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Earthworm mediated dispersal of baculovirus occlusion bodies: Experimental evidence from a model system

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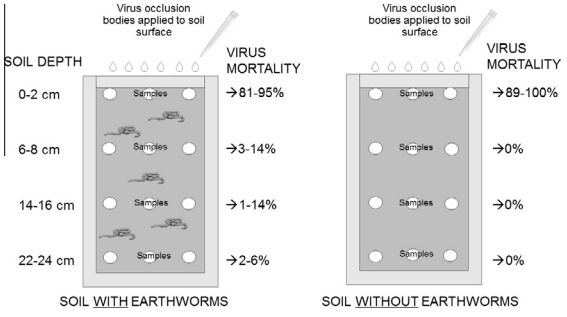
HIGHLIGHTS

- We demonstrate earthworm mediated transport of baculovirus occlusion bodies (OBs).
- *Eisenia fetida* transported 10^4–10^5 OBs/g to depths of 6–24 cm.
- Incubation of soil with earthworms was not detrimental to OB virulence.
- The earthworm intestine was found to be slightly acidic.

GRAPHICAL ABSTRACT



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ABSTRACT

The soil is the most important reservoir of baculovirus occlusion bodies (OBs) in the environment. The ability of the earthworm *Eisenia fetida* to transport OBs of *Spodoptera frugiperda* multiple nucleopolyhedrovirus was examined in laboratory terraria filled with an artificial soil. OBs were detected in soil samples using a soil-diet incorporation bioassay, for which the 50% lethal concentration was estimated at 2.7×10^6 OBs/g soil in *S. frugiperda* second instars. Incubation of earthworms in soil containing 10^9 OBs for 7 days did not result in a significant loss of OB virulence compared to soil without earthworms. The earthworm intestine was found to be slightly acidic, with acid-base indicators applied to lengths of dissected intestine suggesting a pH of 6.0–6.3. Despite their epigean habits, *E. fetida* individuals were observed to form burrows up to 22.5 cm deep in laboratory terraria. Soil-diet bioassays indicated the presence of OBs at depths of 6–8, 14–16 and 22–24 cm in samples taken at 1, 7 and 14 days following the application of 10^9 OBs to the surface of terraria containing earthworms. In contrast, OBs were only detected in samples from the soil surface in terraria without earthworms. We conclude that earthworms likely affect the distribution and dynamics of OB populations in soil habitats.

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1. Introduction

The baculovirus occlusion body (OB) is the key structure that allows these viruses to persist in an infective state outside of the insect host. The OB is also the unit of transmission of baculoviruses, as susceptible insect larvae must consume at least one, and possibly many OBs to acquire an infection (Harrison and Hoover, 2012). Once released from the cadaver of an infected insect, OBs contaminate plant foliage and ultimately arrive at the soil surface, mainly by the action of rainfall (D'Amico and Elkinton, 1995; Fuxa and Richter, 1996). OBs in the environment can be dispersed by abiotic

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factors, particularly rain and wind blown dust (Fuxa and Richter, 2001; Olofsson, 1988; Young and Yearian, 1986). Biotic factors can also be involved in OB dispersal. Predatory arthropods and birds that consume infected cadavers can excrete viable OBs in their feces over considerable distances (Entwistle et al., 1993; Lee and Fuxa, 2000a). This is because the acidic pH of the gut of these organisms does not degrade the occlusion matrix of the OB, so that occlusion derived virions (ODVs) remain viable following passage through the gut (Entwistle et al., 1978; Lee and Fuxa, 2000b).

Considering that the soil is the most important reservoir of OBs in the environment, the number and breadth of studies of OB populations in the soil are relatively sparse (reviewed by Fuxa, 2004). Early studies underlined the longevity of OBs in the soil (Fuxa and Geaghan, 1983; Hukuhara and Namura, 1972; Jaques, 1967; Thompson et al., 1981), and the importance of factors such as soil pH (Thomas et al., 1973), whereas later studies were able to quantify the movement of OBs from the soil on to surfaces of field crops (Fuxa, 2008; Fuxa and Richter, 2001, 2007). However, apart from some studies on predatory carabids (Capinera and Barbosa, 1975; Vasconcelos et al., 1996), the role of the soil fauna in the dispersal of OBs has been largely overlooked.

Given the paucity of studies on the role of soil invertebrates in baculovirus dispersal, the present study aimed to examine the potential of earthworms in the dispersal of OBs in the soil ecosystem. Ever since the pioneering studies by Darwin (1881), the role of earthworms in the breakdown of organic matter, nutrient cycling and the redistribution of soil particles has been recognized as a key contribution to defining soil texture, aeration, drainage, microbiota and soil fertility (Satchell, 1983). The part of the soil ecosystem that is influenced by earthworm activities is known as the drilosphere. One of the most studied species of earthworm is *Eisenia fetida*, a European species that is now distributed worldwide and commonly used for vermicomposting (Edwards et al., 2010). It is an epigean species that usually lives at the interface between the soil and the leaf litter or compost. It is readily cultivated in a diversity of organic wastes and is amenable to laboratory rearing using established techniques (Edwards and Bohlen, 1996).

To evaluate the ability of earthworms to transport OBs we used a model system consisting of *E. fetida* individuals from a vermicompost colony that were allowed to inhabit an artificial soil developed for testing the toxicity of pesticides to earthworm populations (OECD, 1984). The use of an artificial soil allowed us to overcome repeatability issues arising from the enormous variability in the composition and physico-chemical characteristics of natural soils (Edwards and Bohlen, 1992). The soil was contaminated with *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV) OBs. The fall armyworm, *S. frugiperda* (Lepidoptera: Noctuidae), is a major pest of maize from the southern United States to northern Argentina. SfMNPV OBs can be readily isolated from the soil of maize fields in the United States and Mexico (Fuxa et al., 1985; Rios-Velasco et al., 2011), and this virus has attracted the attention of biocontrol researchers as a potential biological insecticide in this region (Barrera et al., 2011; Williams et al., 1999). Laboratory studies were used to examine the effects of earthworm activity on the viability and vertical distribution of OBs.

