

Article

Discovery of *Oscheius myriophilus* (Nematoda: Rhabditidae) in Gastropods and Its Similar Virulence to *Phasmarhabditis papillosa* against *Arion vulgaris*, *Deroceras reticulatum*, and *Cernuella virgata*

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Abstract: Between July and September 2021, researchers collected 100 specimens of the Spanish slug, *Arion vulgaris*, and dissected their cadavers to examine them for parasitic nematodes. Molecular techniques were used to identify the nematodes, which confirmed the presence of *Oscheius myriophilus*, marking the first recorded instance of this nematode in a gastropod host. To test the virulence of Slovenian strains of *O. myriophilus* and *Phasmarhabditis papillosa* and their effects on the feeding behavior of the Spanish slug, grey field slug (*Deroceras reticulatum*), and vineyard snail (*Cernuella virgata*), laboratory bioassays were conducted using nematodes grown in vivo. Nematodes were applied at various doses ranging from 10 to 500 nematodes/gastropod. The results showed that *O. myriophilus* and *P. papillosa* caused significant mortality ($82.5\% \pm 2.5\%$ at 15°C) of the Spanish slug while being less effective against the vineyard snail and grey field slug. Nematodes were more virulent at a lower temperature (15°C) than at the higher temperature (20°C) tested in the experiment. Additionally, both nematode species significantly reduced gastropod herbivory. The potential use of *O. myriophilus* and *P. papillosa* as biological control agents against gastropods is discussed.

Keywords: slug parasitic nematodes; the Spanish slug; the grey field slug; the vineyard snail; mortality; feeding inhibition; biological control



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1. Introduction

Slugs and snails (Mollusca: Gastropoda) are a major concern for horticulturists worldwide, as they are among the most destructive pests in greenhouses, home gardens, nurseries, landscapes, and field crops [1]. These generalist feeders have a wide range of diet preferences and tend to target succulent foliage, making them serious pests for seedlings, herbaceous plants, and fruit ripening close to the ground [2]. Some gastropod species are even known to damage stored products [1]. In Europe, 11 species of gastropods need to be controlled in cultivated plants, including *Deroceras reticulatum* (Müller), *Arion distinctus* Mabile, *Arion hortensis* (Férussac), *Arion intermedius* Normand, *Arion rufus* (L.), *Arion vulgaris* Moquin-Tandon, *Tandonia budapestensis* Hazay, *Cornu aspersum* (Müller), *Cepaea hortensis* (Müller), *Cepaea nemoralis* (L.), and *Cernuella virgata* (Da Costa) [1–3].

Chemical molluscicides are currently the primary means of controlling gastropod pests. Three types of pellets based on carbamates, metaldehyde, or iron phosphate are commonly used [4]. However, carbamates, such as methiocarb, are effective but also kill beneficial insects, such as carabid beetles [5]. Metaldehyde targets the mucus-producing

cells of slugs and snails and causes their death through contact or ingestion. Iron phosphate pellets, on the other hand, are approved for organic farming and interfere with calcium metabolism in the gut of the target host, leading to death within a few days [6]. Although iron phosphate is less toxic to animals than other molluscicides, it has been observed to cause increased mortality, decreased mass, and reduced surface foraging in earthworms [7]. Chemical control options for gastropod pests are becoming more limited due to the removal of methiocarb from the European market and the ban on the outdoor use of metaldehyde in the UK in 2022, following recommendations from the Health and Safety Executive and the UK Expert Committee on Pesticides [4]. To address this issue, farmers and growers are exploring sustainable control methods, such as biorational products, physical barriers, agronomic practices, cultural control, monitoring, and biological control [8–10].

The use of molluscicidal nematodes as a biological control measure against pest gastropods is a promising approach. The parasitic nematode *Phasmarhabditis hermaphrodita* Schneider (Rhabditidae) has been sold in Europe as Nemaslug® and SlugTech® and has shown great potential as a slug biological control agent for over thirty years [8]. However, regulations and restrictions on the use of only indigenous species in certain countries have limited the use of *P. hermaphrodita*, necessitating the search for alternative nematode species [11–13]. Currently, there are 16 nominal species of *Phasmarhabditis* worldwide, all of which have demonstrated the ability to specifically target and kill gastropods and protect crops [14–18].

