects on plants.

Most studies evaluating effects of non-native earthworms, including effects on plant growth, diversity, and species composition, have been conducted in regions and ecosystems that were historically earthworm-free, such as previously glaciated northern forests (Bohlen et al., 2004; Eisenhauer et al., 2019; Frelich et al., 2019). However, non-native earthworms can also significantly impact ecosystems where natives are present (Winsome et al., 2006; Szlavecz et al., 2011; Snyder et al., 2013; Xu et al., 2013; Clause et al., 2015). Changes to ecosystems can occur due to non-native earthworms either decreasing (James and Seastedt, 1986; Winsome et al., 2006; Kim et al., 2017) or increasing (Callaham et al., 2001) plant growth compared to sites with only native earthworms. Therefore, in addition to controlled research evaluating mechanisms underlying invasive earthworm effects on plants, additional research is needed to capture and evaluate effects of non-native earthworms on plants from regions and ecosystems where native earthworms are also present.

To address these research gaps, we conducted a mesocosm experiment in which we manipulated earthworm community composition to evaluate combined and separate effects of non-native and native earthworms on growth of *Andropogon gerardii* (big bluestem), a dominant and ecologically important species in tallgrass prairies of North America, where both native and non-native earthworms are present. Specifically, among four earthworm treatments (native only, non-native only, combined native and non-native, and no-earthworm control), we compared: 1) relative abundance of soil microbial functional groups, including AM fungi, and 2) plant growth, including above- and belowground biomass, and root architecture (proportions of fine and coarse root growth). We hypothesized that both non-native and native earthworms would independently reduce AM fungal abundance and decrease *A. gerardii* biomass, including fine roots, compared to the no-earthworm control, and these disruptions would be greater in the presence of non-native, compared to native earthworms, and the presence of both native and non-native earthworms would have intermediate effects between those of only native or only non-native earthworms.

METHODS

Earthworm and Plant Species Selection

To replicate tallgrass prairies in the southern Great Plains region as closely as possible, our mesocosms were established with earthworm taxa that occur in tallgrass prairies of this region, as well as much of North America. These taxa included the native genus *Diplocardia* and the non-native species *Aporrectodea trapezoides*. *Diplocardia* is one of two earthworm tribes/genera native to glacier-free portions of North America

(James, 1991). All members of this genus are endogeic, meaning they live and feed in mineral soil layers, ingesting mineral soil within a network of non-permanent and horizontal burrows and digesting soil organic material and microorganisms (Edwards & Bohlen, 1996). In Oklahoma, there have been 15 *Diplocardia* species reported (Reynolds and Damoff, 2010), of which *D. kansensis*, *D. longiseta*, and *D. singularis/caroliniana* have been reported to occur at the Oklahoma State University Range Research Station (OSURRS) (Malyutina, Ch1; Loss et al. 2017), which was the source of soil used in our mesocosms. *Aporrectodea trapezoides* is in the Lumbricidae family; similar to *Diplocardia* spp., it is endogeic, and although it is of Palearctic origin, this species can be found outside of its native range worldwide, likely the result of its widespread introduction and transport through use in agriculture and as fishing bait (Fernandez et al. 2010). *A. trapezoides* has been reported in most U.S. states (Reynolds and Wetzel, 2012), and is one of the most widespread non-native species in Oklahoma (Reynolds and Damoff, 2010), including at OSURRS (Malyutina, Ch1; Loss et al. 2017). *Andropogon gerardii* is a dominant C_4 grass species found in Oklahoma tallgrass prairies. *A. gerardii* is a mycorrhizal-obligate grass (Wilson and Hartnett, 1998), therefore, changes to AM fungal abundance is expected to impact plant growth.

Experimental Design

To assess native and non-native earthworm effects on plant growth and soil microbial communities, including AM fungi, we conducted a growth-chamber experiment using a fully randomized block design in which all mesocosms contained one *A. gerardii* plant and received one of four earthworm treatments (15 replicates per treatment; 60 total replicates). These treatments included: native earthworms only, non-

native earthworms only, both native and non-native earthworms, and a no-earthworm control (hereafter, we refer to these treatments as native, non-native, combined, and control). Each non-control treatment included two total earthworms regardless of the taxa present (i.e., 2 *Diplocardia spp.* in the native treatment, 2 *A. trapezoides* in the non-native treatment, and 1 of each species in the combined treatment). These abundances were selected to reflect real-world densities of earthworms per unit volume of soil, including observed relative densities when both earthworm taxa are present together, based on our previous field sampling at OSURRS (Loss et al., 2017; Malyutina, Ch1).