2. Methods

2.1. Insects, earthworms, virus and soil

Larvae of *S. frugiperda* were obtained from a laboratory colony reared on a semisynthetic diet based on soya flour, wheat germ, yeast, agar, and vitamins (adapted from Greene et al., 1976). The colony was started using insects from a colony reared in the Universidad Michoacana de San Nicolás de Hidalgo, Michoacán, Mexico. Our colony was reared in an insectary at 25 ± 2 °C, 70–90% relative humidity and 16:8 h L:D photoperiod.

Specimens of *E. fetida* were obtained from a small scale vermicomposting plant used for processing coffee berry pulp in the Instituto de Ecología AC, Xalapa, Mexico. Earthworms were maintained in the insectary in plastic containers with ~50% coffee berry pulp supplemented with ~50% cow manure for 3–4 weeks prior to use in experiments.

A Nicaraguan wild-type isolate of SfMNPV (Simón et al., 2004, 2008) was amplified in *S. frugiperda* by the diet surface contamination technique. For this, fourth instar larvae were individually placed in 15 ml plastic cups and allowed to feed on a 2 g cube of diet contaminated with 5×10^8 OBs, at 25 ± 2 °C, 70–90% relative humidity and 16:8 h L:D photoperiod. The diet was replaced with non-contaminated diet after 4 days and virus-induced mortality was checked daily for the following 6 days. Virus killed insects were triturated in 0.1% (vol./vol.) Tween 80, filtered through a 80 μm pore nylon mesh, centrifuged at 2300*g* for 5 min and washed three times in sterile distilled water. The resulting OB suspension was counted in triplicate using a Neubauer chamber under a phase contrast microscope and stored at 4 °C for up to one month prior to use in experiments, without significant loss of OB activity.

An artificial soil was prepared according to the OECD pesticide testing protocol (OECD, 1984). Briefly, the dry components (70% wt/wt washed sand, 20% wt/wt kaolin, 10% wt/wt sphagnum peat) were mixed thoroughly using a kitchen spatula in a bucket and passed through a 2 mm pore sieve. The pH was measured and found to be within recommended limits (pH 7.0 ± 0.5). The soil was stored dry in the laboratory for up to one month until required in experiments.

2.2. Calibration of the OB detection technique

The presence of OBs in soil was detected using the soil-diet incorporation technique in which samples are mixed with insect diet and fed to early instar larvae that may become infected if OBs are present (Richards and Christian, 1999). To estimate the sensitivity of the OB detection technique, 10 g samples of soil were mixed with 1 ml of one of the following concentrations of SfMNPV OBs: 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8, 1×10^9 OBs/ml, resulting in a range of concentrations of 10^1–10^8 OBs/g of soil.

Semisynthetic diet was prepared and when cooled to 45 °C each 10 g soil sample was incorporated into a 10 ml volume of diet. Each mixture was divided among 30 *S. frugiperda* second instars from the laboratory colony. After feeding individually on contaminated diet for 4 days, larvae were transferred to non-contaminated diet, reared individually and checked daily for virus-induced mortality until death or pupation. The procedure was performed on six occasions for all concentrations. Polyhedrosis disease deaths were confirmed by observation of Giemsa-stained smears using a phase contrast microscope (Lacey, 1997). The OB concentration-mortality response was estimated by logit regression in GLIM 4 (Numerical Algorithms Group, 1993).

2.3. Estimation of earthworm intestinal pH

As OBs are sensitive to alkaline conditions, the pH of earthworm gut was estimated using the following acid-base indicators: 0.1% methyl orange (pH 2.5–4.4), 0.5% wt/vol Congo red (pH 3.0–5.0), 0.1% bromocresol green (pH 4.5–5.5), 0.1% methyl red (pH 4.8–6.0), 0.1% bromothymol blue (6.0–7.6), 0.1% indigo carmine (pH 11.4–13.0). Earthworms were incubated in Petri dishes with moist filter paper discs for 24 h prior to evacuate most of the intestinal tract. Each earthworm was then sacrificed by immersion for 5 s in hot water and a section comprising the foregut (posterior to

the gizzard) and midgut was removed by ventral dissection and placed in a drop of indicator solution for 5 min. After this period the gut was examined using a binocular microscope and the color present in the lumen was determined. A total of 20 clitellate earthworms were used to evaluate intestinal pH for each indicator.

