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Non-native earthworm influences on ectomycorrhizal colonization and growth of white spruce¹

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Abstract: Exotic earthworms are entering previously uninhabited soils of boreal forests, their invasion largely facilitated through human activities. As ecosystem engineers, earthworms are capable of causing dramatic changes in above- and belowground forest composition, but whether they have the same effects in all forests remains unclear. Forest compositional changes caused by earthworms may be mediated by interactions between earthworms and mycorrhizal fungi. Specifically, tree seedling growth may be altered by the presence of exotic earthworms and their subsequent impact on mycorrhizal fungi. In this study, we investigate the effects of exotic earthworms on ectomycorrhizal colonization and seedling growth of the conifer *Picea glauca* (white spruce) in gray luvisolic soils from the Boreal Plains. Anecic *Lumbricus terrestris* and epigeic *Dendrobaena octaedra* earthworms were added to mesocosms each containing a white spruce seedling in a greenhouse experiment. Impacts on the composition of ectomycorrhizal fungi in the mesocosms were determined using a combination of morphological and molecular techniques, and effects on seedling growth were assessed through above- and belowground measurements. The proportion of ectomycorrhizal root tips and ectomycorrhizal community composition did not vary as a function of earthworm species or density. Similarly, exotic earthworms had no significant effect on spruce seedling growth or survival.

Keywords: boreal forest, earthworms, ectomycorrhizal fungi, invasion, white spruce.

Résumé: En forêt boréale, des vers de terre exotiques envahissent des sols précédemment inoccupés et leur invasion est facilitée en grande partie par les activités humaines. Les vers de terre sont des ingénieurs d'écosystèmes, ils peuvent causer des changements majeurs dans la composition de la forêt et du sol, mais il n'est pas clair s'ils ont les mêmes effets dans toutes les forêts. Les changements dans la composition forestière causés par les vers de terre peuvent se produire par l'intermédiaire d'interactions entre ceux-ci et des champignons mycorrhiziens. Spécifiquement, la croissance des semis d'arbres peut être altérée par la présence de vers de terre exotiques ainsi que par leur impact sur les champignons mycorrhiziens. Dans cette étude, nous examinons les effets de vers de terre exotiques sur la colonisation ectomycorrhizienne et la croissance de semis du conifère *Picea glauca* (épinette blanche) dans des sols luvisoliques gris des plaines boréales. Dans une expérience en serre, des vers de terre anéciques *Lumbricus terrestris* et des vers épigés *Dendrobaena octaedra* ont été ajoutés à des mésocosmes contenant chacun un semis d'épinette blanche. Les impacts sur la composition des champignons ectomycorrhiziens dans les mésocosmes ont été déterminés en utilisant une combinaison de techniques morphologiques et moléculaires. Les effets sur la croissance des semis ont été évalués par des mesures prises en surface et dans le sol. La proportion des apex racinaires ectomycorrhizés et la composition de la communauté ectomycorrhizienne ne variaient pas en fonction de l'espèce de vers de terre ou de leur densité. De même, les vers de terre exotiques n'avaient aucun effet significatif sur la croissance des semis d'épinette ou leur survie.

Mots-clés: champignons ectomycorrhiziens, épinette blanche, forêt boréale, invasion, vers de terre.

Nomenclature: Reynolds, 1977; Flora of North America Editorial Committee, 1993.

Introduction

Exotic earthworms are currently spreading, largely as a result of human activities, in forests in the northern United States and Canada (Gundale, Jolly & DeLuca, 2005; Cameron, Bayne & Clapperton, 2007), where native earthworm species are hypothesized to have been extirpated during the Pleistocene glacial period (Gates, 1982). Exotic earthworms can cause dramatic changes to above- and belowground forest composition due to their effects on leaf litter and mixing of soil layers, but there is limited understanding of their impacts in many forest types

(Bohlen *et al.*, 2004; Frelich *et al.*, 2006; Eisenhauer *et al.*, 2007; Addison, 2009). In heavily invaded areas, tree seedling numbers have significantly decreased, while diverse understory herb communities have shifted to simpler communities dominated by only a few species (Frelich *et al.*, 2006; Nuzzo, Maerz & Blossey, 2009). Mycorrhizal-dependent species tend also to decline following earthworm invasion, leaving these simplified communities dominated by non-mycorrhizal plants (Gundale, 2002; Frelich *et al.*, 2006; Nuzzo, Maerz & Blossey, 2009). Consequently, a change in the mycorrhizal community is one of the mechanisms suggested to be driving the shifts in plant communities that occur when earthworms invade (Frelich *et al.*, 2006).

Mycorrhizas occur in the vast majority of plant families and play important roles in the provision of nutrients for

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trees, and the balance of carbon in trees (Lilleskov *et al.*, 2002; Lambers *et al.*, 2008; McLean, Migge-Kleian & Parkinson, 2006). The limited amount of research available on the interactions between exotic earthworms and mycorrhizal fungi focuses almost exclusively on arbuscular mycorrhizas (Lawrence *et al.*, 2003; McLean, Migge-Kleian & Parkinson, 2006). This research indicates that the presence of exotic earthworms decreases colonization rates, probably as a result of physical disruption of fungal mycelium by the earthworms (Lawrence *et al.*, 2003; McLean, Migge-Kleian & Parkinson, 2006). Unlike arbuscular mycorrhizal fungi, ectomycorrhizal fungi form a sheath around root tips and have a complex extraradical mycelium system (Smith & Read, 2008). Consequently, the effects of exotic earthworms may differ considerably between these 2 groups. Ectomycorrhizal fungi also mainly colonize the root systems of conifers, as compared to arbuscular mycorrhizas, which are common in most plant families (Smith & Read, 2008; Wagg *et al.*, 2008). Despite the importance of ectomycorrhizas in some ecosystems due to their colonization of the dominant tree species (Smith & Read, 2008), the response of ectomycorrhizal communities to exotic earthworms is effectively unknown (Baxter *et al.*, 1999; McLean, Migge-Kleian & Parkinson, 2006; Addison, 2009).

