Native and exotic earthworms affect orchid seed loss

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Abstract. Non-native earthworms have invaded ecosystems around the world but have recently received increased attention as they invaded previously earthworm-free habitats in northern North America. Earthworms can affect plants by ingesting seeds and burying them in the soil. These effects can be negative or positive but are expected to become increasingly negative with decreasing seed size. Orchids have some of the smallest seeds of any plants, so we hypothesized that earthworm consumption of seeds would decrease seed viability and lead to burial of ingested seeds. We used a combination of mesocosms and field measurements to determine whether native and non-native earthworms would affect Goodyera pubescens seed germination by decreasing seed viability through digestion or burial. To determine soil depths at which seed burial would decrease chances of germination, we used field measurements of the abundance of mycorrhizal fungi needed for G. pubescens germination at different soil depths. We found that the combined effects of earthworm ingestion and burial would be expected to result in a loss of 49 % of orchid seeds in mature forests and 68 % of those in successional forests over an average year. Differences in seed ingestion and burial among soils from mature and successional forests were probably driven by differences in their ability to support earthworm biomass and not by differences in earthworm behaviour as a function of soil type. The combined effects of earthworm ingestion and burial have the potential to result in substantial loss of orchid seeds, particularly in successional forests. This effect may slow the ability of orchids to recolonize forests as they proceed through succession. Determining whether this strong effect of earthworms on G. pubescens viability and germination also applies to other orchid species awaits further testing.

Keywords: Earthworms; forest succession; mycorrhizae; orchids; seed burial; seed viability.

Introduction

Worldwide, the role of invasive species in contributing to the decline of rare native species is receiving increased attention (e.g. Augustine and Frelich 1998; Mack et al. 2000; Gundale 2002; Pawson et al. 2010). Many invasions, especially those in the soil, are cryptic and can proceed unnoticed for many years. Despite the fundamental ecosystem services that soil biota provide (Lavelle et al. 2006; Wall et al. 2012), belowground biodiversity is still inadequately studied in many regions (Hagvar 1998). Native soil-dwelling species are often poorly known (André et al. 1994; Decoëns et al 2006), so it is not surprising that non-native species that invade the soil environment often go unnoticed. Non-native earthworms have invaded ecosystems around the world but have recently received increased attention as they invaded previously earthworm-free habitats in northern North America (Hendrix et al. 2008). There they have been shown to dramatically reduce leaf litter cover on the forest floor and endanger many
understorey herbaceous species (Gundale 2002; Bohlen et al. 2004). In many other areas, non-native earthworms have invaded habitats that have native earthworm communities and the effects of these invasions have been less clear-cut (Hendrix et al. 2006).

Earthworms are well known to alter the conditions for plant seed germination and can affect plant community composition through both direct and indirect interactions at all stages from seed through mature plant (Lawrence et al. 2003; Eisenhauer and Scheu 2008; Regnier et al. 2008; Eisenhauer et al. 2009a, b; Forey et al. 2011). Two major ways earthworms can affect plants directly are by ingesting seeds and burying them in the soil (e.g. Willems and Huijsmans 1994; Milcu et al. 2006; Eisenhauer and Scheu 2008). These activities can have either positive or negative effects on seed germination. Seed burial may increase seed survival by reducing exposure to aboveground seed predators (e.g. Regnier et al. 2008) and passage through the earthworm digestive tract may break seed dormancy, increasing germination (e.g. Eisenhauer et al. 2009b). However, seeds may also be digested and viability of undigested seeds may decrease as a result of damage during earthworm gut passage (Shumway and Koide 1994; Eisenhauer et al. 2009b). Viability of seeds that have passed through earthworm digestive systems has been found to decrease with seed size (Milcu et al. 2006) such that viability decreases much more in small than in large seeds. Accordingly, Eisenhauer and Scheu (2008) found that earthworms disproportionately decreased the number and biomass of small-seeded plants establishing from seeds. Because orchid seeds are among the smallest seeds of land plants, we expected that viability of orchid seeds would be greatly affected by being ingested by earthworms.

Seeds of a few plant species are actively collected by earthworms and either ingested or cached, and these species have received the bulk of research attention. Earthworms that cache seeds may bury seeds without ingesting them (Regnier et al. 2008). This burial often protects seeds, especially large seeds, from aboveground seed predators, but it can also result in seeds being buried too deeply to successfully germinate and grow (e.g. Traba et al. 1998; Regnier et al. 2008). Seeds of less preferred plants may be infrequently or incidentally ingested (e.g. Willems and Huijsmans 1994; Zaller and Saxler 2007; Regnier et al. 2008) and may only be buried as a consequence of ingestion and deposition in casts. As earthworm species differ in feeding and casting behaviour and where they are distributed within the soil (e.g. Bouché 1977; Curry and Schmidt 2007; Zicsi et al. 2011), both the ingestion of seeds from the soil surface and the depth at which ingested seeds are deposited may also differ.