2.4. Effect of incubating earthworms with OB-contaminated soil

To determine whether earthworm feeding or mucus secretions affected the viability of OBs in soil, individual clitelliate earthworms (mean live weight ± SE: 193.5 ± 9.1 mg) were each placed in a 7 cm plastic Petri dish with moist filter paper in darkness for four days at 25 ± 1 °C to allow earthworms to empty their intestine. Following this earthworms were assigned to one of two treatments: (i) OBs in soil with an earthworm, (ii) OBs in soil with no earthworm present, (iii) soil with an earthworm but without OBs (control). For the first treatment, 10 g of soil was placed in a 20 ml plastic cup, 10 ml potable water was added and 1 ml of a suspension of 1×10^9 OBs. These substances were mixed thoroughly for 2 min using a disposable plastic teaspoon that was subsequently discarded. A clitelliate earthworm was placed in the cup, which was sealed with a perforated lid for aeration and incubated in darkness at 25 ± 1 °C for 7 days. The procedure was repeated using sterile water for the control. After this period, the earthworm was removed and placed individually in a Petri dish with moist filter paper for 4 days to collect feces, that were dried for 24 h at 50 °C and weighed using an electronic balance. The soil that had been inhabited by the earthworm for 7 days was mixed with 50 ml of diet and subjected to the soil incorporation bioassay procedure using a group of 30 *S. frugiperda* second-instars that were reared individually as described in Section 2.1. Virus-induced mortality was recorded and confirmed as described previously. Each treatment was replicated 15 times in total (5 times on each of three occasions). The results were used to fit a generalized linear model in GLIM 4 with a binomial error structure specified. The significance of treatment effects was determined by *t*-test.

2.5. Burrowing behavior of earthworms

To determine the range of depths to which *E. fetida* might disperse OBs in soil, it was necessary to estimate the depth to which earthworms would burrow under laboratory conditions. For this, experimental units (terraria) were constructed using two glass plates 30 cm height × 15 cm width. A 1 cm thick × 1 cm wide U-shaped piece of expanded polystyrene was cut using a hot wire. The polystyrene was sandwiched between the plates of glass to form the sides and base of the experimental unit, that was held in place using four large metal binder clips, one at each corner. The unit was filled with 250 g of dry artificial soil and 250 ml of non-chlorinated potable water was added to moisten the soil. The soil level was approximately 2 cm from the top of the terrarium. Groups of 10 clitellate earthworms were placed on the surface of the soil of each terrarium and allowed to burrow into the soil in a dark bioclimatic chamber at 25 ± 1 °C. The maximum depth of earthworm burrows was observed through the sides of each terrarium and recorded at 2, 24, 48 and 72 h after introducing the earthworms. The process was replicated using eight terraria.

2.6. Earthworm mediated dispersal of OBs

To evaluate the ability of earthworms to disperse OBs, 45 terraria were constructed as described in the previous section. Each terrarium was filled with 250 g artificial soil and 250 ml potable water. Fifteen terraria were each assigned to one of the following three treatments: (i) OBs in a terrarium without earthworms, (ii) OBs in terrarium with earthworms, (iii) a terrarium with earthworms but without OBs (control). In treatments with OBs, a 1 ml suspension of 1×10^9 OBs was applied in drops to the soil surface using a micropipette. In the control treatment 1 ml of sterile water was applied using the same technique. One hour after having applied OB suspension, a group of 30 small earthworms, approximately 5 cm in length and ~300 mg each live weight, was placed on the soil surface of each terrarium. Terraria were placed upright in a bioclimatic chamber in darkness at 25 ± 1 °C. The top of each terrarium was loosely closed with a 1 cm wide strip of polystyrene to reduce evaporation.

To estimate earthworm-mediated movement of OBs, five terraria were selected at random from each treatment at 1, 7 and 15 days after the start of the experiment. On each sample occasion, the terrarium was placed flat on the laboratory bench, the binder clips were removed and the uppermost glass plate was carefully lifted off the terrarium, from left to right (as if turning the page of a book), to avoid any risk of soil from the upper OB contaminated layer falling onto the lower layers during opening. Three subsamples of 2 g of soil were then taken at equidistant points at a depth of 0–2 cm from the soil surface. These subsamples were pooled to produce one 6 g sample at the indicated depth. This procedure was also performed at depths of 6–8 cm, 14–16 cm and 22–24 cm from the soil surface, so that a total of four samples were obtained from each terrarium. Each subsample was taken with a disposable plastic teaspoon that was discarded after taking each subsample. Once sampled, each terrarium was discarded. As such, the experiment consisted of 3 treatments × 3 sample times that was replicated on five occasions over time (45 experimental units in total), and each experimental unit was sampled at 4 soil depths, resulting in a total of 180 samples. Bioassays were performed on each sample using the diet incorporation technique. Each soil-diet mixture was fed to 30 *S. frugiperda* second instars that fed on contaminated diet for 4 days before being reared on non-contaminated diet for a further 6 days. Virus-induced mortality was recorded daily and confirmed by microscopic examination of Giemsa-stained smears. The prevalence of virus-induced mortality in experimental insects was analyzed by fitting a generalized linear model with a binomial error structure specified in GLIM 4. The significance of changes in model deviance resulting from the stepwise removal of treatment terms during model simplification was estimated by reference to the χ^2 distribution. The suitability of the model was determined by examining residuals and the distribution of fitted and experimental values.

3. Results

3.1. Calibration of the OB detection technique

Virus-induced mortality varied from an average of 1.4–99% in diet–soil mixtures treated with 10^4–10^8 OBs/g soil. Virus-killed insects were not observed at lower concentrations in soil–diet mixtures (10^1–10^3 OBs/g). The LC_{50} of OBs in soil was estimated at 2.7×10^6 OBs/g soil (range of 95% confidence interval: 1.8×10^6–4.1×10^6). The slope and intercept of the fitted regression were 0.8564 ± 0.0882 and −14.66 ± 1.510, respectively (Fig. 1). The error distribution was scaled by a factor of 2.07 to account for moderate overdispersion in the results. No virus mortality was observed in control insects that fed on diet + soil mixtures without OBs.