Because ectomycorrhizal colonization and fungal species composition are important determinants of tree seedling growth and survival in some species (Miller *et al.*, 1998; Purdy, Macdonald & Dale, 2002; Nuñez, Horton & Simberloff, 2009), any effects of earthworms on ectomycorrhizas may impact seedling performance. A clear understanding of the impact of exotic earthworms on native tree seedlings is necessary, as changes in seedling success may have long-term implications for the trajectory of ecological succession (Frelich *et al.*, 2006). However, conflicting results exist regarding the response of tree seedlings to earthworm invasion (Welke & Parkinson, 2003; Frelich *et al.*, 2006; Hale *et al.*, 2008), and nothing is known about their response to exotic earthworms in boreal soils that are dominated by mineral horizons with high clay content.

We examined how exotic earthworms impact ectomycorrhizal colonization, community composition of ectomycorrhizal fungi, and growth of tree seedlings through a short-term greenhouse earthworm addition experiment. We observed both the combined and the individual effects of the exotic earthworms *Lumbricus terrestris* and *Dendrobaena octaedra*, 2 species that are invading many North American forests (Bohlen *et al.*, 2004; Frelich *et al.*, 2006; Addison, 2009), on the growth of *Picea glauca* (white spruce) seedlings and their associated ectomycorrhizal fungi. *Dendrobaena octaedra* is an epigeic species found in the upper organic layers of soil profiles (Bouché, 1977; Bohlen *et al.*, 2004). Epigeic earthworms produce litter fragmentation but are capable of only limited mixing of mineral and organic soil. *Lumbricus terrestris* is categorized as an anecic species, forming vertical burrows up to 2 m deep in the soil, and is responsible for more thorough mixing of organic and mineral soils (Bouché, 1977; Bohlen *et al.*, 2004).

Mycorrhizal and seedling growth responses may depend upon which species of earthworms are introduced,

as the degree to which soil horizons are mixed and altered varies depending on the life history strategy of the species of earthworm invading (McLean, Migge-Kleian & Parkinson, 2006). Ectomycorrhizal colonization levels and diversity are predicted to decrease in the presence of earthworms, with a greater impact expected from the addition of *L. terrestris* relative to *D. octaedra*, based on previous studies of other fungal types (McLean & Parkinson, 2000; Lawrence *et al.*, 2003; McLean, Migge-Kleian & Parkinson, 2006). If earthworm introduction simplifies ectomycorrhizal communities and/or reduces their contact with roots, it is expected that white spruce seedlings will exhibit reduced growth and poor health in the presence of earthworms. However, white spruce seedlings might also benefit from earthworm invasion, particularly by the anecic *L. terrestris*, because substrates with high amounts of available mineral soil and thin organic horizons are favourable for white spruce establishment and growth (Purdy, Macdonald & Dale, 2002). Given the relatively high nutrient demands of white spruce (Neinstadt & Zasada, 1990), the increases in nutrient availability often observed during the early stages of earthworm invasion (Bohlen *et al.*, 2004; Frelich *et al.*, 2006) could also result in a positive effect of earthworms on this species.

Methods

MATERIAL COLLECTION

Gray luvisolic soil was collected from a site free of exotic earthworms in northeastern Alberta (54° 36' N, 110° 59' W) in early May 2009. Organic soil horizons were collected as intact cores using a 29-cm-wide by 20-cm-deep coring device, while mineral soil was manually shovelled from holes remaining after organic core collection. One-year-old white spruce seedlings (mean height 26.96 cm ± 0.56 cm, SE) were purchased from Treetime Services Inc. (<http://treetime.ca/>). These seedlings were grown in earthworm-free peat soil and were not subject to mycorrhizal inoculation, but colonization of fungi from spores present in the greenhouse or our soil cores was possible. *Dendrobaena octaedra* were collected from a single location near the soil collection site, while *Lumbricus terrestris* were purchased from a local bait shop. Only sexually mature earthworms, as verified by the presence of a distinct clitellum, were selected for use in this study.

GREENHOUSE SET-UP

The study was conducted over 1 growing season, from 9 May to 20 October 2009. This amount of time is similar to the average length of experiments (21 weeks, range of 8–104 weeks) used to test the effects of ectomycorrhizal inoculation on seedling growth (Karst *et al.*, 2008), and earthworm impacts on plants are also often observed over similar or shorter time periods (Hale *et al.*, 2008; Belote & Jones, 2009). Our experiment consisted of 5 treatments: 1) no earthworms; 2) 30 *D. octaedra*; 3) 2 *L. terrestris*; 4) 30 *D. octaedra* combined with 2 *L. terrestris*; and 5) 3 *L. terrestris*. These densities were chosen based on field densities commonly found in northern forests (Cameron, Bayne & Clapperton, 2007; Eisenhauer *et al.*, 2007; Hale *et al.*, 2008). One *L. terrestris* (~5.3 g) is

approximately equivalent in weight to 30 *D. octaedra* (~0.15 g each), and therefore treatments 4 and 5 contained similar biomasses. The inclusion of treatment 5 in the experiment allowed us to assess whether any effects observed in treatment 4 (both species) *versus* the single species treatments were related only to its higher total biomass or to interactions between species (*i.e.*, synergistic or competitive responses between the 2 species).

