

Article

Nematodes Associated with Terrestrial Slugs in Mid-Atlantic (Delaware, USA) Soybean

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Abstract: Slugs are cryptic terrestrial pests that target a wide range of crops and are especially damaging to seedlings. Management of these invertebrates mostly relies on synthetic chemistry. These molecules can be efficient against slugs and snails but can be toxic to other organisms (e.g., dogs) and harmful to the environment (e.g., leaching into surface and groundwater). The usage of pathogenic nematodes has been effective in several crops and European countries. A survey was conducted to investigate the presence of natural populations of malacopathogenic nematodes in soybean in the mid-Atlantic region. Slugs were sampled in nine fields across Delaware at various distances from the field edges (0 m, 5 m, 10 m, 15 m). Soil cover and soil type were also recorded. Invertebrates were brought back to the laboratory. Slugs were monitored for four weeks, and mortality was classified into one of three categories: (1) death with the presence of nematodes; (2) death with the presence of fungi; (3) death without the presence of nematodes or fungi. Nematodes associated with slugs were identified based on 18S rRNA sequencing. The distance from the field edge did not impact the number of trapped slugs and the incidence of slug death associated with the presence of nematodes. Overall, nematodes were collected from ca. 20% of the slug cadavers, and most have previously been associated with slugs (ca. 35% of deaths associated with fungi and ca. 45% not associated with nematodes or fungi). The number of captured slugs and slug death associated with the presence of nematodes were positively correlated with ground cover. Soil type impacted both the number of captured slugs and the presence of pathogenic nematodes. This survey provides a first insight into the natural populations of mollusk-associated nematodes in the mid-Atlantic region. This knowledge may contribute to implementing cultural practices favoring these natural enemies of slug pests.

Keywords: mollusk-associated nematodes; integrated pest management; abiotic conditions; field crop



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1. Introduction

Slugs and snails are voracious mollusk pests that have the potential to damage virtually all crops, ranging from specialty to field crops. Because they are particularly hard on young and emerging plants, slugs are especially problematic during crop establishment in spring or fall. With the growing popularity of conservation agriculture, mollusk outbreaks have become a serious concern for growers in no-till systems, which provide stable, residue-rich habitats [1–3]. Slug herbivory can damage up to 37% of the total biomass of annual crucifer species [4]. Among mollusks, slugs tend to be more damaging than snails because of their greater resistance to mechanical disturbance (e.g., planting, tillage) and lower need for calcium to build shells [5]. Due to their generalist diets, slugs are among the most

important pests in areas that get frequent rain, such as Northern and Western Europe, and are an increasing challenge in some parts of the United States, such as the Mid-Atlantic region and the Pacific Northwest, that tend to receive ample precipitation [1]. Aside from mechanical control (i.e., tillage) [6], currently, the most widely used slug management practice is the application of granular baits containing active ingredients of metaldehyde or iron phosphate. In addition to the potential leaching of these chemicals into groundwater and streams [7], metaldehyde can be toxic to mammals such as canids [8] or rodents [9]. Furthermore, these baits are expensive (US \$16–20/hectare) yet are not always effective, partly because they are somewhat water-soluble. Fortunately, these active ingredients do not show toxicity to natural enemies of slugs, such as predatory insects [10], meaning they can be used in integrated pest management programs.

Given the limited management options, slug control would undoubtedly benefit from alternative management approaches. Currently, the main cultural approach to controlling slugs is tillage, which can facilitate soil erosion and the transport of nutrients and pesticides into nearby waterways. In the Mid-Atlantic region of the U.S., about 70% of agricultural lands are not tilled to prevent soil erosion and run-off into streams and rivers leading into the Chesapeake Bay, the world's largest estuary [11]. Unfortunately, no-till farming in this region favors the growth of slug populations by providing a stable, relatively undisturbed environment.

One alternative approach to slug management that needs to be better explored is promoting or augmenting populations of natural enemies that can kill slugs, especially ground beetles [12] and malacopathogenic nematodes (MPN) [13,14]. MPNs have received some research attention [15–20], but more research is necessary for them to become both viable and safe candidates for helping to control pest slug populations. These particular nematodes are spread across various taxonomic families (i.e., Alloionematidae, Cosmocercidae, Mermithidae, and Rhabditidae) [21]. While several pathogenic nematode species have been considered in sustainable insect pest management for several years [22], there are only two MPN species registered as commercial products for slug and snail control, (*Phasmarhabditis hermaphrodita* Shneider (Nematoda: Rhabditidae) and *P. californica* De Ley et al.) [13,23–25].