3.2. Estimation of earthworm intestinal pH

The earthworm intestine was found to be slightly acidic. Slightly variable responses were observed following treatment with Methyl red indicating that the majority of earthworms (17 out of 20) had a gut pH in the range 6.0–6.3, and 3 out of 20

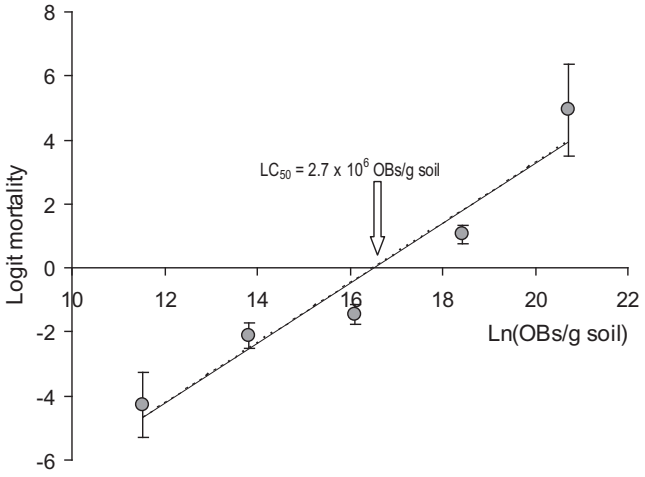

Fig. 1. *Spodoptera frugiperda* multiple nucleopolyhedrovirus occlusion body concentration–mortality relationship in the soil + diet mixture bioassay following logit analysis.

earthworms had a pH of slightly over pH 6.3, which was consistent with the results obtained with Congo red, in which all individuals tested showed a pH > 5.0.

3.3. Effect of incubating earthworms with OB-contaminated soil

The average prevalence of mortality of insects that fed on soil-diet mixtures was 98% (Fig. 2), and did not differ significantly between soil in which earthworms had been active for 7 days and soil without the presence of earthworms ($t = 0.12$, df = 14, $p = 0.45$). No virus-induced mortality was observed in control larvae that fed on the diet-soil mixture without OBs confirming that the bioassay insects were healthy and no cross-contamination had occurred during the bioassay procedure. These results indicated that the OBs that had been exposed to earthworm exudates and/or passage through the earthworm gut did not experience a reduction in viability. The average dry weight (±SE) of feces expelled from earthworm intestines was 15.6 ± 1.9 mg/individual, which was found to be too small a quantity to use in the soil-diet bioassay procedure.

3.4. Burrowing behavior of earthworms

The maximum burrow depth (mean ± SE) observed in terraria was 0 cm at 2 h, 7.3 ± 1.9 cm at 24 h, 18.5 ± 2.4 at 48 h and 22.5 ± 1.7 cm after 72 h. Consequently, the sampling regime in the following experiment was based on samples taken at depths of 0–2, 6–8, 14–16 and 22–24 cm, as described in the Methods section.

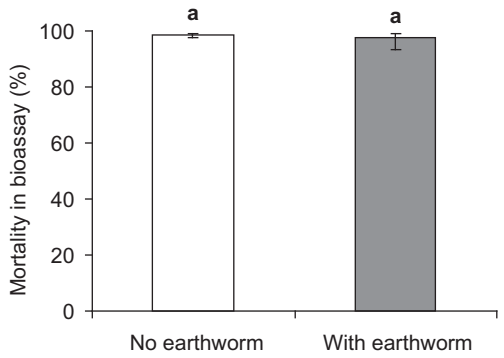

Fig. 2. Mortality of *Spodoptera frugiperda* second instars in bioassay of occlusion body contaminated soil inhabited by *Eisenia fetida* during 7 days compared to control soil without earthworm. Columns labeled with identical letters did not differ significantly (generalized linear model analysis, P > 0.05).

3.5. Earthworm mediated dispersal of OBs: laboratory study

Earthworms were capable of vertical redistribution of OBs in experimental terraria (Fig. 3). Larval mortality due to lethal polyhedrosis was significantly affected by the presence/absence of earthworms ($\chi^2 = 4.35$, df = 1, $p = 0.037$), depth of the soil sample ($\chi^2 = 1324$, df = 3, $p < 0.001$), sample time ($\chi^2 = 8.85$, df = 2, $p = 0.012$), and by the interactions of depth × time ($\chi^2 = 21.1$, df = 6, $p < 0.001$) and earthworms × depth ($\chi^2 = 47.7$, df = 3, $p < 0.001$). Virus induced mortality was very high (>90%) in the uppermost 2 cm of soil at all samples times in the absence of earthworms, reflecting the high quantity of OBs used to inoculate terraria (Fig. 3). Accordingly, OBs were detected in the samples taken at depths of 6–8 and 14–16 cm, and even in samples taken at 22–24 cm, albeit at a low prevalence, as a result of earthworm mediated dispersal of OB-contaminated surface soil. The