We examined the effects of native and non-native earthworms on seeds of the North American native orchid Goodyera pubescens. Orchids are widely threatened and endangered, yet effects of non-native earthworms on orchids have not been considered. Orchids that grow in the forest understorey may be among the plants most likely to be affected by non-native earthworms. Temperate forest orchids often grow within the litter and organic layers of the forest floor, and depend strongly on mycorrhizal fungi (e.g. Preiss et al. 2010). Earthworms, especially in the high abundances sometimes maintained by invasive species, hasten decomposition of leaf litter, revealing bare soil, incorporate organic-rich surface soils into deeper mineral horizons, and alter soil fungal communities (e.g. McLean and Parkinson 2000; Gundale 2002; Bohlen et al. 2004; Hale et al. 2006; Szlavecz et al. 2011) compared with soils without earthworms or with less abundant native earthworms. While direct effects on mature orchids are likely to be minimal, orchid seeds may be vulnerable to earthworm consumption and burial. Goodyera pubescens seeds, like those of most orchids, have little or no nutrients to support embryo development, and germination generally requires specific fungi. Mycorrhizal fungi provide all nutrients, including carbon, at least until leaves are produced, and fungi continue to supplement growth in mature orchids.

We used a combination of laboratory mesocosms and field measurements to determine whether native and non-native earthworms would affect G. pubescens seed germination by decreasing seed viability through digestion or burial. We hypothesized that earthworm ingestion would significantly decrease seed viability and lead to burial of ingested seeds. We also hypothesized that earthworm species would affect orchid seed viability and burial differently, and that these effects would differ among soils from successional and mature forests, which have historically supported different earthworm abundances (high and low, respectively; Szlavecz and Csuzdi 2007). As a minimal estimate of soil depths at which seed burial would decrease chances of seed germination, we used field measurements of the abundance of fungi needed for G. pubescens germination at different soil depths. We then used estimates of earthworm activity to extrapolate the mesocosm results to an average year.

**Methods**

**Species and study site**

Goodyera pubescens is a common, evergreen terrestrial orchid found in mid-late successional forests throughout the eastern USA and into southeastern Canada. Goodyera pubescens rhizomes and roots are present
almost exclusively within the top 2–5 cm of soil (M. K. McCormick, pers. observ.). Goodyera pubescens has a very specific fungal association and protocorms and adults both associate with a small group of closely related Tulasnella spp. (McCormick et al. 2004, 2006).

Soil core collection and field studies of Tulasnella distribution were conducted at the Smithsonian Environmental Research Center (SERC) in Edgewater, MD, USA. Soils at the study sites have been classified as Collington sandy loam (fine-loamy mixed, active, mesic Typic Hapludult) and Monmouth fine sandy loam (fine, mixed, active, mesic Typic Hapludult) (Soil Survey Staff, http://soils.usda.gov/technical/classification/osd/index.html). These two soil types are very similar, with Monmouth series soils containing slightly more fine sand (particle diameter 0.2–0.02 µm) than Collington series soils. Sites where soil cores were collected included mature forest stands (120–150 years old), which had soils in the Collington series, and successional forest stands (50–70 years old), which had soils in the Monmouth series, of the tulip poplar–oak association (Brush et al. 1980; Szlavecz et al. 2011). Although the parent soil types are very similar, differences in land use history, agriculture and earthworm colonization have resulted in soils in mature forests having more carbon, especially in the Oe, layer, that is less associated with aggregates (Ma et al., 2013). Mature forest soils are also more acidic (Szlavecz and Csuodzi 2007). Earthworms, especially non-native species, are abundant in upland forests at SERC where native orchids occur. Twelve earthworm species have been recorded in SERC forests, of which seven are non-native, including Lumbricus rubellus (epi-endogeic), Octolasion lacteum (endogeic) and Lumbricus friendi (anecic), and one is native, Eisenoides innomberi (endo-geic) (Szlavecz and Csuodzi 2007; Szlavecz et al. 2011).