Phasmarhabditis hermaphrodita is a facultative parasite of terrestrial mollusks (Wilson 1993). Similar to entomopathogenic nematodes, this mollusk-killing species evolved a symbiosis with bacteria that partially serve as food sources. After penetrating its mollusk host, *P. hermaphrodita* and its symbiont kill the host within 4 to 21 days [13,25,26]. This symbiosis between *P. hermaphrodita* and bacteria is not as strict as with their entomopathogenic counterparts; rather, it occurs with complex and variable natural assemblages of bacterial species [17,27], a phenomenon that may also occur with other MPN species but remains to be elucidated. After depleting resources from the cadaver, MPNs leave their host and forage for new hosts. As facultative parasites, MPNs can also survive on alternative resources, such as decomposing organic matter or invertebrate feces [13,26,28]. Because of their potential for pest management, efforts to isolate different *Phasmarhabditis* species have been pursued in Europe and elsewhere. Over various field campaigns, the following species have been isolated and described: *P. hermaphrodita* [29], *P. papillosa* Schneider [30] and *P. neopapillosa* Mengert in Osche [31], *P. bonaquaense* Nermut', Půža & Mráček [32], *P. apuliae* Nermut', Půža & Mráček [33], *P. bohemiae* Nermut', Půža & Mráček [34], *P. tawfiki* Azzam [35], *P. huizhouensis* Huang, Ye, Ren & Zhao [36], *P. californica* [37], *P. meridionalis* Ivanova & Spiridonov [38], *P. safricana* Ross, Pieterse, Malan & Ivanova, [39], and *P. thesamica* Gorgadze et al. [40]. Other genera have been largely overlooked.

The present survey contributes to the current global effort to identify distributions of MPNs in gastropod hosts on agricultural soils. Focusing on soybeans, we sampled various farms across Delaware, USA. To get better insights into the impact of the environment on slugs and associated MPNs, we hypothesized that the distance from the edge of the field, providing shelter and resources, negatively impacts the slug density as well as the incidence of MPN infections. In addition, we hypothesized a positive impact of ground cover (weed and crop debris) on both slug populations and MPN incidence.

2. Materials and Methods

2.1. Field Location

We worked in nine soybean fields in Delaware, USA, three sites per County (New Castle, Kent, and Sussex). A brief description of each sampling site is available in Table 1.

2.2. Slug Sampling

In each selected field, we established a transect perpendicular to the field edge, with a trap positioned every 5 m up to 15 m into the field (i.e., 0 m, 5 m, 10 m, and 15 m from the field edge). Slug traps were squares of roofing shingle (1 × 1 m, White Fiberglass Mineral Roll, Home Depot, Inc., Atlanta, GA, USA). This particular shingle was selected for its white/grey color, which reflects some sunlight and helps prevent overheating of the traps. We visited the transects weekly and checked each shingle trap and residues underneath for the number of slugs present for a max. of 1 h. We collected the first 100 slugs that we encountered and brought them back to the laboratory for further processing. Slugs were identified based on Chichester and Getz [41].

2.3. Ground Cover

To evaluate the ground cover along the transects, we captured digital images of the shingle traps from a height of 1.5 m perpendicular to the center of each trap. We analyzed the images with CANOPEO [42] and recorded the portion of ground covered by debris and vegetation.

Table 1. Description of the field conditions.

Field	County	Previous Crop	Soil Type ^a
1	New Castle	Maize	RdA—Reybold-Queponco complex, fine-loamy, very deep well drained
2	New Castle	Maize	WocA—Woodbridge loam, Fine-loamy, very deep moderately well drained
3	New Castle	Maize	ReB—Reybold silt loam, fine-loamy, very deep, well drained
4	Kent	Maize	UIB—Unicorn loam, coarse-loamy very deep, well drained
5	Kent	NA	WocA—Woodbridge loam, fine-loamy, very deep moderately well drained
6	Kent	Maize	IgA—Ingleside sandy loam, coarse-loamy, siliceous, very deep, well drained
7	Sussex	Maize	WddA—Woodstown sandy loam, fine-loamy, very deep moderately well drained
8	Sussex	Maize	DodB—Downer sandy loam, coarse-loamy very deep, well drained
9	Sussex	NA	WddA—Woodstown sandy loam, fine-loamy, very deep moderately well drained

^a Soil types were determined on Web Soil Survey [43] based on field location.