Twenty replicate mesocosms were set up for each treatment in plastic pails (30 cm diameter by 38 cm tall) (Hale *et al.*, 2008; Belote & Jones, 2009). Replicates contained a 3 cm layer of surface leaf litter, a 4- to 6-cm intact organic soil core, and a base 18-cm layer of mineral soil. The mineral soil, which was high in clay content, was mixed together until homogenous and sorted by hand to remove rocks, large roots, and conspicuous macroarthropods. Five evenly spaced holes (7/8" in diameter) were drilled through the bottom of each pail and covered with landscaping fabric to allow water drainage. A 5-cm strip of coarse sand was applied using white glue along the inner rim of each pail to deter the escape of earthworms (Hale *et al.*, 2008). Each pail was randomly placed within the greenhouse and assigned a treatment. A white spruce seedling was planted in the centre of each bucket, with the top of the root plug roughly 1 cm below the organic soil surface.

SEEDLING MAINTENANCE

Naturally occurring forbs and grasses from intact organic soil cores were left to grow within each pail to simulate boreal forest floor conditions. Seedlings were watered 0.5 L every second day (Belote & Jones, 2009). This amount was reduced to approximately one third of a litre every 3 d beginning June 17 and, eventually, one third of a litre every 4 d beginning July 13. Temperature within the greenhouse fluctuated between 14.5 °C and 19.0 °C, with a mean temperature of 15.7 °C. Relative humidity within the greenhouse fluctuated between 32.8% and 88.8%, with a mean relative humidity of 68.6%. No supplemental lighting was used, as our experiment occurred during the growing season.

MORPHOTYPING AND ASSESSING MYCORRHIZAL COLONIZATION

Replicates were processed through destructive sampling between September and October 2009. Forbs and grasses were cut and spruce seedling roots were separated from shoots. The root systems with adhering soil intact were stored at -20 °C until further processing. At that time, roots were thawed in a lukewarm water bath and thoroughly rinsed clean over a 1.4-mm sieve. Washed root systems were cut into approximately 1-cm fragments, which were randomly selected for observation under a dissecting microscope, similar to previously described methods (Welke & Parkinson, 2003). All root tips on each 1-cm root fragment were recorded as mycorrhizal or non-mycorrhizal until 100 tips were counted, excluding dead tips. Only those root tips with a distinct and visible mantle were considered mycorrhizal, and representative mycorrhizal tips were observed under a compound microscope to confirm mantle presence. Ectomycorrhizal colonization was calculated as the number of ectomycorrhizal root tips

divided by the sum of living mycorrhizal and non-mycorrhizal tips. Ectomycorrhizas were morphotyped based on differences in tip colour, texture, thickness, shape, and presence of hyphae (Jones *et al.*, 1997). Following mycorrhizal characterization, root systems were dried for a minimum of 48 h at 60 °C and weighed to obtain belowground biomass measurements.

MOLECULAR CONFIRMATION OF ECTOMYCORRHIZAL MORPHOTYPES

DNA from 3 root tips representing each morphotype was extracted using a Sigma Extract-N-Amp Tissue Kit following the manufacturer's protocol (Sigma, Gillingham, Dorset, UK). Polymerase chain reaction (PCR) amplification was carried out using the fungal-specific primer combination NS11 and NLB4 (Martin & Rygiewicz, 2005). An aliquot of 0.5 µL of extracted DNA was combined with 4 µL of Extract-N-Amp PCR solution in an 8 µL reaction. Amplifications were performed with an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 72 s, with a final extension of 72 °C for 10 min. Successful PCR products were purified using ExoSAP-IT (USB, Cleveland, Ohio, USA). Cycle sequencing was conducted using BIGDYE v3.1 (Applied Biosystems, Foster City, California, USA) with the ITS1F primer, and the resulting products were precipitated following the manufacturer's instructions for EDTA/ethanol. Uni-directional sequences were analysed on an ABI Prism 3730 Genetic Analyzer (Applied Biosystems) and edited with Sequencher (GeneCodes, Ann Arbor, Michigan, USA). DNA sequences were checked against the GenBank sequence database to assign a taxonomic name to each morphotype.

SEEDLING GROWTH MEASUREMENTS

Aboveground spruce seedling measurements, including seedling height, stem diameter, primary branch number, and branch length, were made at the beginning and end of the experiment. Primary branches were defined as those branches growing off of the main shoot and included new branch buds without needles. For branch length, we marked the first branch from the top of the seedling that exceeded 1 cm in length and measured it.

After separation from the roots, the shoots were qualitatively ranked for dead needles and then dried for a minimum of 48 h at 60 °C before being weighed. To assess dead needle profiles, each seedling was separated into 3 distinct regions: the lower half of the seedling, the upper half of the seedling, and new apical stem growth. Division of the seedlings into these regions provided a more precise estimate of dead needle occurrence than simply treating each tree as a whole. Each segment was then visually ranked for the presence of dead needles on the following scale: 0 = no dead needles; 1 = < 1% of needles dead; 2 = 1–50% of needles dead; and 3 = 51–100% needles dead. Finally, we also determined N concentration in the organic horizon, where most of the spruce roots were located, using a CEC Model 440 Elemental Analyzer (Control Equipment Corporation, Cazenovia, New York, USA).