Seed viability
Effects of earthworm ingestion on seed viability were tested on three batches of field-collected G. pubescens seeds. Seeds were collected from local plants in 1997, 2001 and 2003, and stored dry at 4 °C until used for this experiment in 2007. We mixed 0.5 ± 0.025 mg of G. pubescens seeds with damp crushed tulip poplar leaf litter and placed the mixture on damp plaster of paris in 10-cm-diameter x 10-cm-deep plastic jars with screw caps. We then introduced one earthworm to each jar. Three species of earthworms that were collected from the field were starved for 24 h and used in this study. In each case we used mature earthworms to ensure accurate species identification. Ten jars each received one native earthworm, E. innomberi, and 30 received one non-native earthworm, L. rubellus (10), L. friendi (10) or O. lacteum (10). Earthworms were allowed to feed on the orchid seeds for 48 h. After 48 h, we removed the earthworms, rinsed them in tap water to avoid transferring seeds that had not passed through the earthworm’s digestive system and placed the earthworms in clean jars with damp plaster for 72 h. We then collected seeds excreted into these fresh jars. We also collected seeds from each original jar that had not been ingested for comparison. Excreted and uningested seeds were placed in seed packets made of 50-µm nylon mesh in 35-mm slide mounts, stained for 48 h using the triphenyltetrazolium chloride (TTC) method (Rasmussen 1995; Baskin and Baskin 1998; Ramsey and Dixon 2003) and scored for viability. Viability was calculated as the percentage of seeds staining red in TTC. Decrease in viability was calculated as the per cent of ingested seeds that were viable divided by the per cent of uningested seeds that were viable.

Effects of earthworm species and ingestion on seed viability were tested using an analysis of variance (ANOVA) with Species and Ingestion as fixed effects, and Seed Batch as a random effect. All analyses were conducted using Systat 11 for Windows (Systat Software Inc, San Jose, CA, USA).

Seed burial
We used a combination of mesocosm experiment and field measurements of the abundance of mycorrhizal fungi required by G. pubescens to estimate the effect of earthworm seed burial on seed germination. We collected 16 intact soil cores of 10 cm diameter × 20 cm depth from each of three mature forest stands (~120–150 years old) with a few, primarily native, earthworms and 24 from each of two mid-successional forest stands (~50–70 years old) with abundant non-native earthworms, to use as mesocosms (N = 48 per forest age). Corers were constructed of 10-cm-diameter aluminium irrigation pipe with teeth sharpened into one end and a hole drilled through the top to allow insertion of a steel bar for a handle. Corers were inserted into the soil by hand and each soil core was extruded out of the top of the corer, pushing from the base to minimize compaction of surface soil, into a 25-cm-long section of a 10.1-cm-diameter PVC pipe. A plastic cap was then added to the bottom of the PVC pipe to contain the core. Cores were transferred to the laboratory within 2 h of collection and soil moisture was measured for each core using a soil moisture sensor (Dynamax ML2 ThetoProbe). Sufficient tap water was then slowly added over several hours to bring each core to 30 % (by volume) moisture to promote earthworm activity and facilitate electroshocking. Once 30 % moisture was maintained for 2 h, we weighed each core and throughout the duration of the experiment we maintained this
mass by adding water weekly. We removed existing earthworms from each core by electroshocking (Thielemann 1986; Weyers et al. 2008; Szlavecz et al. 2012) each core twice for 20 min.

We added ∼10 g of pre-moistened leaf litter and 2 ± 0.1 g (fresh weight) of one earthworm species to each mesocosm (except controls). This earthworm biomass was similar to what we have found in field sites with very abundant earthworms (∼250 g m⁻², Szlavecz and Csuzdi 2007; K. Szlavecz, unpubl. res.) and leaf litter input was similar to high natural litter inputs (G. G. Parker, pers. comm.). We used three earthworm species (two non-natives: O. lacteum, L. rubellus; and one native: E. lonnbergi), each of which was added to 24 mesocosms. Each mesocosm received either tulip poplar (Liriodendron tulipifera) or oak leaves (a mixture of primarily Quercus falcata and Q. velutina) to determine whether earthworm activity and seed burial differed when palatable (tulip poplar) or unpalatable (oak) food was available on the soil surface. Each leaf litter type was added to six mesocosms per earthworm species (three species + control) per soil type (two), for a total of 48 mesocosms.

Goodyera pubescens seeds are very small, ∼0.35 mm in diameter (Whigham et al. 2002), and it is impossible to track them in the soil. We used fluorescent latex beads (green fluorescent polymer microspheres, Duke Scientific Corp., Palo Alto, CA, USA) that were similar in size (157 μm) to G. pubescens seeds as a proxy for seed movement. In preliminary tests neither orchid seeds nor fluorescent beads were actively ingested by earthworms, but rather both were ingested in the process of feeding at the soil surface, so we considered the beads to be an accurate representation of movement of orchid seeds. We added 1.5 ± 0.1 mg of fluorescent beads to the soil surface of each mesocosm before adding leaf litter and covered each PVC tube with a plastic wrap fastened with two rubber bands to prevent earthworm escape. All mesocosms were then placed in a dark incubator at 17°C for 6 weeks. Mesocosms were removed weekly for weighing and water addition but were otherwise undisturbed until harvest.