2.4. Soil Type

Based on the GPS coordinates, soil types of each site were extracted from the website Web Soil Survey managed by the USDA-NRCS [43]. Soil types encountered in the current study are briefly described in Table 1.

2.5. Slug Parasitic Nematodes

We individually placed the slugs taken from the field in Petri dishes (10 cm diameter) with moist filter paper and provided small slices of organic carrots as diet; water and food were provided as needed. We weekly checked each slug for one month (i.e., four weeks) or until death. Dead slugs were placed in White traps [44] and monitored every other day for nematode emergence. Emerging nematodes were prepared for molecular identification [18]. Briefly, we placed aliquots of max. 15 nematodes into 1 mL of 70%

ethanol in a 1.5 mL sample tube (ThermoFisher Scientific, Waltham, MA, USA). Tubes were stored at -80°C until processing with molecular identification. We classified dead slugs into three categories; (1) death with the presence of nematodes; (2) death with the presence of fungi; (3) death without nematodes and fungi. We discarded slugs still alive four weeks after capture.

2.6. Molecular Identification of Slug Parasitic Nematodes

After removing the storage ethanol, each sample was washed twice with sterile water, and then the nematodes were transferred to a proteinase-K-based lysis buffer for DNA extraction [45]. We performed PCR amplification of the 18S ribosomal DNA region as previously described using the primer set 18A (5'-AAAGATTAAGCCATGCATG-3') and 26R (5'-CATTCTTGGCAAATGCTTTCG-3') [46,47]. The resulting ~ 800 bp amplicons were purified using a modified SPRI method [48] and direct end sequenced using primer 18A. All 18S rDNA sequences were compared against GenBank's non-redundant (nr) database using blast [49]. Nematode BLAST matches with a percent identity of 98–100 were used for species identification. One representative sequence for each nematode species identified in this study was submitted to GenBank (see Table 2 for accession numbers).

2.7. Statistical Analysis

We tested statistical differences in the number of slugs collected between sampling dates with an RM-ANOVA. As there were no differences between sampling dates, we applied GLMs to fields, distance from the field edge, soil type, and the number of dead slugs associated with the presence of nematodes with a QuasiPoisson distribution. Further differences between factors were tested with Tukey HSD post-hoc tests.

We tested for interactions between the number of slugs and ground cover as well as between the number of slug deaths with the presence of nematodes and ground cover with Spearman correlations.

All tests were conducted in R Statistical Software (v4.2.1; [50]).

Table 2. Nematodes associated with dead slugs trapped in soybean fields.

Field	Nematode Species	Accession number ^a	Nematode Order: Family	Association with Slugs	References
3, 4, 5, 6	<i>Cosmocercoides dukae</i>	FJ516753	Ascaridia: Cosmocercidae	Parasitic, final host	[51–55]
8	<i>Oscheius</i> sp. 4	MN082320	Rhabditida: Rhabditidae	Necromenic, saprophitic	[56]
4	<i>Panagrolaimus</i> sp. 1	MN082328	Rhabditida: Panagrolaimidae	Phoretic, bacterivorous	[57,58]
3, 9	<i>Panagrolaimus</i> <i>artyukhovskii</i>	MK636578	Rhabditida: Panagrolaimidae	Phoretic, bacterivorous	[57,58]
5, 8, 9	<i>Panagrolaimus</i> cf. <i>rigidus</i>	DQ285636	Rhabditida: Panagrolaimidae	Phoretic, bacterivorous	[57,58]
1, 2, 7, 9	<i>Panagrolaimus</i> <i>subelongatus</i>	KY119431	Rhabditida: Panagrolaimidae	Phoretic, bacterivorous	[57,58]
6	<i>Panagrolaimus</i> <i>trilabiatus</i>	KF011487	Rhabditida: Panagrolaimidae	Phoretic, bacterivorous	[57,58]
9	<i>Pristionchus lheritieri</i>	AY284690	Rhabditida: Diplogastridae	Necromenic, saprophitic	[59]
3, 6, 9	<i>Rhabditis</i> sp.	MH608279	Rhabditida: Rhabditidae	Parasitic?	[60]
7, 8	<i>Rhabditoides</i> <i>inermiformis</i>	AF083017	Rhabditida: Rhabditidae	Opportunistic	[60]
5, 9	Unidentified nematode isolate	EU541352	??	??	NA

^a One representative sequence was submitted to GenBank for each nematode taxon identified in this study.