Mesocosms were plastic pots (20 cm in diameter and 20 cm in height). For all pots, we sprayed inside surfaces with polyurethane and coated them with coarse sand to prevent preferential earthworm movement along the inner sides. We drilled holes in the bottom of pots and covered them with mesh to allow water drainage, prevent waterlogging of the soil, and prevent earthworms from escaping. Mesocosms were filled with fresh soil with a silty clay loam texture that was collected from OSURRS, and which was mixed with fine sand in a 4:1 soil-to-sand ratio. To preserve microbial communities, the soil was not treated.

A. gerardii seeds were germinated in vermiculite in a greenhouse and allowed to grow for three weeks (second-leaf stage). Three seedlings were then transplanted into each mesocosm and allowed to establish for one week, after which extras were removed, leaving one plant per mesocosm. Including extra plants prevented loss of replicates that might otherwise occur due to plant stress and death associated with transplantation.

Earthworms were also collected from field sites at OSURRS and identified under a dissecting microscope as either native *Diplocardia* spp. or *Aporrectodea trapezoides* using published keys (James, 1990; Schwert, 1990) and with guidance and feedback on identifications provided by an earthworm taxonomic expert (S. James). Prior to adding earthworms to the respective pots, we measured earthworm fresh biomass on an electronic balance, after which we randomly assigned earthworms to mesocosms on May 22-23, 2020. Mesocosms were maintained in a growth-chamber with controlled light and temperature regimes. The light regime was 16 hours of light starting at 0600 hr each day; the temperature regime was 20°C between 0600 and 2200 hr each day and 18°C during all other periods. The light regime represented mid-summer light at mid-to-high latitude locations, such as the North American Great Plains region, and the temperature regime reflected optimum earthworm environmental conditions allowing for maximum activity and survival (Lowe & Butte, 2005). Mesocosms were watered two times a week, and every two weeks we added 1645 mg of Nitrogen (N) to each mesocosm. At this application rate the plant is experiencing an N supply rate at levels typically encountered in tallgrass prairie ecosystems over a growing season and includes N mineralization as well as N from dry deposition sources (Seastedt et al., 1991). Mesocosms were rearranged every four weeks to minimize potential effects caused by localized conditions in the growth-chamber.

Sample Collection

Mesocosms were harvested after 16 weeks of exposure to earthworm treatments. Aboveground biomass of *A. gerardi* plants was clipped, with reproductive parts counted and collected separately. Roots were also collected and washed free of all soil. The

remaining soil in the mesocosm was sifted for earthworms, after which the soil was mixed and a subsample was collected for microbial analyses.

We weighed each recovered earthworm on an electronic top-loading balance to determine fresh weights, after which earthworms were euthanized in 70% isopropyl alcohol and preserved in 10% buffered formalin. Across treatments, 68 of 90 earthworms (76%) were recovered, with the lowest earthworm survival occurring in the non-native treatment (47%). To ensure that each replicate used in analysis included earthworms throughout the course of the experiment, we only retained replicates from which we recovered at least one earthworm. Due to unequal numbers of successful replicates across treatments, including only 8 successful replicates for the non-native treatment, we used only 8 randomly chosen replicates from each of the other treatments (32 total replicates), with preference given to replicates with both earthworms recovered.

Above- and Belowground Plant Assessments

We collected two subsamples from each root, including a small portion of fine roots used for measurement of intra-radical AM fungal colonization (IRC), a key representation of the plant-AM fungal symbiotic relationship, and three representative primary roots were collected for analysis of root architecture. Root subsamples collected for IRC were soaked in 10% KOH for 1 hr at 90°C, after which they were acidified with dilute 1% HCL for a few minutes and then stained by soaking the roots in 0.05% Trypan Blue and Lacto-Glycerol at room temperature for 24 hrs. The excess stain was then drained and roots were kept in 30% lactic acid until they were analyzed for AM fungal root colonization. Roots were scored for AM colonization using the magnified gridline

intersect method (McGonigle et al., 1990) using a digital microscope (HiroxKH 7700). Total percent colonization was determined by combining the percent root length colonized by hyphae, vesicles, coils, and arbuscules.

The root subsamples prepared for root architecture analysis were detangled and dyed with Basic Fuchsin diluted with water. Once dyed, the roots were scanned (Epson Perfection V800 Photo) and analyzed using WinRHIZO software (WinRHIZO, V.2016, Regent Instruments, Canada) to estimate average root diameter (mm), total root length, and root length for the following diameter (mm) classes: 0-0.1, 0.1-0.2, 0.2-0.3, 0.3-0.4, 0.4-0.5, 0.5-0.6, 0.6-0.7, 0.7-0.8, 0.8-0.9, 0.9-1.0, 1.0-2.0, and >2.0. To further assess relative changes in roots, fine:coarse root ratio was calculated by dividing root length of diameter classes 0-1.0 by root length of diameter classes >1.0 (Klopf & Baer, 2011).