After 6 weeks, we removed the mesocosms from the incubator, removed the remaining surface litter and any surface casts, and sliced the remainder of each core into four sections according to depth (0–2, 2–5, 5–10 and 10–20 cm depth). During sectioning, all earthworms were also removed and weighed to verify that the earthworms remaining were those we added and to determine whether earthworms survived. Each section was then placed in a paper bag in a 45°C drying oven until processing (2–14 days). After drying, each section was placed in a large tray and aggregates were broken apart with a pestle to allow access to fluorescent beads. We then methodically moved through soil from each section in a dark room with a black light and counted all fluorescing beads.

**Analysis of depth distribution**

The number of beads detected in each core section was expressed as a proportion of the total beads found within the soil to account for differences in bead recovery and number of beads added among mesocosms, and to avoid confounding patterns of burial depth with different amounts of bead ingestion. We assumed that all beads buried deeper than 2 cm were moved by ingestion and subsequent egestion, and not by active burial or transport in mucus, which would be very unlikely to happen with species that do not form permanent burrows. Effects of Soil Type, Earthworm Species and Litter type on Bead Depth Distribution were tested using two repeated measures ANOVAs with Species, Forest age and Litter as fixed effects, and measurements at different depths as the repeated measure. The first ANOVA compared Bead Distribution in control cores with the cores with earthworms. The second ANOVA compared Bead Distribution in cores with different species of earthworms added. All analyses were conducted using Systat 11 for Windows (Systat Software Inc.). We also calculated the proportion of beads ingested by earthworms in each mesocosm as the sum of all buried beads and all beads recovered from surface costs divided by the total number of beads recovered from the mesocosm.

**Mycorrhizal distribution**

To understand the effects of seed burial at different depths on orchid seed germination, we quantified the distribution of the Tulasnella spp. needed to support G. pubescens germination and growth. We selected 12 mature G. pubescens plants spread as widely as possible across the 3600 ha of SERC property. All plants were at least 10 m apart. At each plant we removed one 2.5-cm-diameter × 30-cm-deep soil core from immediately adjacent to a root. We then divided each core into 0–5–, 5–10–, 10–20– and 20–30-cm-deep sections. Each core section was lyophilized (Labconoco 195 freeze dry system, Kansas City, MO, USA), mixed, ground and DNA was extracted from 20 ng of DNA extracted from each soil sample using the polymerase chain reaction (PCR) primers ITS5 (White et al. 1990)/ITS4-tul (Taylor and McCormick 2008), which are specific to Tulasnella spp., on an MJ Research Opticon DNA Engine with Continuous Fluorescence Detection (MJ Research, now
Bi-Rad Laboratories, Hercules, CA, USA). Each 25-μL reaction mixture contained 12.5 μL of iQ SYBR Green PCR Super Mix (Bi-Rad Laboratories), 20 ng of DNA template in 10 μL of H2O and 1.25 μL (10 mM) of each of the primers. Amplifications were run as follows: initial denaturation for 5 min at 95 °C followed by 41 cycles of 15 s denaturation at 94 °C, 30 s annealing at 53 °C and 30 s elongation at 72 °C. Each sample extract was amplified in triplicate and quantified using a standard curve. Four serial dilutions of genomic DNA from a pure culture of a Tulasnella sp. (isolate # M109; AY373263) isolated from G. pubescens were used to construct a standard curve (range: 0.001–1 ng of target genomic DNA). A melting curve analysis was performed after each analysis to confirm the specificity of the quantitative PCR (qPCR). Reactions with no template DNA were performed with each amplification to ensure that no contaminants were present.

DNA abundance data were square-root transformed prior to analysis to improve normality. We compared Tulasnella abundance at different depths using an ANOVA with depth as a categorical fixed main effect. We then fit an exponential curve to the points describing fungal abundance change with depth (SigmaPlot 11.0) to convert the point values to a continuous distribution. We then used this distribution to determine the depth below which Tulasnella abundance was <0.5 ng/g dry soil. The cut-off of 0.5 reflected the Tulasnella abundance below which McCormick et al. (2012) found that field soils did not support G. pubescens germination.