STATISTICAL ANALYSES

To test for significant differences in proportion of root tips colonized by ectomycorrhizal fungi between earthworm treatments, we used a Kruskal–Wallis test because this variable had a non-normal residual distribution according to the Shapiro–Wilks test. Non-metric multidimensional scaling (NMDS) was used to qualitatively determine the effects of each treatment on ectomycorrhizal morphotype diversity and abundance, as this method is compatible with data sets containing multiple zeroes (McCune & Grace, 2002). Replicates were colour-coded by treatment and positioned on 3 axes, which provided a less stressed configuration than 2 axes. Replicates with 0% colonization were excluded from the ordination matrix, as these replicates were unable to be spatially plotted. The multi-response permutation procedure (MRPP), a non-parametric form of MANOVA, was used to assess if morphotype composition differed between earthworm treatments (McCune & Grace, 2002).

To examine differences in seedling growth between earthworm treatments, we performed ANOVAs on above- and belowground measurements of seedling biomass. A Kruskal–Wallis test was used to analyze change in branch length, which had a non-normal residual distribution. Seedling diameter, height, and branch number were tested using ANCOVAs, in order to control for initial size of the seedlings. An ordered logistic regression was applied to dead needle ranks to determine if significant differences were present in seedling needle profiles between treatments. Needle ranks were clustered by replicate ID, and each treatment was compared to the no worms control group. Exact logistic regression was used to test whether the proportion of seedlings dying differed among treatments. Exact logistic regression can produce more reliable results than logistic regression for small sample sizes or unbalanced data by using the conditional distribution of the parameter sufficient statistics to estimate the logistic model parameters (Cox & Snell, 1989; Hilbe, 2009). Such estimations use exact probability statements that are valid for any sample size, whereas logistic regression using maximum likelihood is based on asymptotic theory and thus is more likely to be unreliable at small sample sizes.

For all other statistical analyses, replicates where the seedling died or earthworm contamination occurred (see discussion) were excluded. This resulted in final sample sizes of 12, 18, 17, 19, and 17 for treatments 1 to 5, respectively. As well, for mycorrhizal colonization and composition and spruce biomass (*i.e.*, those variables measured only at the end of the experiment), we randomly selected 50 replicates for measurement in order to maintain a balanced sample size in each treatment. We also

assessed differences in aboveground forb biomass and species composition, aboveground grass biomass, soil nitrogen concentrations, and earthworm survival, as these factors could also influence spruce growth and mycorrhizal colonization (Appendix I; Cameron & Bayne, 2011). Analyses were conducted using Stata 11 (StataCorp, College Station, Texas, USA) and R version 2.8.1 (R Foundation for Statistical Computing, Vienna, Austria) and significance was set to $P < 0.05$.

Results

ECTOMYCORRHIZAL FUNGAL COMMUNITY COMPOSITION AND COLONIZATION

Ectomycorrhizas were categorized into 8 identifiable groups (Table I). We were able to amplify DNA and obtain sequences for only 3 of these morphotypes. Query matches to Genbank accessions did not provide species-level information. All 3 amplified morphotypes were described as uncultured ectomycorrhizas from different families. Specifically, morphotypes were identified from the clades Pyronemataceae (gb | GU452518.1; 703/704; 99%) (accession number, identities/length, %), *Thelephora* (gb | EF218819.1; 771/787; 98%), and *Tomentellopsis* (emb | AJ893354.1; 679/682; 99%).

Proportion of root tips colonized did not differ significantly across treatment groups for our 8 morphotypes, as indicated by a Kruskal–Wallis ranks test ($\chi^2_4 = 1.164$, $P = 0.8840$; Figure 1). Similarly, multi-response permutation procedures (MRPPs) revealed no significant differences were present in morphotype composition among treatments (Distance measure = Sorensen, $A = -0.0278$, $P = 0.951$). Congruent with the MRPP results, no clear patterns were detectable within the non-metric multidimensional scaling ordination plots (Figure 2). A 3-dimensional solution provided the lowest value for stress (stress = 0.129). Analysis of the 3 sequenced morphotypes alone produced similar results in all cases.

IMPACT OF EARTHWORMS ON WHITE SPRUCE GROWTH

Earthworms had a limited impact on indicators of spruce seedling growth (Figure 3). One-way ANOVAs for both aboveground ($F_{4, 45} = 1.14$, $P = 0.352$) and belowground biomass ($F_{4, 45} = 0.99$, $P = 0.424$) revealed no significant differences among treatments. No significant differences were observed in change in branch length ($H_4 = 1.505$, $P = 0.837$). After controlling for initial size, no significant differences were found for primary branch number ($F_{4, 76} = 1.90$, $P = 0.120$), height ($F_{4, 76} = 1.95$, $P = 0.111$), and diameter ($F_{4, 76} = 0.37$, $P = 0.829$). Needle

TABLE I. Mean (± 1 SE) number of root tips colonized for each ectomycorrhizal morphotype across the 5 treatments. For each *Picea glauca* seedling, 100 root tips were examined.

Treatment	Morphotype							
	<i>Tomentellopsis</i> sp.	Pyronemataceae sp.	<i>Thelephora</i> sp.	Unknown A	Unknown B	Unknown C	Unknown D	Unknown E
No worms	11.6 \pm 4.57	0.7 \pm 0.70	0.0 \pm 0.0	0.2 \pm 0.20	3.3 \pm 2.06	0.4 \pm 0.40	0.0 \pm 0.0	0.3 \pm 0.30
<i>Dendrobaena octaedra</i>	14.3 \pm 5.51	1.3 \pm 0.94	0.0 \pm 0.0	0.3 \pm 0.21	0.3 \pm 0.30	1.4 \pm 1.29	0.0 \pm 0.0	0.0 \pm 0.00
<i>Lombricus terrestris</i>	13.4 \pm 5.43	1.3 \pm 0.90	0.0 \pm 0.0	0.2 \pm 0.20	2.1 \pm 1.67	0.0 \pm 0.00	0.7 \pm 0.7	0.0 \pm 0.00
3 <i>Lombricus terrestris</i>	6.0 \pm 1.59	1.7 \pm 1.19	1.0 \pm 1.0	0.1 \pm 0.10	0.5 \pm 0.50	0.2 \pm 0.20	0.0 \pm 0.0	0.0 \pm 0.00
Both species	9.5 \pm 3.98	1.1 \pm 0.69	0.0 \pm 0.0	0.0 \pm 0.00	1.8 \pm 1.14	1.5 \pm 1.39	0.0 \pm 0.0	2.1 \pm 2.10