3. Results

3.1. Slug Sampling

From 36 traps deployed across three counties, we collected 1808 slugs, representing only three species. The majority of individuals were *Deroceras laeve* (98.3%), whereas grey garden slug (*Deroceras reticulatum*; 1.6%) and leopard slug (*Limax maximus*; 0.1%) were rare

by comparison. Without a formal population estimate, the data suggests seasonality in slug population densities, with most (38.4%) captured on 19 April 2018 and fewer collected on other dates (19.9% on 12 April 2018, 19.9% on 26 April 2018, 21.8% on 3 May 2018; $F_{3-140} = 2.426$, $p = 0.06$).

The distance from the field edge did not influence the number of collected slugs ($F_{3-140} = 0.108$, $p = 0.956$). However, the number of slugs collected per trap (4) and sampling date (4) varied among fields (Figure 1, $F_{8-135} = 20.034$, $p < 0.001$) and soil types (Figure 2, $F_{6-137} = 10.693$, $p < 0.001$).

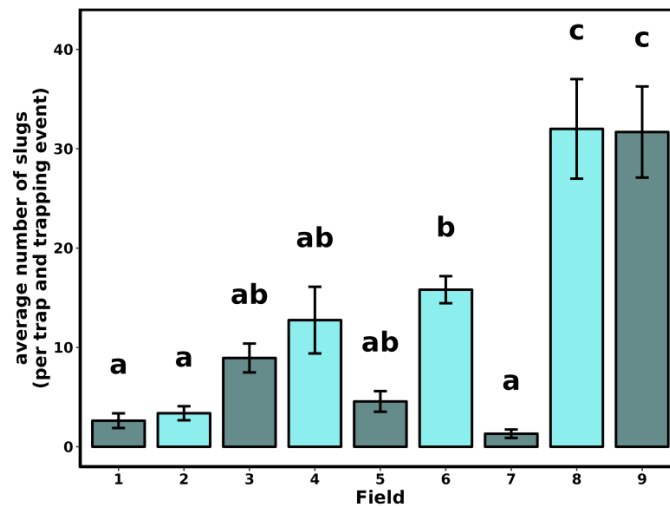


Figure 1. Average number of slugs per trap (4 traps of 1 m²) and per trapping event (4) in each survey field. Details on the field conditions are provided in Table 1. Bars indicate SEM. Letters indicate statistical differences among fields.

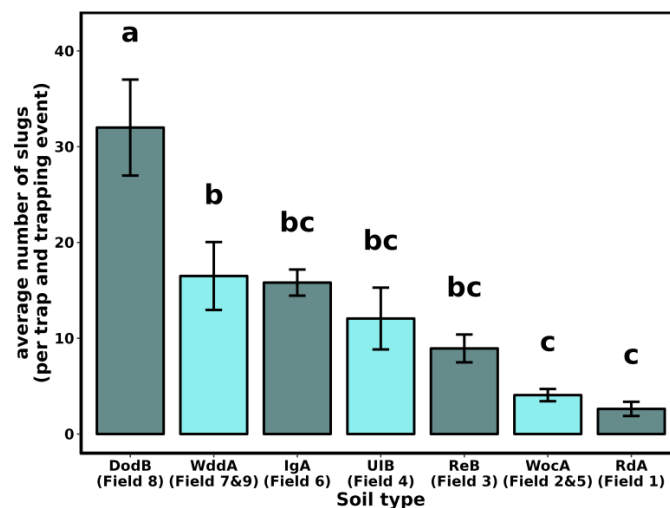


Figure 2. Average number of slugs collected per trap (4 traps of 1 m²) and per trapping event (4) by soil types. Soil types were identified on Web Soil Survey [43], based on field location, as Downer sandy loam (DodB), Ingleside sandy loam (IgA), Reybold-Queponco complex (RdA), Reybold silt loam (ReB), Unicorn loam (UIB), Woodstown sandy loam (WddA), Woodbridge loam (WocA). Short descriptions of the soil types are provided in Table 1. Bars indicate SEM. Letters indicate statistical differences between soil types.