The gastropod-parasitic nematode *Phasmarhabditis papillosa* (Schneider) Andr ssy, 1983 has been isolated from various gastropod species, such as *Arion circumscriptus* Johnston, *Arion ater* L., *Deroceras laeve* (M ller), *Limax cinereoniger* Wolf, *Limax tenellus* (M ller) [19], *Deroceras invadens* Reise, Hutchinson, Schunack and Schlitt [11], *D. reticulatum* [13], and *A. vulgaris* [12]. Recent studies have shown that *P. papillosa* is capable of causing significant mortality in *Deroceras panormitanum* (Lessona and Pollonera) and *A. vulgaris*, which highlights its potential as a new biological control agent against gastropods and warrants further investigation.

The Rhabditidae family comprises free-living nematodes that feed on bacteria, and *Oscheius* is a genus within this family [20]. The genus is divided into two groups, Insectivora and Dolichura [21], and it includes 45 described species [22]. Studies have shown that *Oscheius* nematodes exhibit entomopathogenic or scavenging behavior and can serve as effective biological control agents against various invertebrate pests [22–24]. Recently, *Oscheius* has gained increasing attention. For example, *Oscheius myriophilus* Poinar was found to be associated with garden millipedes in California, as well as millipedes in South Australia, the European mole cricket in Turkey, and sugar cane crop soil in Mexico [24–27].

The opportunity to investigate the potential biocontrol efficacy of *O. myriophilus* against various gastropod species arose when it was isolated from a Spanish slug (*A. vulgaris*). In this study, we also included the recently confirmed slug parasitic nematode *P. papillosa* from Slovenia [12]. Our objective was to evaluate and compare the pathogenicity of Slovenian native strains of *O. myriophilus* and *P. papillosa* against three gastropod species (*A. vulgaris*, *D. reticulatum*, and *C. virgata*) under controlled laboratory conditions.

2. Materials and Methods

2.1. Nematode Isolation from Slugs and Culture Maintenance

Between July and September 2021, *Arion vulgaris* specimens were collected in Ljubljana, Slovenia, near the river Glin  ica (46 03' N, 14 28' E). This species is known to be the most common and harmful slug pest in Europe [28]. Species identification of collected slugs ($n = 100$) was performed using identification charts [2]. The slugs were then rinsed with 0.9% saline solution using the protocol described by Pieterse et al. [11] and dissected alive to isolate nematodes. Recovered nematodes were maintained in a laboratory culture on freeze-killed *A. vulgaris* slugs, and nematodes needed for identification were preserved in 80% ethanol for molecular analysis [11]. The nematode cultures were washed after 10 days using a sodium hypochlorite solution (5%) and distilled water, with the goal of obtaining

infective juveniles (IJs) for use in the experiment. The IJs were stored in M9 buffer at 4 °C, and only those less than two weeks old with a viability of over 95% were utilized. Native populations of *Phasmarhabditis papillosa* (GenBank accession number MT800511.1) were also subcultured for use in this study, as the species was recently confirmed in Slovenia [12].

2.2. Molecular Identification

Individual nematodes were used for genomic DNA extraction, and the internal transcribed spacer (ITS) region and small subunit rRNA gene (SSU) were amplified through polymerase chain reaction (PCR) using primers TW81 and AB28 per Hominick et al. [29] and Liu et al. [30], respectively. The PCR products were then purified using the QIAquick Gel Extraction Kit (Qiagen) from a 1% TAE-buffered agarose gel. The purified samples were sent to the laboratory of Microsynth AG for sequencing. The obtained DNA sequences were compared with the National Centre for Biotechnology Information (NCBI) database using the BLAST search tool.