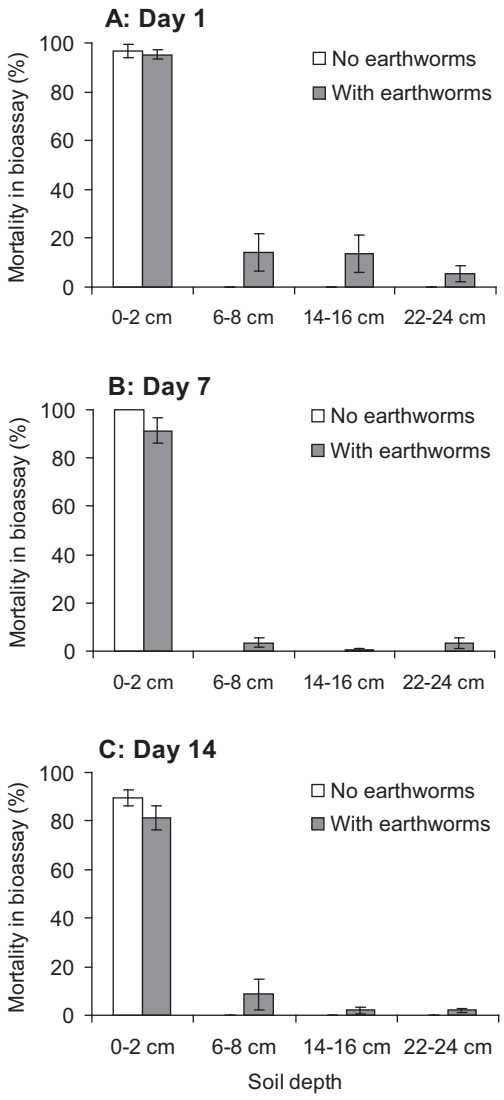

Fig. 3. Influence of the presence of earthworms in laboratory terraria surface contaminated with *Spodoptera frugiperda* multiple nucleopolyhedrovirus occlusion bodies.

prevalence of between 2 and 14% virus mortality in the subsurface samples taken at depths of 6–24 cm is consistent with OB concentrations of approximately 10^4–10^5 OBs/g soil, as indicated by the results of the soil bioassay calibration. No virus deaths were observed in control larvae that fed on the diet-soil mixture without OBs confirming that the bioassay insects were healthy and no cross-contamination had occurred during the bioassay procedure.

4. Discussion

Laboratory studies revealed that the earthworm *E. fetida* is capable of redistributing nucleopolyhedrovirus OBs in a soil habitat. To our knowledge this is the first attempt to demonstrate earthworm mediated OB dispersal. In this respect, the present study represents a proof-of-concept study rather than a quantitative estimate of OB dispersal by earthworms.

The findings of our study highlight the likely importance of the earthworm fauna in modulating the distribution and dynamics of virus OB populations in the soil. This is because of the combination of the quantities of OBs arriving at the soil surface and the abundance and activity of earthworm populations in agricultural and forest ecosystems. The majority of OBs produced in infected phytophagous insects is likely to arrive at the soil surface having been washed by rainfall or by OB-contaminated leaves that senesce and fall from the plant onto the soil. Those OBs that have not been previously inactivated by exposure to ultraviolet solar radiation (Jones et al., 1993), or chemically inactivated by plant secondary chemicals (Hoover et al., 1998), are likely to persist in the soil for extended periods (Jaques, 1974; Thompson et al., 1981).

Our findings have a number of implications for soil OB dynamics that require further study. First, earthworms may assist in the establishment and persistence of soil OB populations by covering the soil surface with castings, thus protecting surface OBs from exposure to UV radiation and the extremes of temperature. Second, the habits of some earthworm species to take surface leaf litter into their burrow likely further contribute to the dispersal of OBs within the drilosphere. Moreover, for soil-dwelling insect larvae such as *Tipula* spp. or *Wiseana cervinata* that can experience epizootics of nucleopolyhedrosis disease (Carter et al., 1983; Fleming et al., 1986), earthworm mediated changes to the vertical distribution of OBs could have a marked influence on the probability of acquiring a lethal infection particularly when the distribution of OBs and of susceptible insects is vertically stratified in some types of soil habitat (Kalmakoff and Crawford, 1982).

Earthworms are also likely to interact frequently with soil OB reservoirs due the abundance of these organisms in agricultural and forest ecosystems. Earthworm populations are typically present at densities of 10–200 individuals/m^2 in agricultural and forest soils or 100–1000 individuals/m^2 in undisturbed soils, such as those of pastures (Edwards, 2004). Moreover, such populations can turnover 5–100 tonnes of soil/year in temperate zones and considerably more in tropical regions, resulting in a 2–5 cm thick layer of earthworm casts each year (Edwards and Bohlen, 1996).

The use of *E. fetida*, a composting earthworm with very limited burrowing habits, as the study species was due mainly to the fact that this species is easily reared and manipulated in the laboratory. Despite its limited burrowing habits, earthworms burrowed to depths of > 22 cm in the laboratory terraria. This species also survived in the nutritionally poor artificial soil during experiments. The artificial OECD-defined soil, designed for comparison of the results of pesticide tests on earthworm fauna between different laboratories, allowed accurate replication of experimental conditions, albeit with clear limitations regarding the similarity of earthworm behavior in the artificial substrate compared with that of a natural soil. In this respect, the findings of the present study should be validated using a range of natural soils, given that the composition and physical and chemical properties of natural soils can have an important influence on earthworm behavior (Edwards and Bohlen, 1992) and OB stability (Jaques, 1985).

The soil-diet bioassay technique for detection of SfMNPV OBs was found to result in 50% mortality of in second instar *S. frugiperda* larvae at a concentration of approximately 3×10^6 OBs/g soil. This was clearly an approximation as the range of OB concentrations used was very large, but allowed us to identify potentially informative concentrations of OBs for use in the remaining studies. The soil-diet technique applied to SfMNPV was markedly lower than that reported with other nucleopolyhedroviruses. The technique developed by Richards and Christian (1999) for detection of HearNPV using first instar *Helicoverpa armigera* larvae reported an LC_{50} of 2.1×10^3 OBs/g soil. Similarly, when this technique was employed for detection of SeMNPV in first instar larvae of *Spodoptera exigua*, Murillo et al. (2006) reported an LC_{50} value of 2.7×10^3 OBs/g in greenhouse soil substrates. The higher LC_{50} value of the SfMNPV soil-diet bioassay reflects a combination of two factors: (i) the lower virulence of SfMNPV OBs compared to that of the other viruses and (ii) the use of second instars rather than first instars in our bioassay, as the amount of inoculum required to initiate a lethal nucleopolyhedrovirus infection increases with instar in SfMNPV and most other nucleopolyhedroviruses (Escribano et al., 1999). However, the SfMNPV soil-diet bioassay provided consistent results and provided clear evidence of earthworm-mediated dispersal of OBs in laboratory terraria.