Combining effects and scaling up
To predict the combined effects of viability decline and seed burial on G. pubescens seeds over the course of a year, we needed to estimate the length of time that earthworms would be active. Using data from Gongalsky et al. (2008), we assumed that earthworm activity would be greatest in soils above 5 °C and 20 % moisture. Using soil temperature and moisture data collected continuously from a wireless sensor network in SERC forests (Terzis et al. 2009; Szlavecz et al. 2012), we estimated that the duration of most earthworm activity would be ~32 weeks out of each year.

Because seeds were not actively sought out, but rather ingested as a function of consuming surface soil and leaf litter, and because we had no evidence that earthworms preferentially avoided consuming previously ingested soil, we used an exponential decay function (e.g. Manzoni et al. 2012) to estimate seed ingestion in an average year from that observed over the 6 weeks of the experiment. This equation took the form \( N(t) = N_0 \times e^{-\lambda t} \), where \( N(t) \) is the per cent of uningested seeds remaining at time \( t \), \( N_0 \) is the initial per cent of seeds (100) and \( \lambda \) is the exponential decay function. We calculated \( \lambda \) using the proportion of seeds remaining uningested at the end of the experiment (\( t = 6 \) weeks) for the average, high and low rates of seed ingestion observed for each earthworm species in each soil type, and used that to calculate the expected per cent of seeds ingested after 32 weeks of earthworm activity (100 – \( N_{32} \)). We then calculated the per cent of ingested seeds that would become non-viable using the observed decreased viability. We assumed that the remaining ingested but viable seeds would be buried to the same depths as observed over the experiment by each of the three earthworm species in each soil type (mature or successional forest soils). We used the depth distribution of Tulasnella abundance to determine when buried seeds were probably too deep to establish mycorrhizal associations and so were rendered unlikely to germinate. We then combined the per cent of seeds ingested with the proportion of ingested seeds that each earthworm species buried deeper than the point at which host Tulasnella abundance declined to <0.5 ng g\(^{-1}\) dry soil. We used this to calculate the per cent of seeds that would not germinate (NV) as a result of earthworm activity by adding the per cent of seeds lost to non-viability after 32 weeks of earthworm activity:

\[
NV = (I_{32} \times F_{nv}) + B.
\]

In this equation, \( I_{32} \) is the per cent of seeds ingested after 32 weeks, \( F_{nv} \) is the proportion of ingested seeds rendered non-viable and \( B \) is the per cent of ingested seeds that remained viable but would be ‘lost’ by being buried too deeply. \( B \) is calculated as \( B = I_{32} \times V \times D \), where \( V \) is the proportion of ingested seeds that remained viable and \( D \) is the proportion of ingested seeds buried deeper than the depth at which host fungus abundance declined to <0.5 ng g\(^{-1}\) dry soil.

We assumed that the observed seed burial depth would be similar over time but that more seeds would be present at each depth. Although it is likely that buried seeds would eventually be re-ingested and might then be buried deeper or, conversely, deposited in casts on the surface, we assumed that these effects would be dominated by the viability effects of being ingested and so re-distribution of seeds would not be a significant occurrence.

Results

Seed viability
Earthworm ingestion strongly decreased seed viability (\( P < 0.001 \)). The viability of G. pubescens seeds decreased by 79 ± 4 (SE) % when ingested by earthworms. The
effects of different earthworms ($P = 0.30$) and different batches of seeds ($P = 0.29$, not shown) were similar (Fig. 1).

**Seed burial**
Earthworms had a significant effect on the depth distribution of beads in the soil mesocosms ($P < 0.001$, Table 1). Bead burial in soils from different forest stands within an age category (mature or successional) were always similar ($P > 0.5$) and hence were combined in the final analysis. Earthworm species buried beads differently ($P < 0.001$, Table 1, Fig. 2) and ingested different proportions of beads in soils from different age forests ($P = 0.004$, Fig. 3) but this was not affected by leaf litter type (the main effect $P = 0.96$, all interactions $P > 0.15$). In particular, *O. lacteum* ingested fewer beads in mature compared with successional forest soils ($P = 0.02$, Fig. 3). On average, $19.2 \pm 1.4 \%$ of beads were ingested and $6.0 \pm 0.6 \%$ of beads ($31 \%$ of those that were ingested) were buried deeper than 5 cm in the 6-week experiment (Table 2). We used post hoc comparisons with Scheffe critical values (Ruxton and Beauchamp 2008) to test for differences in how earthworm species ingested beads, and how deeply they buried beads in mature compared with successional forest soils, to better understand what was driving the significant interaction between earthworm species and site age in bead burial depth and differences between earthworms in bead ingestion. Significant differences are indicated by asterisks in Figs 2 and 3.