3.2. Ground Cover

Ground cover (before planting) was variable among fields and transects. We found a strong positive relationship between the % of ground cover and the number of slugs under

shingles ($r_s(19) = 0.74, p < 0.001$). Similarly, the total number of nematodes associated with dead slugs was positively correlated with ground cover (Figure 3, $r_s(19) = 0.70, p < 0.001$)

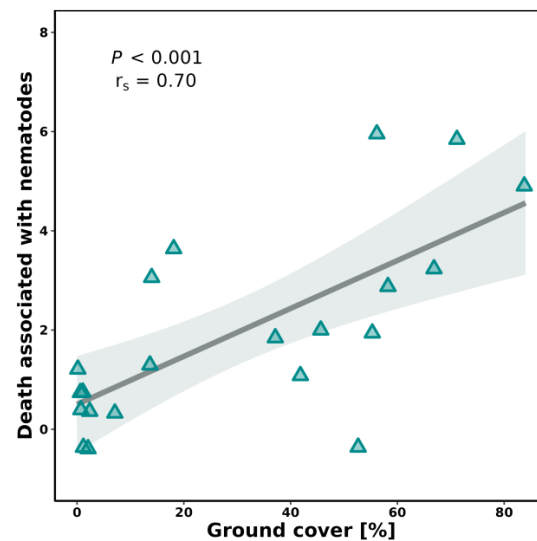


Figure 3. Spearman correlation between ground cover and the number of dead slugs with the presence of killed by nematodes.

3.3. Slug Parasitic Nematodes

Of the slugs we collected, 616 died within four weeks after collection, and 122 deaths were associated with the presence of nematodes (Table 2). The mortality of slugs associated with the presence of nematodes varied among fields ($F_{8-135} = 3.181, p < 0.01$) but was not influenced by the distance from the field edge ($F_{3-140} = 2.172, p = 0.094$) nor the trapping events ($F_{3-140} = 1.356, p = 0.07$). Soil type also affected slug mortality associated with nematodes (Figure 4, $F_{6-137} = 2.505, p = 0.025$).

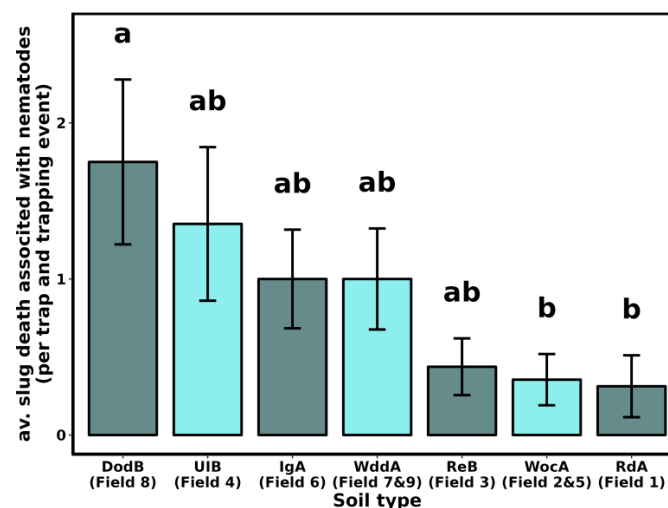


Figure 4. Average number of slugs death associated with the presence of nematodes per trap (4 traps of 1 m²) and per trapping event (4) by soil types. Soil types were identified on Web Soil Survey [43], based on field location, as Downer sandy loam (DodB), Ingleside sandy loam (IgA), Reybold-Queponco complex (RdA), Reybold silt loam (ReB), Unicorn loam (UIB), Woodstown sandy loam (WddA), Woodbridge loam (WocA). Short descriptions of the soil types are provided in Table 1. Bars indicate SEM. Letters indicate statistical differences between soil types.

Overall, ca. 19% of the slug death was associated with the presence of nematodes. Fungi were associated with 35%, and 46% of the dead slugs were not associated with the

presence of nematodes or fungi. These mortality rates varied across the field, but these percentages were quite constant across the state, even though Field 7 had >50% of death associated with the presence of nematodes (Figure 5B).