Both above- and belowground samples and subsamples were oven-dried to constant mass for 48 h at 60°C, after which final dry biomass weights were recorded. Root subsamples were weighed separately with the weights combined for total belowground biomass. Root subsample biomass was used to calculate specific root length (SRL) by dividing each subsample's root length by dry biomass.

Measurement of Microbial and AM Fungal Abundance

For each treatment, 7 of the 8 replicates were randomly selected for analysis of relative abundances of soil microbial functional groups, including AM fungi, and total microbial biomass, as alterations in soil microbial communities can indicate alterations in nutrient availability and soil organic carbon (You et al. 2014; Ma et al. 2018). Microbial functional groups included gram positive and gram negative bacteria, saprophytic and

AM fungi, and were assessed using phospholipid fatty acid (PLFA) and neutral lipid fatty acid (NLFA) (Olsson et al. 1995). PLFAs are used to analyze microbial community composition and biomass while NLFAs are used to measure the biomass of fungal energy storage units and have been used in studies measuring AM fungal density (Frostegård et al., 2011; Ngosong et al., 2012; Sharma and Buyer, 2015; Chang et al., 2018; Blume-Werry et al., 2020). Soil subsamples were freeze-dried and finely ground, and 5 g of each subsample were mixed with a phosphate buffer, methanol, and chloroform. The soil-solvent mixture was separated with a centrifuge and afterwards decanted with a mix of chloroform and methanol (1:2). We added a phosphate buffer to the solution and left it overnight for phase separation. The chloroform layer containing lipids was then recovered and reduced by nitrogen flow at 50°C. Lipids were separated into neutral lipids, glycolipids, and phospholipids by solid-phase extraction, which was done by eluting with chloroform, acetone, and methanol, respectively. Lipids were hydrolyzed and methylated, and the methylated fatty acids were extracted with hexane and evaporated under nitrogen at 37°C. The PLFA/NLFA analyses were performed using an Agilent 7890A gas chromatograph with an Agilent 5975C series mass selective detector. The biomarkers i-15:0, a- 15:0, i-17:0, and i-16:0 were used to select for the functional group of gram-positive bacteria (GPB). For gram-negative bacteria (GNB), selected biomarkers were 16:1ω7, cy19:0, cy17:0ω9, 2-OH 14:0, 2-OH 16:0, 3-OH 14:0, and 18:1ω9 trans. For AM fungal extra-radical hyphae (ERH) and energy storage units, biomarkers consisted of 16:1ω5c, 20:1ω9, and 22:1ω13, and for saprophytic (SAP) fungi, the selected biomarkers were 18:2ω9,12 and 18:1ω9c. Using the abundances for each biomarker, we were able to calculate the total nmol per gram of soil for each functional

group, which were all combined with non-specific markers consisting of 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0 biomarkers to determine the total microbial biomass. Total bacterial biomass was calculated by combining GPB and GNB functional groups (PLFA only), and total fungal hyphae (PLFA) and storage unit biomass (NLFA) were calculated by combining AM and SAP fungal functional groups.

Statistical Analysis

We conducted preliminary analyses using Shapiro-Wilk (shapiro.test function) and Levene's tests (leveneTest function) to determine if data met assumptions of normality and homogeneity of variance. Parametric one-way ANOVAs (aov function) were used to test treatment effects on below- and aboveground plant biomass, IRC, root diameter class lengths (except 0-0.1 and >2mm) and percent (except 0.5-0.6, 0.6-0.7, 0.9-1, >2mm), fine to coarse root ratio, and PLFA variables, because data satisfied assumptions of normality and homogeneity of variance for all of these variables. For analyses with a significant overall ANOVA result ($p<0.05$), we conducted multiple comparisons of group means using the Tukey post-hoc test (glht function). We used non-parametric Kruskal-Wallis tests (kruskal.test function) to assess treatment effects on fine:coarse root ratio, average root diameter, SRL, and NLFA variables, because data did not satisfy normality assumptions for these variables. For analyses with a significant Kruskal-Wallis test result, we used post-hoc Dunn's tests (dunnTest function) to evaluate pairwise differences among treatments. For all above pairwise comparisons of group means, we used a Holmes sequential Bonferroni correction when assessing statistical significance to account for the large number of comparisons made and to reduce the probability of Type I error.

As described above, we held earthworm abundance and density constant among the various treatments and replicates; however, due to some variation in earthworm biomass between and within the three treatments with earthworms, we assessed whether initial earthworm biomass had effects independent of treatment effects. This was conducted using linear regressions (lm function) with a Gaussian distribution for the above variables that met normality and homogeneity of variance assumptions, and using generalized linear models regressions (glm function) with a Gamma distribution for variables that did not meet these assumptions.