**Mycorrhizal distribution**
*Tulasnella* spp. were most abundant in the top 5 cm of the soil (Fig. 4). The equation of the exponential curve describing the abundance of fungi with depth was $\text{abundance} = 1.551 \times e^{0.2217 \times \text{Depth}}$ ($r^2 = 0.994$, $P = 0.003$). Using this curve, we predicted that the abundance of *Tulasnella* would decline to 0.5 ng g$^{-1}$ dry soil at 5 cm, so seeds buried deeper than 5 cm would have little chance of developing the mycorrhizal association they would need to germinate and grow.

**Combining effects and scaling up**
The proportion of beads that we estimated would be ingested over a year by each earthworm species in each soil type ranged from as low as 5 % up to 99 % (Table 2) but was lower in the mature than the successional forest soils ($50.8 \pm 3.6 \%$ vs. $68.8 \pm 4.0 \%$, $P = 0.001$). Only *O. lacteum* differed significantly between the two soil types ($40.6 \pm 5.9$ vs. $78.8 \pm 5.0$, $P = 0.02$). The other two species were estimated to ingest slightly but not significantly more beads in the successional forest soils. Similarly, significantly more seeds were estimated to be lost as a result of non-viability and burial combined in successional compared with mature forest soils ($56.7 \pm 3.3$ vs. $41.1 \pm 3.0$). Again, only *O. lacteum* differed significantly among soil types ($32.0 \pm 4.5$ in mature vs. $63.2 \pm 3.9$ in successional forest soils, $P = 0.03$), although all species produced somewhat greater losses in successional than in mature forest soils.

Differences in seed ingestion and losses in successional and mature forest soils may have been driven by differences in earthworm abilities to maintain biomass over the course of the experiment. Native and non-native earthworms differed in ability to maintain biomass in soils from different age forests. Earthworms in all mesocosms lost biomass (a combination of weight loss and mortality) in mature forest soils and gained biomass in successional forest soils, but the native earthworm, *E. lonnbergi*, lost the least biomass in mature forest soils, where they are commonly found, and gained the least in successional forest soils (Fig. 5), which are dominated by non-native earthworms at SERC. Both non-native species had a much greater difference in biomass between successional and mature forest soils than the native earthworm species we studied ($P = 0.001$).

**Discussion**
We found that *G. pubescens* seeds that were ingested by earthworms had 79 % lower viability than uningested seeds and this effect was similar among earthworm species. This represents a much greater decrease in viability than has been found in studies with larger seeds, some of which have even found increased seed viability after egestion (e.g. Milcu et al. 2006; Eisenhauer et al. 2009b), but it is still likely to be an underestimate of the actual decline in viability for this species, as any seeds that were fragmented or consumed were not
Table 1. Effects ($F$ values and significance) of statistical analyses examining the distribution of fluorescent beads in mesocosms. Effects significant at $P < 0.05$ are indicated in bold.

| Between-subject effects | Presence/absence | Species |
|--------------------------|------------------|---------|
|                         | $F$ value        | $P$ value | $F$ value | $P$ value |
| Site Age                | 2.709            | 0.103    | 0.001     | 0.976     |
| Earthworms              | 10.528           | **0.002** | 2.796     | 0.069     |
| Litter Type             | 1.353            | 0.248    | 0.650     | 0.423     |
| Site Age $\times$ Earthworms | 3.383         | 0.069    | 0.339     | 0.714     |
| Site Age $\times$ Litter | 1.295            | 0.258    | 0.012     | 0.913     |
| Earthworms $\times$ Litter | 0.231          | 0.632    | 1.624     | 0.205     |
| Site Age $\times$ Earthworms $\times$ Litter | 1.271 | 0.263    | 1.341     | 0.269     |

| Within-subject effects | Presence/absence | Species |
|-------------------------|------------------|---------|
|                         | $F$ value        | $P$ value | $F$ value | $P$ value |
| Depth                   | 1241.029         | <0.001   | 1310.422  | <0.001    |
| Depth $\times$ Site Age | 0.267            | 0.824    | 3.006     | 0.029     |
| Depth $\times$ Earthworms | 19.197         | <0.001   | 4.770     | <0.001    |
| Depth $\times$ Litter   | 3.480            | 0.021    | 5.362     | 0.001     |
| Depth $\times$ Site Age $\times$ Earthworms | 3.210 | 0.029    | 7.195     | <0.001    |
| Depth $\times$ Site Age $\times$ Litter | 0.713           | 0.528    | 0.983     | 0.405     |
| Depth $\times$ Earthworms $\times$ Litter | 0.680           | 0.55     | 0.887     | 0.511     |
| Depth $\times$ Site Age $\times$ Earthworms $\times$ Litter | 0.530 | 0.639    | 1.453     | 0.192     |

Figure 2. Proportions of ingested seed analogue beads distributed in different depth sections of soils from mature (black) and successional (grey) forests. Asterisks indicate significant differences ($P < 0.05$) in burial of beads by an earthworm species among soil types.