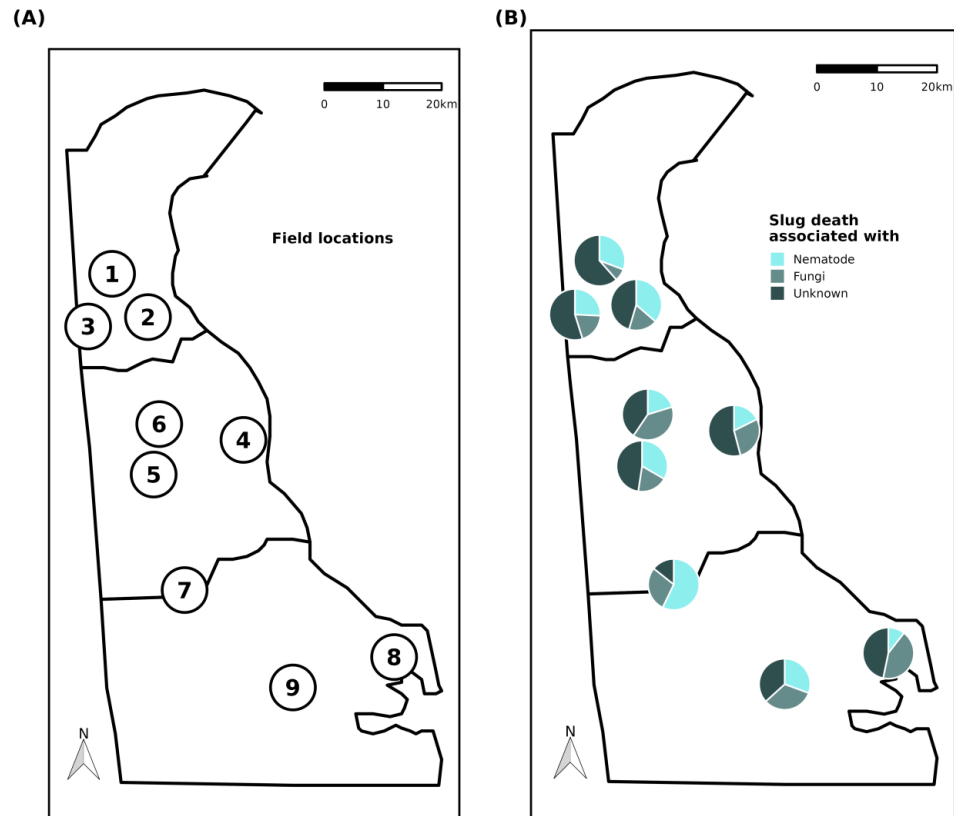


Figure 5. (A) Geographical locations of the surveyed fields across Delaware. Details on the field conditions are provided in Table 1. (B) Slug death associated with the presence of nematodes, fungi, or unknown causes at the different surveyed sites. Pie charts illustrate the proportion of slug death associated with the different factors measured among all individuals dead within 4 weeks after capture (4 traps per field and 4 sampling dates).

4. Discussion

We conducted this study to isolate MPN from slugs in Delaware, with the hope of finding *Phasmarhabditis*, which may be able to facilitate sustainable control of pest slugs in North America. Failing to achieve this ambitious aim, the present study still offers interesting insights into the ecology of slugs, slug-associated nematodes, and other potential pathogens.

The three slug species that we captured are invasive species. Exotic populations of *D. laeve* were probably established in North America in the 1700s but this Holarctic species also has native populations in the U.S. [61]. *Deroceras reticulatum* was introduced into the USA in the 1800s [62]. *L. maximus* was introduced into the USA in the late 1800s [62], yet this, to our knowledge, is its first record in Delaware [63]. For slugs to be abundant, an area must receive adequate moisture, which helps them avoid desiccation [64]. However, on a local level, crop residue and soil coverage by weeds can influence slug abundance. Increased soil coverage likely retains soil moisture, favoring slug development and survival [64]. Even though we found no differences in the number of slugs collected from the edge of the fields and along transects, there were differences among fields with the highest number of slugs found in southern Delaware. This result is counterintuitive because soil conditions tend to be dryer in southern Delaware than in the north; perhaps this difference can partly be explained by the greater atmospheric humidity in southern Delaware.