All analyses were conducted in R version 3.6.1 (R Core Team 2013) using the following packages: 'lme4' (Bates et al., 2015) for GLMs and LMs, 'car' (Fox & Weisberg, 2018) for leveneTest, 'FSA' (Ogle et al., 2021) for dunnTest, and 'multcomp' (Hothorn et al. 2008) for glht.

RESULTS

Native earthworms were characteristically lower in biomass (0.48g) compared to non-native earthworms (1.03g), presumably due to inherent species differences. Therefore, differences in earthworm biomass, rather than aspects related to species feeding and burrowing habits, may drive changes to plant growth or microbial communities. However, neither initial nor final earthworm biomass, irrespective of species, significantly influenced plant or microbial variables. Of the 16 earthworms added to each mesocosm, the treatment with non-native earthworms only had 13 recovered earthworms, while all 16 earthworms were recovered from mesocosms

established with native earthworms, or mesocosms with both non-native and native earthworms.

Neither above- (Figure 5a; $p = 0.18$) nor belowground (Figure 5b; $p = 0.17$) biomass differed in response to earthworm treatments. Similarly, specific root length (Figure 5c; $p = 0.149$) or shoot to root ratio (Figure 5d; $p = 0.438$) did not significantly differ between earthworm treatments. Earthworm treatment did not affect sexual reproduction of *A. gerardii* ($p = 0.189$).

Root architecture of *A. gerardii* was assessed at the conclusion of the study. *A. gerardii* was generally characterized by two hierarchical levels of roots, with the majority of fine roots 0.1 – 0.2 mm in diameter, and course roots 1-2 mm in diameter. While the presence of earthworms did not affect overall specific root length, average root diameter was marginally affected by presence of native earthworms ($p = 0.053$). Roots in mesocosms containing only native earthworms were larger in diameter, or more coarse, compared to roots in mesocosms containing both native and non-native earthworms. Roots from mesocosms with only non-native earthworms or with no earthworms were intermediate in diameter (Figure 6). Presumably due to the greater relative abundance of fine roots (relatively fewer course roots) in mesocosms containing both native and non-native earthworms, these roots were characterized by a greater fine:coarse root ratio, compared to roots associated with native earthworms only (Figure 7; $p = 0.043$).

Although the presence of earthworms did not affect overall root length, there were earthworm effects on the proportion of root length of each diameter class. Plant roots in mesocosms containing only native earthworms had significantly lower percentages of

fine roots (0 – 0.1 mm: $p = 0.018$) and higher percentages of course roots (0.2 – 0.3 mm: $p = 0.028$; and 0.5 – 0.6 mm: $p = 0.085$), compared to roots associated with both native and non-native earthworms. While not statistically significant ($p < 0.05$), mesocosms containing native only earthworms tended to have lower percentages of 0.1 – 0.2 mm ($p = 0.080$) diameter roots and higher percentages of 1 – 2 mm ($p = 0.087$) diameter roots than mesocosms with both native and non-native earthworms. Relative proportion of all root diameter classes were similar for control and non-native only earthworm treatments (Table 3).

The presence of earthworms did not affect relative abundance of soil microbial communities (determined by PLFA analyses): saprophytic fungal hyphae (p-value = 0.157) or spore abundance (p-value = 0.630), total fungal biomass (p-value = 0.217), or bacterial biomass (p-value = 0.655). Similarly, earthworm presence did not affect AM fungal root colonization (p-value = 0.692), extraradical AM fungal hyphae (determined by PLFA: p-value = 0.236), or AM fungal spore abundance (determined by NLFA: p-value = 0.306).

DISCUSSION

Earthworms Effects on Root Architecture

Despite finding no apparent effect of native and non-native earthworms, either separately or in tandem, on microbial abundances, including AM fungi, or on several plant growth-related variables, our controlled experimental study illustrated earthworm effects on root architecture. Specifically, native and non-native earthworms differentially

affected root architecture of *A. gerardii*, as roots of plants grown in mesocosms with only native earthworms had proportionally fewer fine roots (lower fine:coarse root ratio) than plants in the combined native and non-native earthworm treatment. Root architecture was similar among control and non-native earthworm treatments and was intermediate between native and combined treatments. These effects were driven by alterations in fine (< 1 mm) rather than coarse (> 1 mm) root abundance, specifically 0-0.1 and 0.2-0.3 root diameter classes. Our results related to earthworm-mediated changes to root architecture are consistent with previous studies indicating earthworms affect root diameter, as well as proportional abundance of fine vs coarse roots (Jana et al., 2010; Agapit & Blouin, 2018; Agapit et al., 2018; Nahberger et al., 2021).