The use of acid-base indicators revealed that the *E. fetida* intestine was slightly acidic. This is compatible with the passage of OBs that can withstand exposure to the acidic gut conditions of predatory insects (Abbas and Boucias, 1984; Beekman, 1980; Vasconcelos et al., 1996), and birds (Entwistle et al., 1993). We previously used a similar range of acid-base indicators to estimate the gut conditions of earwig and neuropteran predators of *S. frugiperda* in southern Mexico (Castillejos et al., 2001). Although it provides only an approximate estimate of gut pH, this technique has the advantage of being very straightforward and allows a representative number of samples to be assessed in a short period of time.

Petri dish tests with OB contaminated soil revealed no significant reduction in OB virulence, as determined by the soil-diet bioassay. This indicates that a combination of exposure to earthworm exudates and passage through the gut (for that fraction of the soil that was consumed by earthworms), was not detrimental to OB populations during the 7-day experimental period. Exposure of OBs to earthworm exudates was an issue of interest given that *E. fetida* mucus secretions have antimicrobial activity (Wang et al., 2011), and its coelomic fluid has antiviral activity (Wang et al., 2007). The activity of OBs in earthworm feces was not determined directly as the very small quantities of fecal material produced by each earthworm precluded the use of the diet incorporation bioassay, which requires larger quantities of substrate to produce sufficient inoculum to feed to suitable numbers of larvae (~30) for an informative bioassay of OB activity. In a recent study on dispersal of the fungal pathogen *Beauveria bassiana* by *Lumbricus terrestris* in a natural soil, earthworm casts were shown to contain viable conidia using a combination of selective media and insect bioassay techniques (Shapiro-Ilan and Brown, 2013). Similarly, studies on the interaction of *Bacillus thuringiensis* var. *kurstaki* and common earthworms in a temperate region revealed the presence of vegetative bacterial cells in earthworm guts followed by sporulation in earthworm feces, suggesting a role of earthworm activities in the environmental persistence of the entomopathogen (Hendriksen and Hansen, 2002). In contrast, a study on the relationship between *E. fetida* and the nematode *Steinernema feltiae* indicated that the capacity of infective juveniles to establish infection in

Spodoptera littoralis larvae was markedly decreased following passage through the earthworm gut, presumably due to the action of digestive enzymes (Campos-Herrera et al., 2006). Given these findings it is clear that earthworms have the potential to modulate the pest control capacity of various entomopathogens, although in all cases the results of laboratory experiments require validation in field studies.

In laboratory terraria, OBs were detected at low concentrations at depths of up to 22–24 cm in samples taken 1, 7 and 15 days after the beginning of the experiment, although no consistent pattern was evident in the accumulation of OBs at different depths over time. This likely reflects the poor burrowing habits of *E. fetida* that normally resides on, or close to, the soil surface (Edwards, 2004). OB contamination was detected at depths greater than observed in preliminary trials as the density of earthworms used in OB dispersal studies was higher than that used in initial observations on earthworm burrowing activity. The transport of OBs through the soil likely involved a combination of adhesion to the external surfaces or mucus secretions of the earthworm and the release of OB-contaminated feces at different depths in the soil. Previous studies on two common earthworm species, demonstrated improved vertical movement of several entomopathogenic nematode species in soil columns in the laboratory, which was attributed to a phoretic association between earthworms and infective juvenile nematodes (Shapiro et al., 1993, 1995). Clear evidence of vertical dispersal of viable *B. bassiana* conidia in laboratory soil columns was also observed in the presence of *L. terrestris* (Shapiro-Ilan and Brown, 2013).

We conclude that laboratory studies indicate that earthworms could contribute to the dynamics of baculovirus OB populations in soils. Future studies in natural soil habitats should focus on the local earthworm communities present and their capacity to disperse OBs, particularly in agricultural settings in which baculovirus-mediated pest mortality can contribute to pest control.

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References

Abbas, M.S.T., Boucias, D.G., 1984. Interaction between nuclear polyhedrosis virus-infected *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) larvae and predator *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae). Environ. Entomol. 13, 599–602.

Barrera, G., Simón, O., Villamizar, L., Williams, T., Caballero, P., 2011. *Spodoptera frugiperda* multiple nucleopolyhedrovirus as a potential biological insecticide: genetic and phenotypic comparison of field isolates from Colombia. Biol. Control 58, 113–120.

Beekman, A.G.B., 1980. The infectivity of polyhedral of nuclear polyhedrosis virus (N.P.V.) after passage through the gut of an insect predator. Experientia 36, 858–859.

Campos-Herrera, R., Trigo, D., Gutiérrez, C., 2006. Phoresy of the entomopathogenic nematode *Steinernema feltiae* by the earthworm *Eisenia fetida*. J. Invertebr. Pathol. 92, 50–54.

Capinera, J.L., Barbosa, P., 1975. Transmission of nuclear-polyhedrosis virus to gypsy moth larvae by *Calosoma sycophanta*. Ann. Entomol. Soc. Am. 68, 593–594.

Carter, J.B., Green, E.I., Kirkham, A.J., 1983. A *Tipula paludosa* population with a high incidence of two pathogens. J. Invertebr. Pathol. 42, 312–318.