In several instances, slug mortality was associated with the presence of fungal hyphae and spores on the surface of cadavers. Attempts to isolate these fungi were unsuccessful, but further investigations on pathogenic fungi against slugs could be a worthwhile area for future research. Literature on this topic is extremely scarce, yet fungi seem to have the potential to produce metabolites having repellent or even molluscicide effects [65]. It is also possible that the fungi we observed were secondary colonizers and not responsible for slug death.

Our study also highlighted aspects of the ecology of nematodes associated with dead slugs. Associations between nematodes and mollusks likely vary along a continuum of intimacy. Certain vertebrate nematode species use mollusks as transitional hosts (paratenic hosts) in which little or no development takes place [66]. Other nematode species complete part of their life cycle in the mollusk [67]. Finally, mollusks can be definitive hosts of nematodes, with some species only developing partially in the host (non-pathogenic) while finishing their life cycle as free-living organisms. Other nematode species that use mollusks as definitive hosts are pathogenic and kill their hosts at the completion of their life cycle. From the nematode species that we isolated, *Cosmocercoides dukae* belongs to the class of nematodes using slugs as definitive hosts [51–55]. Other nematode species isolated from soybean fields, such as *Rhabditoides inermiformis*, also closely interact with slug species [60], yet their lifestyle may or may not be parasitic. We also found several individuals from the genus *Panagrolaimus*, associated with mollusks in a phoretic interaction [57,68]. Nematodes from the genus *Panagrolaimus* are bacterivorous and can be found in various niches, from the soil in Antarctica to arid soil in Iran [57,58]. Some species use mollusks as final hosts [67], yet the nematodes we isolated have not been described to have such an association. We isolated nematodes from the genus *Rhabditis*, which has been associated with slugs and mollusks [60], but it is unclear whether they were parasites even if there is evidence of parasitism of mollusks within the genus [30,60].

Overall, regardless of the type of association that nematode species have with mollusks, soil type influenced nematodes associated with slugs. The highest densities of nematodes were found in soils that better retain water and provided suitable conditions for nematodes and their gastropod hosts. Related to moisture (and, to some extent, the soil's capacity to retain moisture), the instances when a nematode was associated with a slug increased with soil cover, suggesting that, in addition to favoring slugs, ground cover would also favor the presence of slug-associated nematodes.

Even though we failed to isolate *Phasmarhabditis* from Delaware, this genus has previously been collected in North America. *Phasmarhabditis hermaphrodita* was found in a number of pest slug species in California and Oregon [69,70], and these US strains were subsequently shown to be lethal to a number of invasive gastropod species in lab assays [14,71,72], suggesting that they may have a role to play in biological control of mollusks [73]. Furthermore, *P. papillosa* [37] and *P. californica* were also collected from multiple invasive gastropod species in both California and Oregon [37,74]. More recently, *P. californica* was found in Canada for the first time [60], and this, combined with its known lethality to pest slugs [71], has led to suggestions that it may have the potential to be used as a biological control agent in different areas of North America, as congener *P. hermaphrodita* was used in Europe. It should be noted that our survey focused on soybeans. More comprehensive surveys should include other crops and habitats, including horticultural sites and/or natural habitats. In addition, the methods used to collect slugs could be widened (e.g., use of soil cores) to increase the probability that mollusks infected by *Phasmarhabditis* sp. are sampled. Indeed, it has been suggested that these nematodes manipulate the behavior of their infected host, as infected slugs tend to be found underneath the soil surface, which prevents the parasite-infected host from being predated or scavenged [75]. The trapping method used herein sampled slugs from the soil surface only.

To the best of our knowledge, our MPN survey was the first on the east coast of North America. While the slug-associated nematode species we found are not obvious candidates for biological control of pest slugs, our data demonstrate that a diversity of nematodes

can be found along with slugs in crop fields. Even though we did not find *Phasmarhabditis*, this first effort has highlighted some pitfalls that could be avoided in future surveys and demonstrated the importance of soil and ground cover on MPNs and their hosts.

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Conflicts of Interest: R.J.M.D. declares he is a co-inventor on a patent application entitled Mollusk-killing Biopesticide (U.S. application Serial No. 62/236,674). B.K., W.J.C., J.F.T., D.K.H., D.R.D. and I.H. declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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