Soil microbial communities were not directly or indirectly influenced by the presence of earthworms, therefore, alterations in root architecture may have occurred due to a variety of other factors. For example, these patterns may have been the result of differential earthworm feeding or damage to roots, possibly due to differences in body size, or due to earthworm activity indirectly affecting *A. gerardii*. Additionally, previous research has reported that earthworm activities, such as feeding, excretion, secretion of mucus, and burrowing, increase soil nutrient availability, especially of immobile nutrients such as P, N, and K (Callaham & Hendrix, 1998; Welke & Parkinson, 2003; Domı´nguez et al., 2004; Fisk et al., 2004; Blume-Werry et al., 2020). It is also known that root architecture of warm-season grasses, such as *A. gerardii*, may be plastic and adjust in response to alterations in nutrient availability (Miller et al. 2012). Different root architectures have varying nutrient acquisition efficiencies and capabilities. Fine roots are more efficient at nutrient uptake and allow the plant to access a greater amount of

available resources in the rhizosphere (Hetrick, 1991) while larger diameter roots increase the plant's access to total soil volume by lateral and vertical spread of their root system (Eissenstat, 1992). Increases in plant-available nitrogen (nitrate) can increase production of plant-growth hormones, inhibiting lateral root elongation and transport of shoot-derived auxin to roots (Zhang et al., 1999; Jana et al., 2010). Therefore, in addition to potential differences in the sizes of roots consumed by native and non-native species, earthworms may be indirectly influencing root architecture by increasing nitrate availability. In our study, native and non-native earthworms may affect nitrate similarly when independent of each-other, but differently when interacting with each-other, as earthworm effects on nitrate availability is species specific, and can change with cross-species interactions (Postma-Blaauw et al., 2006). Interactions between native and non-native earthworms have been observed in Puerto Rico, as mineralization by non-native earthworms was partially mitigated when native earthworms were also present (Lachnict et al. 2002). However, it should be noted that in the Lachnict et al. (2002) study, native earthworms were anecic (occupying permanent burrows as deep as 3 m below soil surface), while non-native earthworms were endogeic, indicating the non-native earthworms occupied a niche unique from the native species.

Plant-growth hormones exuded from earthworm casts have also been proposed as a mechanism by which earthworms are driving alterations in root architecture. Humic substances isolated from earthworm casts exhibit hormone and auxin-like activity that induce lateral root emergence and increase fine root proportions (Muscolo et al., 1999; Canellas et al., 2002; Quaggiotti et al., 2004; Agapit et al., 2018). The emission of plant growth-regulating substances by earthworms is also species-specific and is not a

characteristic shared by all earthworms (Tomati et al., 1988). In our study, native and non-native earthworms may have emitted different levels of growth-promoting hormones, resulting in greater increased fine root development when plants were associated with both native and non-native earthworm species.

Earthworms Effects on Plant Productivity and Microbial Communities

Although root architecture varied across earthworm treatments, our study did not directly discern mechanisms driving increases in coarse roots in plants grown with only native earthworms, and/or decreased root coarseness in the presence of both native and non-native earthworms. Previous research suggests alterations in microbial communities, in particular AM fungi, are likely the mechanism for alterations in plant growth following introduction of non-native earthworms (Drouin et al., 2016; Paudel et al., 2016; Chang et al., 2017; Dobson et al., 2019). However, in our study, presence of either native or non-native earthworms had no significant effects on soil microbial communities, as relative abundance of major microbial functional groups (gram negative or gram positive bacteria, saprophytic fungi, total microbial biomass) were not significantly different between earthworm treatments, and extra-radical AM fungal abundances and AM fungal root colonization were similar across treatments. Previous studies have also reported that non-native earthworms reduced plant biomass production compared to native earthworms (James & Seastedt, 1986; Callaham & Blair, 2001). Therefore, we initially hypothesized native and non-native earthworms would differentially affect plant production and soil microbial communities. However, earthworms did not affect *A. gerardii* above- or belowground biomass production, sexual reproduction, or specific root length. Therefore, our data do not support our initial hypothesis that non-native earthworms would have a

greater impact on plant production and soil microbial communities. In fact, there were no differences in any of the plant response variables, including root architecture, between the native or non-native earthworm treatments and the no-earthworm control treatment.

Because we sought to capture real-world earthworm abundances and densities observed in a complimentary field study conducted in the same tallgrass prairie site (Malyutina, Ch1) as soil and earthworms were collected for this mesocosm study, our overall earthworm populations in each mesocosm were relatively low. This ecosystem may be in the early stages of invasion (lag phase), and rapid increases in non-native earthworms may occur (log phase), as native earthworms were present in the majority of these grassland sites, and no areas contained only non-native species (Malyutina, Ch1). Earthworm abundances at this stage of invasion might not be sufficiently high enough for significant ecosystem effects and could be a reason for the lack of significance on plant growth and microbial abundances. However, a rapid increase in earthworm abundance as non-native invasion progresses into a log phase may result in substantial increases in deleterious effects on plant and soil microbial communities.