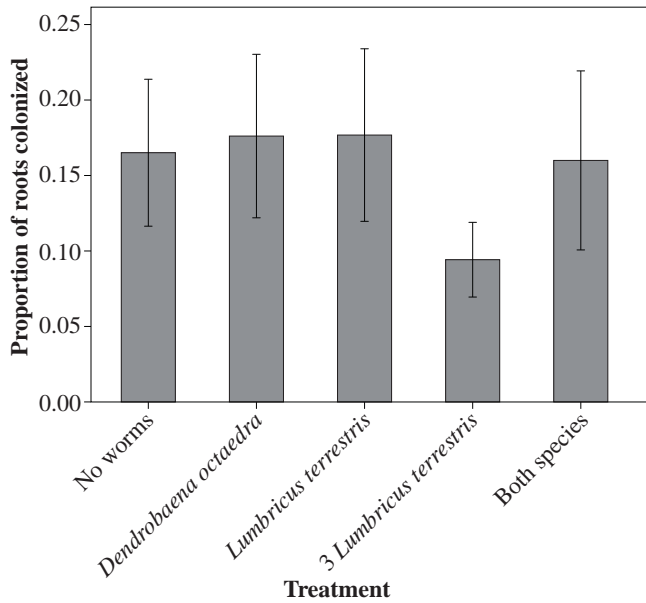


FIGURE 1. Means (± 1 SE) for proportion of root tips of *Picea glauca* colonized by ectomycorrhizal fungi within each treatment (no worms, *D. octaedra*, *L. terrestris*, 3 *L. terrestris*, both species) for all 8 morphotypes.

death was unaffected by earthworm treatment (*D. octaedra* only: odds ratio = 1.005, $P = 0.978$; *L. terrestris* only: odds ratio = 0.909, $P = 0.320$; 3 *L. terrestris*: odds ratio = 1.109, $P = 0.429$; both species: odds ratio = 0.855, $P = 0.269$). The proportion of dead seedlings did not differ significantly between treatments (model score = 8.348, $P = 0.0733$).

Aboveground grass biomass (mean ± 1 SE = 9.8 g \pm 0.44), aboveground forb biomass (mean ± 1 SE = 15.6 g \pm 0.40), and forb species composition did not differ significantly between treatments, and heights of these species were similar to those typically observed in the understory in this region at the end of the growing season (Appendix I; Cameron & Bayne, 2011). Leaf litter depth decreased significantly during the experiment, and the survival rates for each earthworm species were insensitive to treatments (Cameron & Bayne, 2011). Survival of *D. octaedra* was lower than that of *L. terrestris*, although we observed high reproductive success of *D. octaedra* and continued earthworm activity in an extra replicate throughout the experiment, suggesting that most survived until near the end of the experiment. Nitrogen concentration in the organic horizon differed significantly among treatments, according to an ANOVA (Figure 4, $F_{4, 45} = 3.08$, $P = 0.0250$); nitrogen was significantly higher in the *L. terrestris* treatment than the treatment containing both species ($P = 0.034$).

Discussion

No major differences were observed among any of the earthworm treatments across all measured variables for both ectomycorrhizas and seedlings. This suggests that increased earthworm biomass or multiple species invasions do not have an added impact on mycorrhizas or spruce seedlings relative to single species invasions, at least over a single growing season. In our study, no significant effects were