Castillejos, V., García, L., Cisneros, J., Goulson, D., Caballero, P., Cave, R.D., Williams, T., 2001. The potential of *Chrysoperla rufilabris* and *Doru taeniatum* as agents for dispersal of *Spodoptera frugiperda* nucleopolyhedrovirus in maize. Entomol. Exp. Appl. 98, 353–359.

D'Amico, V., Elkinton, J.S., 1995. Rainfall effects on transmission of gypsy moth (Lepidoptera: Lymantriidae) nuclear polyhedrosis virus. Environ. Entomol. 24, 1144–1149.

Darwin, C., 1881. The Formation of Vegetable Mould, Through the Action of Worms, With Observations on Their Habits. John Murray, London.

Edwards, C.A. (Ed.), 2004. Earthworm Ecology. CRC Press, Boca Raton, FL.

Edwards, C.A., Bohlen, P.J., 1992. The effects of toxic chemicals on earthworms. Rev. Environ. Contam. Toxicol. 125, 23–99.

Edwards, C.A., Bohlen, P.J., 1996. Biology and Ecology of Earthworms, third ed. Chapman & Hall, London.

Edwards, C.A., Arancon, N.Q., Sherman, R.L. (Eds.), 2010. Vermiculture Technology: Earthworms, Organic Wastes, and Environmental Management. CRC Press, Boca Raton, FL.

Entwistle, P.F., Adams, P.H.W., Evans, H.F., 1978. Epizootiology of a nuclear polyhedrosis virus in European spruce sawfly (*Gilpinia hercyniae*): the rate of passage of infective virus through the gut of birds during cage tests. J. Invertebr. Pathol. 31, 307–312.

Entwistle, P.F., Forkner, A.C., Green, B.M., Cory, J.S., 1993. Avian dispersal of nuclear polyhedrosis viruses after induced epizootics in the pine beauty moth, *Panolis flammea* (Lepidoptera: Noctuidae). Biol. Control 3, 61–69.

Escribano, A., Williams, T., Goulson, D., Cave, R.D., Chapman, J.W., Caballero, P., 1999. Selection of a nucleopolyhedrovirus for control of *Spodoptera frugiperda* (Lepidoptera: Noctuidae): structural, genetic, and biological comparison of four isolates from the Americas. J. Econ. Entomol. 92, 1079–1085.

Fleming, S.B., Kalmakoff, J., Archibald, R.D., Stewart, K.M., 1986. Density-dependent virus mortality in populations of *Wiseana* (Lepidoptera: Hepialidae). J. Invertebr. Pathol. 48, 193–198.

Fuxa, J.R., 2004. Ecology of insect nucleopolyhedroviruses. Agric. Ecosyst. Environ. 103, 27–43.

Fuxa, J.R., 2008. Threshold concentrations of nucleopolyhedrovirus in soil to initiate infections in *Heliothis virescens* on cotton plants. Microb. Ecol. 55, 530–539.

Fuxa, J.R., Geaghan, J.P., 1983. Multiple-regression analysis of factors affecting prevalence of nuclear polyhedrosis virus in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations. Environ. Entomol. 12, 311–316.

Fuxa, J.R., Richter, A.R., 1996. Effect of agricultural operations and precipitation on vertical distribution of a nuclear polyhedrosis virus in soil. Biol. Control 6, 324–329.

Fuxa, J.R., Richter, A.R., 2001. Quantification of soil-to-plant transport of recombinant nucleopolyhedrovirus: effects of soil type and moisture, air currents, and precipitation. Appl. Environ. Microbiol. 67, 5166–5170.

Fuxa, J.R., Richter, A.R., 2007. Effect of nucleopolyhedrovirus concentration in soil on viral transport to cotton (*Gossypium hirsutum* L.) plants. Biocontrol 52, 821–843.

Fuxa, J.R., Warren, G.W., Kawanishi, C., 1985. Comparison of bioassay and enzyme-linked immunosorbent assay for quantification of *Spodoptera frugiperda* nuclear polyhedrosis virus in soil. J. Invertebr. Pathol. 46, 133–138.

Greene, G.L., Leppla, N.C., Dickerson, W.A., 1976. Velvetbean caterpillar: a rearing procedure and artificial medium. J. Econ. Entomol. 69, 487–488.

Harrison, R., Hoover, K., 2012. Baculoviruses and other occluded insect viruses. In: Vega, F.E., Kaya, H.K. (Eds.), Insect Pathology, second ed. Academic Press, San Diego, CA, pp. 73–131.

Hendriksen, N.B., Hansen, B.M., 2002. Long-term survival and germination of *Bacillus thuringiensis* var. *kurstaki* in a field trial. Can. J. Microbiol. 48, 256–261.

Hoover, K., Kishida, K.T., DiGiorgio, L.A., Workman, J., Alaniz, S.A., Hammock, B. D., Duffey, S.S., 1998. Inhibition of baculoviral disease by plant-mediated peroxidase activity and free radical generation. J. Chem. Ecol. 24, 1949–2001.

Hukuhara, T., Namura, H., 1972. Distribution of a nuclear-polyhedrosis virus of the fall webworm, *Hyphantria cunea*, in soil. J. Invertebr. Pathol. 19, 308–316.

Jaques, R.P., 1967. The persistence of a nuclear polyhedrosis virus in the habitat of the host insect, *Trichoplusia ni* II: polyhedra in soil. Can. Entomol. 99, 820–829.

Jaques, R.P., 1974. Occurrence and accumulation of viruses of *Trichoplusia ni* in treated field plots. J. Invertebr. Pathol. 23, 140–152.

Jaques, R.P., 1985. Stability of insect viruses in the environment. In: Maramorosch, K., Sherman, K.E. (Eds.), Viral Insecticides for Biological Control. Academic Press, San Diego, pp. 285–360.