Figure 3. The proportion of fluorescent beads that earthworms removed from the soil surface or deposited in casts (interpreted as ingested) in soils from mature (black bars) or successional (grey bars) forests. Asterisks indicate significant differences ($P < 0.05$) in the proportion of beads that were ingested by an earthworm species among soil types.
counted. However, the declines in viability we found for *G. pubescens* may not be representative of orchid seeds as a group. While many orchids require appropriate mycorrhizal fungi to initiate germination and can remain dormant for many years (Whigham et al. 2006), seeds of *G. pubescens* initiate germination in response to moisture (Whigham et al. 2002), which may predispose them to damage during gut passage. Earthworms did not appear to actively seek out orchid seeds or our seed analogue beads, but rather ingested them incidentally with soil and leaf litter, so beads that were buried had almost certainly been ingested and subsequently egested in casts, rather than being cached. In mesocosms with field soil and earthworm biomass approximating field conditions, the three earthworm species we studied ingested an average of 10–29 % of our seed analogues over 6 weeks, although individual mesocosms ranged from 1 to 55 % of beads ingested. On average, 31 % of ingested beads were subsequently buried deeper than 5 cm, the depth below which we

| % Ingested | % Ingested | % Loss |
|------------|------------|--------|
| Average    | Low        | High   | Average | Low | High | Average | Low | High |
| Mature     |            |        |         |      |      |         |      |      |
| *L. rubellus* | 19.1 | 7.0  | 52.3  | 22.6 | 59.0 | 32.1  | 98.1  | 47.0  | 25.5 | 78.0 |
| *O. lacteum* | 10   | 3.6  | 20.8  | 19.2 | 40.6 | 17.7  | 71.2  | 31.9  | 13.9 | 56.0 |
| *E. loennbergi* | 15.5 | 2.6  | 55.1  | 37.7 | 51.0 | 43.2  | 98.6  | 43.4  | 36.1 | 82.3 |
| Mean       | 15.5       | 26.5  | 50.8  |     |     |       |      | 41.4  |      |      |
| Young      |            |        |         |      |      |         |      |      |
| *L. rubellus* | 21.7 | 5.7  | 33.8  | 29.1 | 67.9 | 26.9  | 88.9  | 55.2  | 21.8 | 72.2 |
| *O. lacteum* | 28.6 | 7.4  | 48.0  | 24.6 | 78.8 | 33.6  | 96.9  | 63.3  | 26.9 | 77.5 |
| *E. loennbergi* | 19.2 | 1.1  | 28.5  | 50.3 | 59.8 | 5.3   | 83.3  | 52.1  | 4.6  | 72.3 |
| Mean       | 23.2       | 34.7  | 68.8  |     |     |       |      | 56.7  |      |      |

Figure 4. Abundance of *Tulasnella* spp. at different soil depths. Points are mean ± 1 SE abundance for the four depth ranges. Abundance decreased exponentially with depth. Curve fit to the points is abundance = 1.551 × e⁻⁰·⁰²²¹⁷ × Depth \(r² = 0.999, P = 0.003\). The dashed vertical line indicates the depth below which *Tulasnella* abundance is too low to support *G. pubescens* germination.

Figure 5. Per cent change in the biomass of three earthworm species that was recovered at the end of the 6-week mesocosm experiment in soils from successional (grey bars) and mature (black bars) forests.

Table 2. Mean, high and low per cent loss of orchid seeds as a result of decreased viability and burial depth combined, by earthworm species (*L. rubellus*, *O. lacteum* and *E. loennbergi*) and soil type (mature or successional (young) forest soils).
found that host mycorrhizal fungus abundance decreased to ~0.5 ng g\(^{-1}\) dry soil. This is particularly important because McCormick et al. (2012) found that the abundance of orchid host fungi was critically important for determining whether they were able to support orchid germination and protocorm growth. The proportion of ingested beads buried in different sections differed among earthworm species and in different soils. In particular, E. lonnbergi distributed beads more equally among the depth sections in successional forest soils than the two non-native species, resulting in more beads being deposited both on the surface and more than 10 cm deep. In contrast, in soils from mature forests, E. lonnbergi egested fewer beads on the surface and more in the 2–5-cm section compared with other species (Fig. 2). This may suggest that E. lonnbergi is more plastic in its behaviour in response to soil characteristics than the two non-native species.