Conclusions

Our study did not observe significant alterations in plant biomass or reproduction or soil microbial communities, including AM fungi, by native and non-native earthworms. However, our study represents current earthworm abundances in Oklahoma grasslands, which are subject to change as non-native earthworm invasion persists. Our study did find root architecture is impacted by native and combined native and non-native earthworms, suggesting earthworms may influence variables beyond the scope of our

study. For example, potential alterations in plant-available nutrients, such as nitrate, should be assessed following introduction of non-native earthworms, as non-native plant species tend to have increased abilities for nutrient uptake in these grassland ecosystems. Additional studies might also examine casts of native and non-native earthworm species for presence of growth promoting hormones to determine if plants respond directly to earthworm activity. While more research is needed on the specific mechanisms by which earthworms affected plants, our study presents evidence for differences between native and non-native earthworms that, as earthworm abundances increase, could have detrimental ecosystem implications for these highly endangered grasslands.

TABLES

Table 3. Relative total root length (%) of each diameter (mm) class for earthworm treatments (native earthworms, non-native, both native and non-native, or no earthworms (control)). Values in parentheses represent standard error of the mean (n = 8). Within each column, means followed by the same letter indicate no significant difference ($p < 0.05$), bold values indicate a p-value of less than 0.1.

Root Length (%)	Treatment			
Root Diameter (mm) Class	Native	Non-Native	Native + Non-Native	Control
0-0.1	**10.6 (1.06)^{a}**	13.8 (1.15)^{ab}	**16.4 (1.49)^{b}**	15.0 (0.98)^{ab}
0.1-0.2	**41.4 (2.03)^{a}**	44.4 (1.56)^{a}	**48.8 (2.07)^{a}**	45.4 (1.72)^{a}
0.2-0.3	**13.5 (0.36)^{b}**	12.2 (0.54)^{ab}	**11.3 (0.48)^{a}**	12.1 (0.52)^{ab}
0.3-0.4	6.88 (0.34)^{a}	6.55 (0.68)^{a}	5.07 (0.46)^{a}	6.72 (0.34)^{a}
0.4-0.5	4.15 (0.30)^{a}	4.26 (0.58)^{a}	3.23 (0.36)^{a}	3.91 (0.37)^{a}
0.5-0.6	**3.23 (0.37)^{a}**	2.96 (0.40)^{a}	**2.13 (0.27)^{a}**	2.42 (0.31)^{a}
0.6-0.7	2.54 (0.36)^{a}	1.97 (0.19)^{a}	1.60 (0.21)^{a}	1.90 (0.33)^{a}
0.7-0.8	2.09 (0.23)^{a}	1.68 (0.13)^{a}	1.38 (0.23)^{a}	1.48 (0.22)^{a}
0.8-0.9	1.92 (0.28)^{a}	1.61 (0.14)^{a}	1.26 (0.23)^{a}	1.37 (0.16)^{a}
0.9-1	1.56 (0.23)^{a}	1.22 (0.13)^{a}	1.07 (0.18)^{a}	1.13 (0.15)^{a}
1-2	**8.12 (0.84)^{a}**	6.63 (0.62)^{a}	**5.57 (0.53)^{a}**	6.17 (0.56)^{a}
>2	3.97 (0.80)^{a}	2.81 (0.50)^{a}	2.15 (0.36)^{a}	2.41 (0.38)^{a}