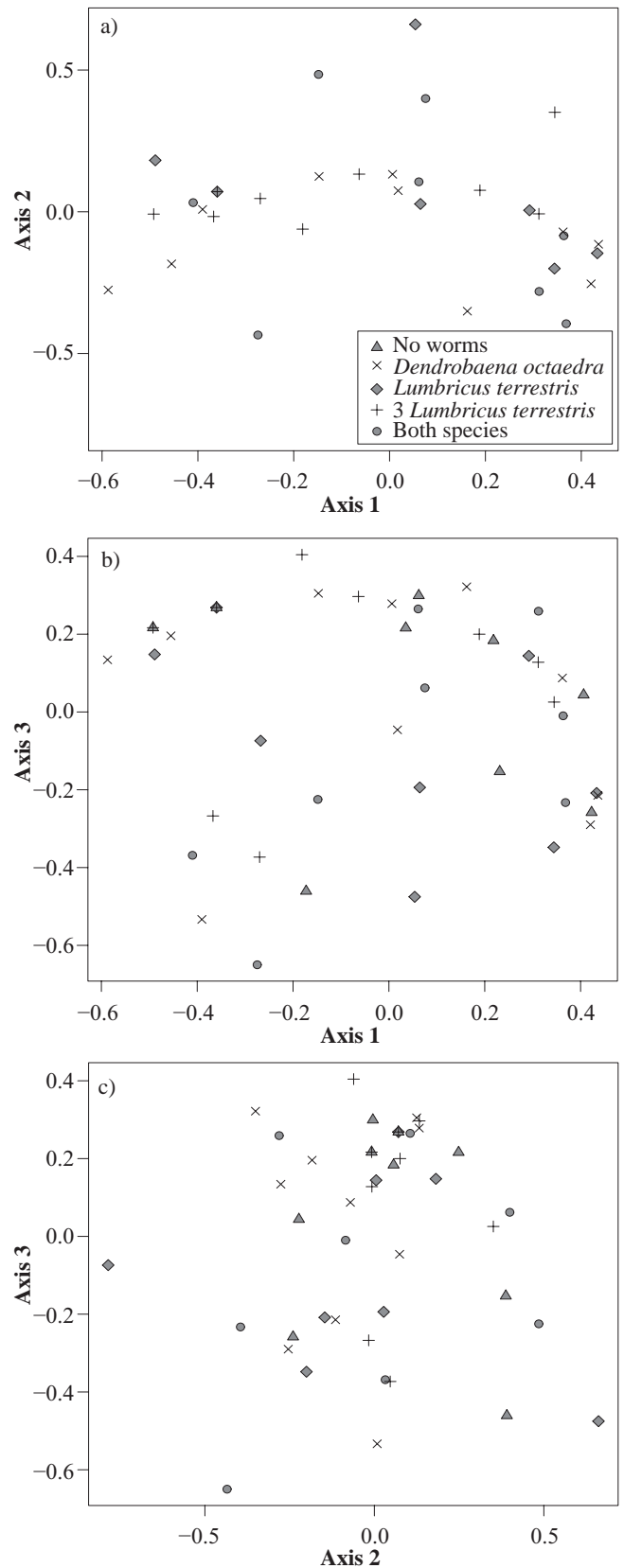


FIGURE 2. Non-metric multidimensional scaling (NMDS) plots of replicates rank-ordered and arranged by ectomycorrhizal morphotype composition for all 8 morphotypes. a) Plot of ordination axes 1 and 2; b) plot of ordination axes 1 and 3; and c) plot of ordination axes 2 and 3.

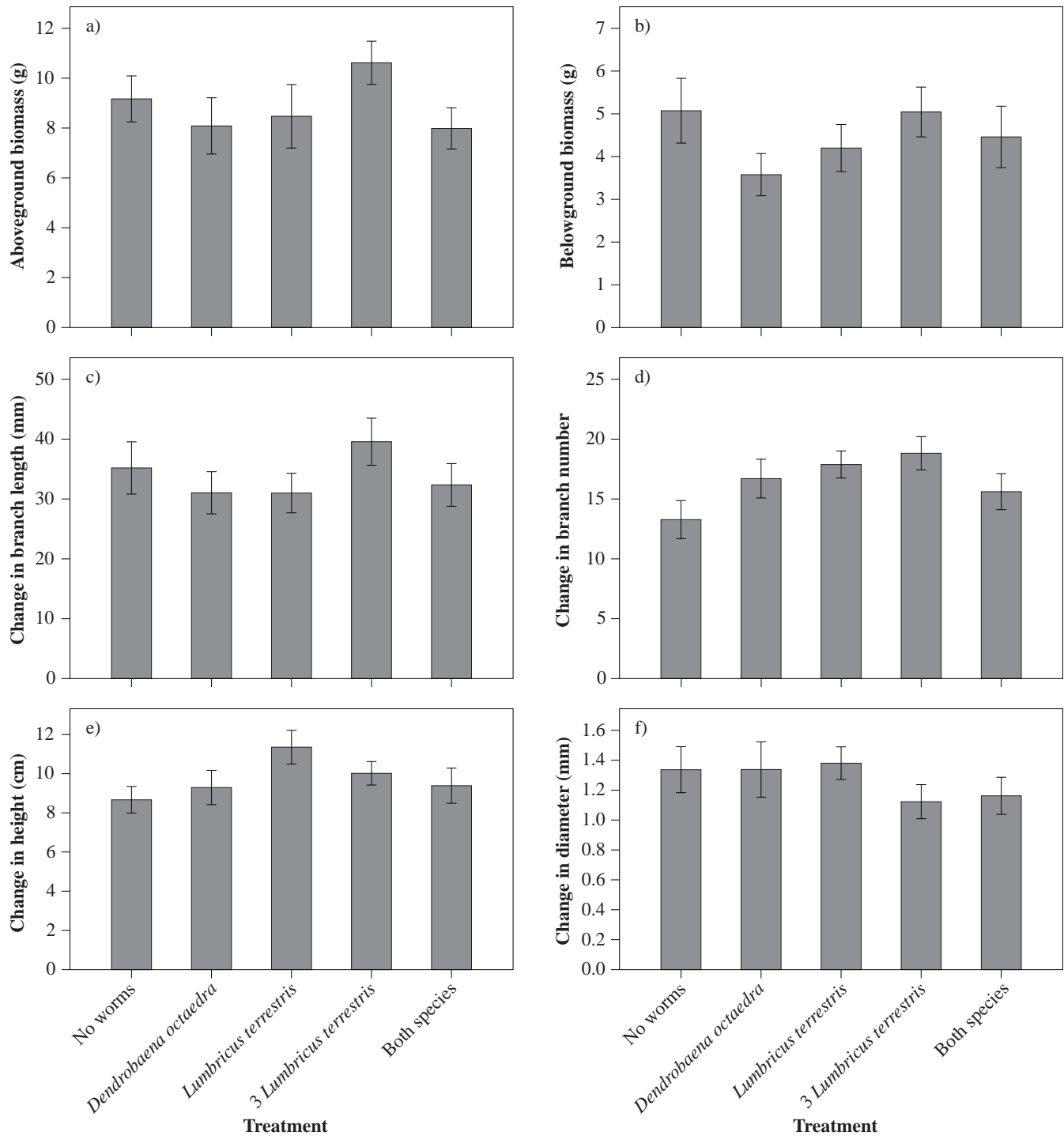


FIGURE 3. Means (± 1 SE) for indicators of white spruce (*Picea glauca*) seedling growth across the 5 treatments (no worms, *D. octaedra*, *L. terrestris*, 3 *L. terrestris*, both species): a) aboveground biomass (g); b) belowground biomass (g); c) change in branch length (mm); d) change in primary branch number; e) change in height (cm); and f) change in stem diameter (mm).