The proportion of beads ingested over the 6 weeks of our mesocosm experiment probably greatly underestimated what would be ingested in the field over the course of a full year. By the end of the experiment, earthworms had only consumed a small portion of the leaf litter we added to each mesocosm. In field sites with abundant earthworms, nearly all palatable leaf litter (petioles often remain) is usually consumed by mid-summer (Szlavecz et al. 2011, 2012), potentially increasing earthworm consumption of soil surface and orchid seeds. Rather than assuming that earthworms would actively seek out as yet uneaten leaf litter and fragments on the soil surface, we chose to represent their foraging activities conservatively, as if they were random. This again probably resulted in our underestimating the proportion of orchid seeds that would be ingested because directed foraging would result in a more thorough coverage of the soil surface and correspondingly higher ingestion of orchid seeds that had fallen there. Combined with the >70 % decrease in viability in seeds that passed through the earthworm digestive tract, burial of the remaining viable seeds could result in a significant loss of orchid seeds in forests supporting abundant earthworms.

Whether these results are applicable to other orchid species will require additional research. Whigham et al. (2006) found that many orchids, although not G. pubescens, can form a long-lived seedbank. If these buried seeds are subsequently moved to shallower soil layers, as through a soil disturbance, then they may germinate. However, if consumption by earthworms decreases the viability of other orchid seeds as much as it does for G. pubescens, it seems unlikely that earthworms moving seeds to the surface will result in significant subsequent germination.

Although mesocosms are artificial habitats, we only examined three earthworm species, and those species only individually, it is worth noting that non-native earthworms retained higher biomass (Fig. 5) in successional forest soils than the native E. lonnbergi did. This result suggests that non-native species would be able to maintain greater biomass than native earthworms in these forests. However, it is also possible that the native species simply responded more slowly to changing conditions, so it maintained closer to the added biomass in both successional and mature forest soils. The greater earthworm biomass supported in successional forest soil mesocosms (Fig. 5) was associated with somewhat greater ingestion of our seed analogue beads and so resulted in greater predicted loss of orchid seeds. Differences in bead burial among the soil types were likely driven more by their differential ability to support earthworm biomass and less by differences in earthworm behaviour as a function of soil type. One effect that may further exacerbate the differences we observed between successional and mature forest soils is that successional forest soils in the field also remained moister than mature forest soils throughout the year. This may allow earthworms to remain active longer during the summer when low soil moisture drives earthworms to aestivate or remain in deeper soils. Successional forests in the mid-Atlantic are also largely dominated by tree species with relatively labile leaf litter, which is preferred by earthworms (Sánchez-de León and Zou 2004). All these effects may act to decrease the abundance of forest orchids, especially in successional forests where non-native earthworms are abundant. Furthermore, while most orchids occur in older forests, much of the current landscape is composed of a mosaic of younger forests with low herbaceous plant diversity. Abundant non-native earthworms in these forests may act to slow the succession process, especially for plants with small seeds.

Conclusions

This study demonstrated that increased seed burial and decreased seed viability after consumption may contribute significantly to the decline of orchids in forests with abundant earthworms. We found that the combined effects of earthworm ingestion and burial have the potential to result in a substantial loss of orchid seeds. Our models estimated that 41 % of orchid seeds in mature forests and 57 % of those in successional forests would be lost to earthworm ingestion over the course of an average year (Table 2). The majority of these losses were predicted to come from decreased viability after passage through the earthworm gut. Between 19 and 50 % of seeds that remained viable were
predicted to be buried too deeply to form successful mycorrhizal associations. Both the proportion of seed analogues that were ingested and the depth at which they were deposited differed among earthworm species. However, our results are limited to a single orchid species and the findings need to be evaluated for other orchids, especially across a range of species that form seed banks. It is not clear whether burial will have the same effect on species with seeds that do not remain viable for long periods of time as on those with extended dormancy.

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**Contributions by the Authors**

All authors contributed significantly to the design of the experiment and all contributed to editing the manuscript. M.K.M. was primarily responsible for data analysis and modelling, wrote the manuscript, and contributed to laboratory and field work. K.L.P. performed most of the laboratory and field work and developed the methods used. K.S. contributed to field work, and she and D.F.W. assisted with site selection, method development and data analysis.

**Conflict of Interest Statement**

None declared.

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