FIGURES

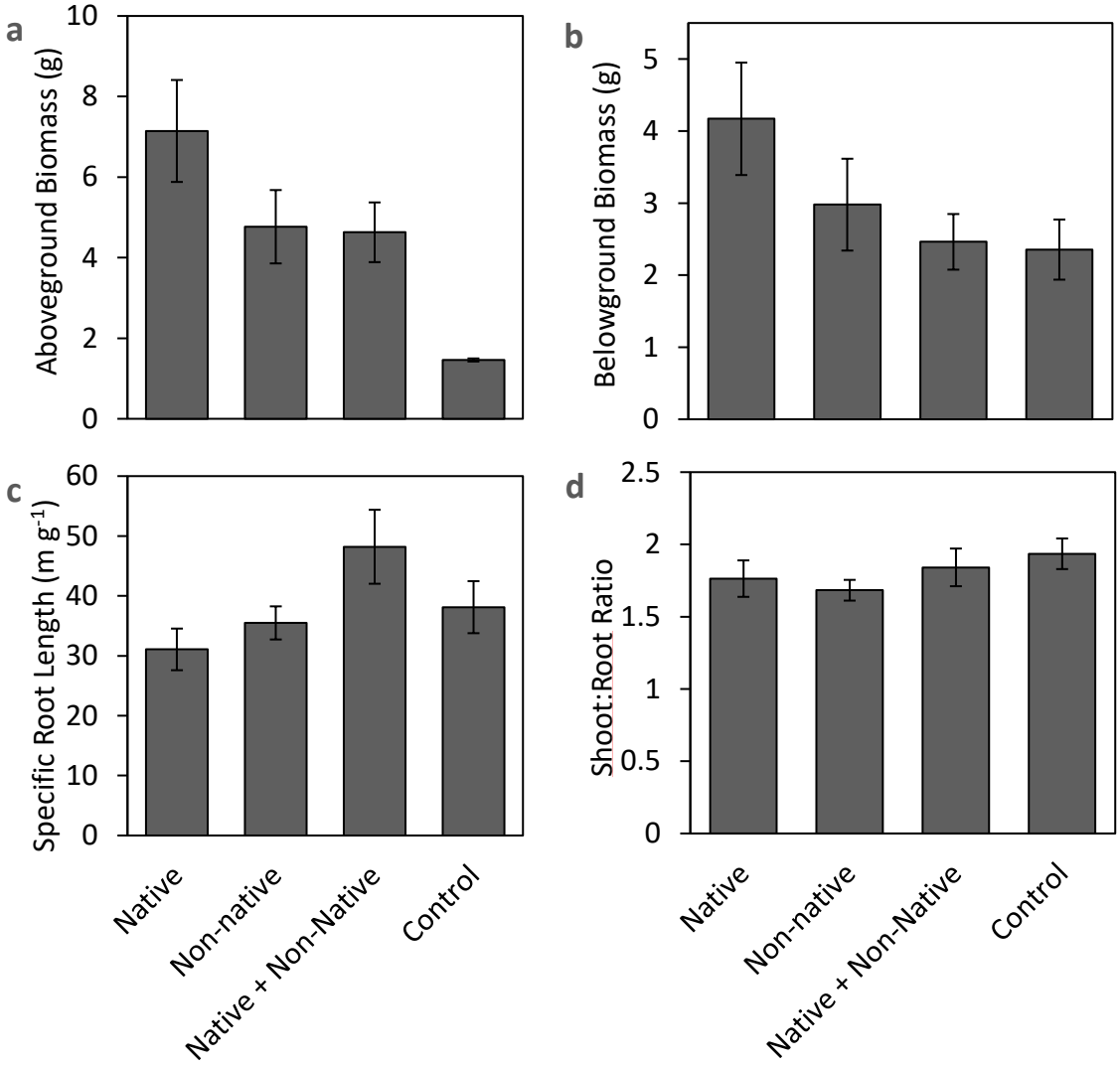


Figure 5. Aboveground (a) and belowground (b) plant biomass (g), specific root length (m g^{-1}) (c), and shoot to root ratio (d) as influenced by native and non-native earthworm treatments (native, non-native, both native and non-native, or no earthworms (control)). No significant differences were observed.