Jones, K.A., Moawad, G., McKinley, D.J., Grzywacz, D., 1993. The effect of natural sunlight on *Spodoptera littoralis* nuclear polyhedrosis virus. Biocontr. Sci. Technol. 3, 189–197.

Kalmakoff, J., Crawford, A.M., 1982. Enzootic virus control of *Wiseana* spp. in the pasture environment. In: Kurstak, E. (Ed.), Microbial and Viral Pesticides. Dekker, New York, pp. 435–438.

Lacey, L.A. (Ed.), 1997. Manual of Techniques in Insect Pathology. Academic Press, San Diego, CA.

Lee, Y., Fuxa, J.R., 2000a. Transport of wild-type and recombinant nucleopolyhedroviruses by scavenging and predatory arthropods. Microb. Ecol. 39, 301–313.

Lee, Y., Fuxa, J.R., 2000b. Ingestion and defecation of recombinant and wild-type nucleopolyhedroviruses by scavenging and predatory arthropods. Environ. Entomol. 29, 950–957.

Murillo, R., Elvira, S., Muñoz, D., Williams, T., Caballero, P., 2006. Genetic and phenotypic variability in *Spodoptera exigua* nucleopolyhedrovirus isolates from greenhouse soils in southern Spain. Biol. Control 38, 157–165.

Numerical Algorithms Group, 1993. In: Francis, B., Green, M., Payne, C. (Eds.), The GLIM System: Release 4 Manual. Oxford University Press, Oxford, United Kingdom.

OECD, 1984. Earthworm, Acute Toxicity Test. Guidelines for Testing of Chemicals N °207. Organization for Economic Cooperation and Development (OECD), Paris, France, 9 pp.

Olofsson, E., 1988. Dispersal of the nuclear polyhedrosis virus of *Neodiprion sertifer* from soil to pine foliage with dust. Entomol. Exp. Appl. 46, 181–186.

Richards, A.R., Christian, P.D., 1999. A rapid bioassay screen for quantifying nucleopolyhedroviruses (Baculoviridae) in the environment. J. Virol. Meth. 82, 63–75.

Rios-Velasco, C., Gallegos-Morales, G., Rincón-Castro, M.C.D., Cerna-Chávez, E., Sánchez-Peña, S.R., Siller, M.C., 2011. Insecticidal activity of native isolates of *Spodoptera frugiperda* multiple nucleopolyhedrovirus from soil samples in Mexico. Fla. Entomol. 94, 716–718.

Satchell, J.E., 1983. Earthworm Ecology: From Darwin to Vermiculture. Chapman & Hall, London, 496 pp.

Shapiro, D.I., Berry, E.C., Lewis, L.C., 1993. Interactions between nematodes and earthworms: enhanced dispersal of *Steinernema carpocapsae*. J. Nematol. 25, 189–192.

Shapiro, D.I., Tylka, G.L., Berry, E.C., Lewis, L.C., 1995. Effects of earthworms on the dispersal of *Steinernema* spp. J. Nematol. 27, 21–28.

Shapiro-Ilan, D.I., Brown, I., 2013. Earthworms as phoretic hosts for *Steinernema carpocapsae* and *Beauveria bassiana*: implications for enhanced biological control. Biol. Control 66, 41–48.

Simón, O., Williams, T., López-Ferber, M., Caballero, P., 2004. Genetic structure of a *Spodoptera frugiperda* nucleopolyhedrovirus population: high prevalence of deletion genotypes. Appl. Environ. Microbiol. 70, 5579–5588.

Simón, O., Williams, T., López-Ferber, M., Taulemesse, J.M., Caballero, P., 2008. Population genetic structure determines the speed of kill and occlusion body production in *Spodoptera frugiperda* multiple nucleopolyhedrovirus. Biol. Control 44, 321–330.

Thomas, E.D., Reichelderfer, C.F., Heimpel, A.M., 1973. The effect of soil pH on the persistence of cabbage looper nuclear polyhedrosis virus in soil. J. Invertebr. Pathol. 21, 21–25.

Thompson, C.G., Scott, D.W., Wickman, B.E., 1981. Long-term persistence of the nuclear polyhedrosis virus of the Douglas-fir tussock moth, *Orgyia pseudotsugata* (Lepidoptera: Lymantriidae), in forest soil. Environ. Entomol. 10, 254–255.

Vasconcelos, S.D., Williams, T., Hails, R.S., Cory, J.S., 1996. Prey selection and baculovirus dissemination by carabid predators of Lepidoptera. Ecol. Entomol. 21, 98–104.

Wang, C., Sun, Z., Liu, Y., Zhang, X., Xu, G., 2007. A novel antimicrobial vermipeptide family from earthworm *Eisenia fetida*. Eur. J. Soil Biol. 43, S127–S134.

Wang, C., Sun, Z., Zheng, D., Liu, X., 2011. Function of mucilaginous secretions in the antibacterial immunity system of *Eisenia fetida*. Pedobiologia 54, S57–S62.

Williams, T., Goulson, D., Caballero, P., Cisneros, J., Martínez, A.M., Chapman, J.W., Roman, D.X., Cave, R.D., 1999. Evaluation of a baculovirus bioinsecticide for small-scale maize growers in Latin America. Biol. Control 14, 67–75.

Young, S.Y., Yearian, W.C., 1986. Movement of a nuclear polyhedrosis virus from soil to soybean and transmission in *Anticarsia gemmatalis* (Hübner) (Lepidoptera: Noctuidae) populations on soybean. Environ. Entomol. 15, 573–580.