seen on colonization and community structure of ectomycorrhizal fungi of white spruce. In contrast, previous studies suggest that exotic earthworms can reduce abundance of arbuscular mycorrhizal fungi (Lawrence *et al.*, 2003; McLean, Migge-Kleian & Parkinson, 2006). These decreases in arbuscular mycorrhizal colonization following earthworm invasion are suggested to be the result of mechanical disruption of hyphal networks as worms

move through the soil (Lawrence *et al.*, 2003; Frelich *et al.*, 2006). However, we did not examine soil hyphal networks in our study, and the effects of the disruption of these networks on colonization of roots by ectomycorrhizal fungi are unclear.

Community structure of ectomycorrhizal fungi did not vary among treatments. The observed taxa of ectomycorrhizal fungi appear insensitive to earthworm modifications

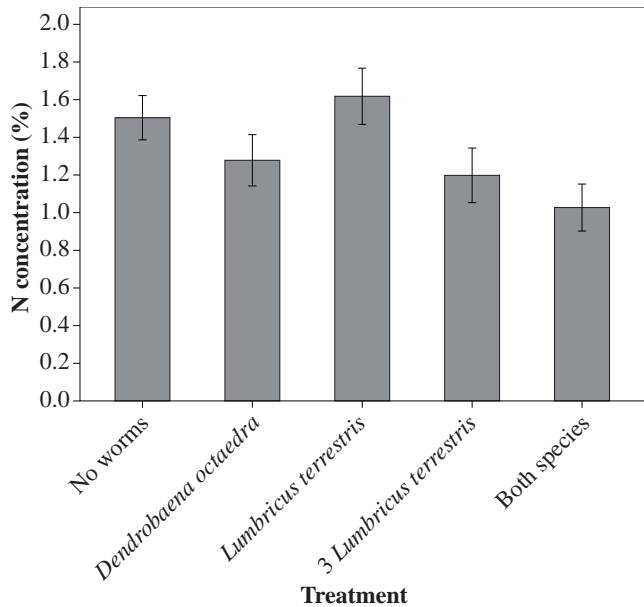


FIGURE 4. Mean (± 1 SE) nitrogen concentration (%) in the organic horizon for the 5 treatments (no worms, *D. octaedra*, *L. terrestris*, 3 *L. terrestris*, both species).

of soil; however, only a subset of the mycorrhizal community was present in our study. Given that we severed all hyphal connections with mature trees when we collected the soils, only those fungal species able to tolerate and recover from such a disturbance would be included in our field collection. Those species of fungi unable to withstand severed connections from a host may be those that are sensitive to earthworm activity. Mycorrhizal colonization of individual root tips in our study was low across all treatments (mean ± 1 SE = 15.5% \pm 0.02), although 90% of the seedlings we examined had at least some mycorrhizal colonization. It is not uncommon to observe low levels of colonization and numbers of fungal morphotypes in young seedlings grown in the greenhouse or the field following disturbance (Jones *et al.*, 1997; Teste *et al.*, 2004; Cline, Ammirati & Edmonds, 2005; Karst *et al.*, 2011).

Greater soil nutrient availability as a result of non-native earthworm activity has also been suggested as a possible mechanism contributing to reduced arbuscular mycorrhizal colonization (Lawrence *et al.*, 2003). Increased soil nitrogen input is strongly and negatively correlated with ectomycorrhizal abundance (Lilleskov *et al.*, 2002). Nitrogen concentration in the organic horizon did not differ among most treatments in our study but was significantly higher in the *L. terrestris* treatment than in the treatment containing both species. Such increases in nutrient flux as a result of earthworm invasion may occur mainly in the early stages of invasion (Bohlen *et al.*, 2004; Frelich *et al.*, 2006), and therefore changes in availability may differ in longer-term studies. In our experiment, ectomycorrhizal abundance was not correspondingly lower in the *L. terrestris* treatment, suggesting that the difference in nitrogen concentration was not large enough to result in a response by ectomycorrhizal fungi.

Spruce seedling performance was unaffected by earthworms, suggesting that this species may be resistant to effects of earthworm invasion over the short term.

Previous work has shown earthworm species can increase tree seedling growth. For example, the addition of the endogeic earthworm *Aporrectodea caliginosa tuberculata* (now known as *Aporrectodea tuberculata*; Pérez-Losada *et al.*, 2009), resulted in increased height and biomass of birch seedlings (Haimi & Einbork, 1992). Similarly, Douglas-fir seedlings had significantly greater root biomasses in endogeic worm-worked soils relative to controls (Welke & Parkinson, 2003). Our results show no effect on seedling mortality in the presence of exotic earthworms, unlike previous studies in sugar maple stands of northern Minnesota (Frelich *et al.*, 2006). This suggests that the responses of tree seedlings to earthworm invasions may be species-specific, and consequently further research examining effects on multiple tree species by multiple earthworm species could be informative.