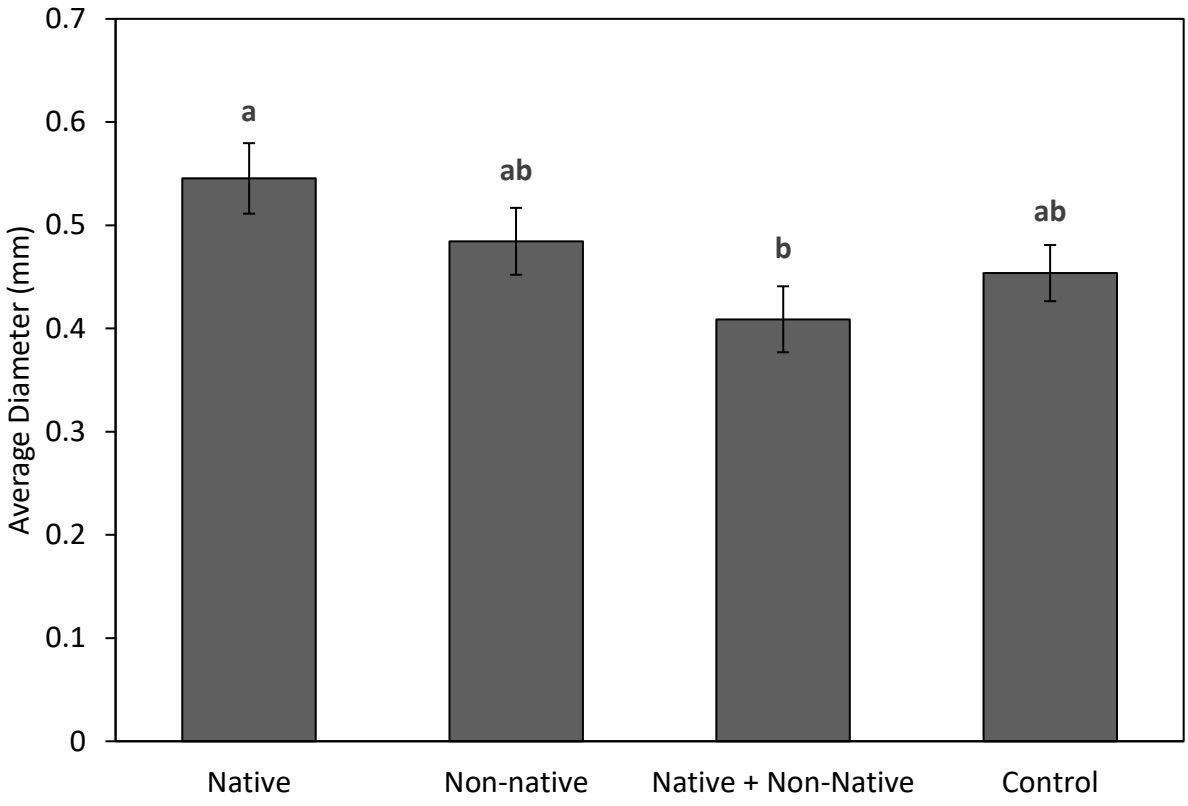


Figure 6. Average diameters (mm) of *A. gerardii* roots in mesocosms with only native earthworms, only non-native, both native and non-native, or no earthworms (control) following 16 weeks of earthworm inoculation.

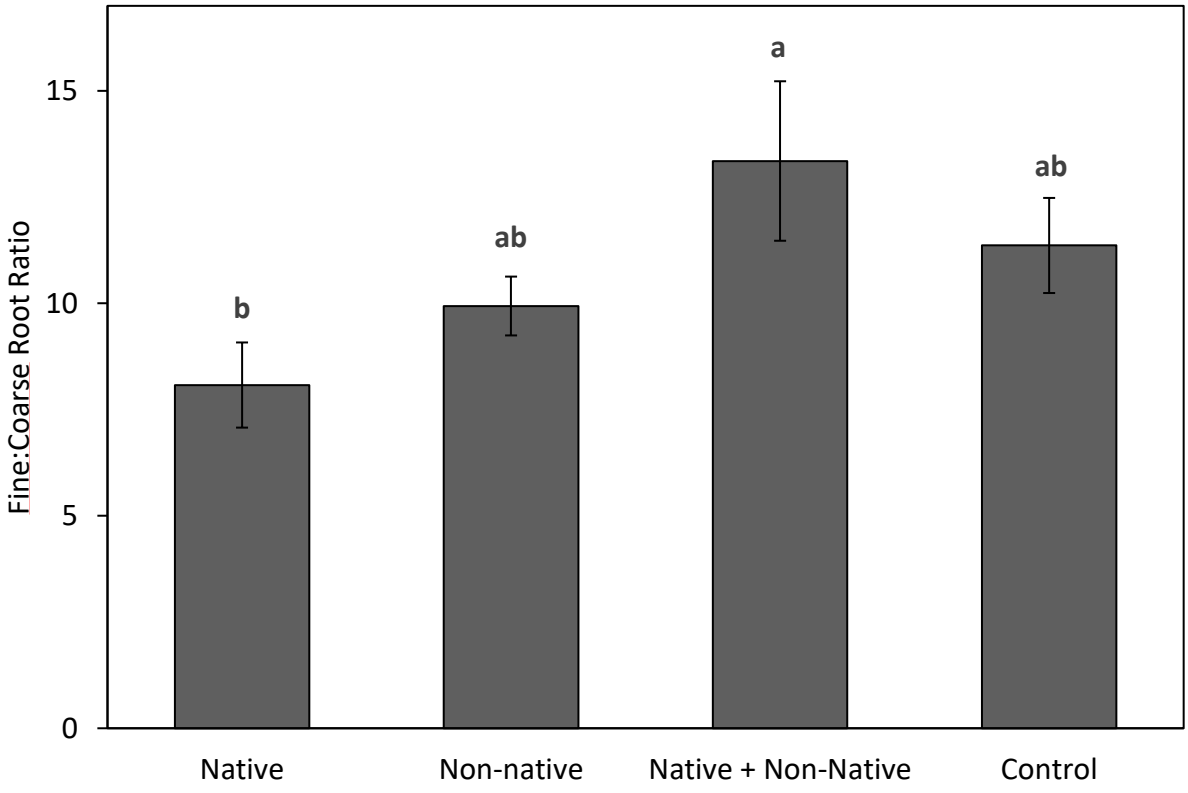


Figure 7. Fine:coarse root ratio of *Andropogon gerardii* grown in mesocosms for 16 weeks with native, non-native, both native and non-native, or no earthworms (control). Fine roots <1 mm diameter and coarse roots >1 mm diameter.

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VITA

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