Seedling mortality was relatively high in this experiment (ranging from 0% in the *L. terrestris* treatment to 30% in the control). There were no significant differences in mortality among treatments. However, mortality did reduce our sample size, resulting in an unbalanced design. Most of the seedling deaths occurred in the second month of the experiment and were in pots that became water logged. This suggests that a better approach would have been to water in an amount that ensured soil moisture was kept constant across all replicates rather than to use a set amount. Also, as is common in many earthworm mesocosm experiments (Fründ *et al.*, 2010), we confirmed the presence of *D. octaedra* (mean ± 1 SE abundance of 2.1 \pm 0.74) in several no-earthworm control and *L. terrestris* replicates. It is unlikely that contamination of these replicates was a result of undetected earthworms initially present in collected soil or root plugs of white spruce seedlings. Instead, some earthworms appear to have been able to overcome our sand strip barriers and escape into adjacent replicates. To maintain the validity of our treatments, contaminated replicates were excluded from analysis.

In our greenhouse experiment, we determined that the exotic earthworm species *L. terrestris* and *D. octaedra* have no direct short-term impact on ectomycorrhizal colonization or spruce seedling growth. As earthworms continue to spread in North American forests, an increased understanding of the impact of earthworm invasion on both mycorrhizal colonization and diversity and seedling growth will be necessary in order to assess the impacts of earthworm invasion on a forest as a whole.

Acknowledgements

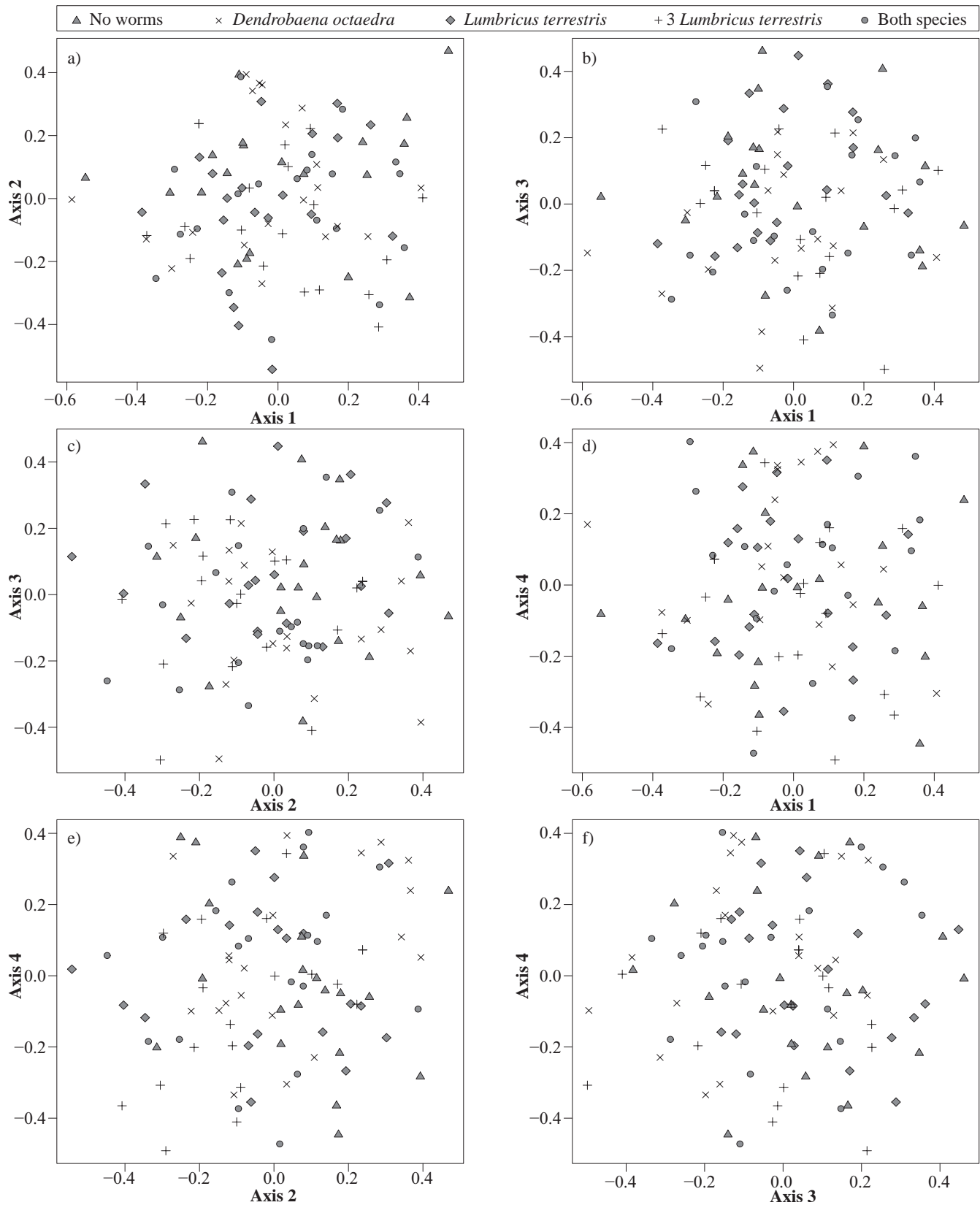
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Appendix I



APPENDIX I, FIGURE 1. Non-metric multidimensional scaling (NMS) plots of replicates rank-ordered and arranged by forb species composition. Plot of ordination axes: a) 1 and 2; b) 1 and 3; c) 2 and 3; d) 1 and 4; e) 2 and 4; and f) 3 and 4. Stress was 0.1629. A multi-response permutation procedure indicated no significant differences between groups ($A = 0.0029$; $P = 0.316$).