2.3. Laboratory Bioassay

The Laboratory of Entomology of the Biotechnical Faculty (Dept. of Agronomy, University of Ljubljana, Ljubljana, Slovenia) was used to conduct experiments to test the potential gastropod control ability of *O. myriophylus* and *P. papillosa*. In June and July 2022, *Arion vulgaris* and *Deroceras reticulatum* slugs were collected from the laboratory field of the Biotechnical Faculty in Ljubljana, Slovenia (46°04' N, 14°31' E, 299 m a.s.l.), while *Cernuella virgata* snails were collected in a vineyard near the city of Miren, Slovenia (45°53' N, 13°35' E, 45 m a.s.l.) in July and August 2022. The collected gastropods were identified using identification charts [2] and were of different sizes and stages to obtain a representative sample of the outdoor population of gastropods. Prior to the experiment, the gastropods were starved for 48 h [12]. The average weight of *A. vulgaris*, *D. reticulatum*, and *C. virgata* used in this study was 6.3 ± 1.3 g, 1.8 ± 0.4 g, and 3.1 ± 0.2 g, respectively.

The study was conducted in plastic Petri dishes (150 × 20 mm) for 28 days. The experiment comprised six different treatments (control, 10, 50, 100, 200, and 500 IJs/gastropod), and each treatment was carried out with 20 slugs (1 slug per Petri dish). The laboratory experiments were performed in a growth chamber (type: RK-900 CH, manufactured by Kambič laboratorijska oprema d.o.o., Semič) at 15 and 20 °C, with 12/12 h photoperiod, and 75% relative air humidity. A total of 480 individuals of each gastropod species (6 treatments × 20 replicates × 2 temperatures × 2 nematode species) were used in the study. The experiment utilized 1440 slugs and snails of various sizes and stages. Moistened filter paper and fresh lettuce leaves were placed in plastic Petri dishes. One ml of the nematode suspension was dripped directly on moistened filter paper, and the nematodes were applied only once during the experiment. The number of nematodes was determined in a previously prepared suspension, and the sample was diluted or concentrated as required to achieve the necessary dose. The control received the same amount of water without nematodes added to the moistened filter paper. Lettuce leaf discs of approximately 5 cm in diameter were offered to the slugs as a food source. The survival and feeding of slugs were monitored on 2, 4, 7, 10, 14, 17, 21, 25, and 28 days after treatment (DAT), while fresh food (i.e., lettuce leaves) and additional moisture were added to the filter paper. The leaf area eaten by the gastropods was visually assessed, and the percentage of gastropods that consumed food was calculated. To confirm the pathogenicity of nematodes, dead gastropods were transferred to modified white traps, and nematode progeny were observed under the microscope [12].

2.4. Statistical Analyses

A multifactor analysis of variance (ANOVA) was conducted to assess the differences in mortality and feeding of gastropods under different variables, such as nematode species, nematode dose, mollusk species, temperatures, days after treatment (DAT), and replication. Data obtained at 0, 7, 14, 21, and 28 DAT were used for statistical analysis after testing each

variable for homogeneity of treatment variances. Mortality in all nematode treatments was higher than that in the control treatments; therefore, the treatment values were corrected for natural mortality. Mean differences among the tested variables were separated using Duncan's multiple range test ($p < 0.05$). The data are presented as the mean $\pm$ S.E. Statistical analyses were performed using Statgraphics Plus for Windows 4.0 (Statistical Graphics Corp., Manugistics, Inc., Rockville, MD, USA). The Kaplan–Meier method and the Mantel–Cox log rank test were used to estimate the gastropod survival rate. GraphPad Prism 9 was used to generate the figures and perform the analyses.

3. Results

3.1. Molecular Identification

The 1510 bp long 18S rRNA gene sequence obtained from the Slovenian nematode isolate shows 100% identity with the U81588 sequence (sequence coverage is 100%). U81588 was deposited as the *Rhabditis myriophila* (syn. *Oscieus myriophilus*) strain DF5020, which was originally isolated by Poinar [25]. The SSU sequence identity with other *Oscieus* species is less than 99%. In the GenBank database, there are 22 different sequences concerning the internal transcribed spacer 1 and 2 regions of *O. myriophilus* showing >90% sequence coverage with the 819 bp long sequence obtained from the Slovenian nematode isolate. The identity values are 99.14–99.88%, while the two sequences representing *O. microvilli*, the closest relative of *O. myriophilus*, show 99.15% and 99.76% identity with the Slovenian isolate. The ITS 1 and 2 sequence similarity with other *Oscieus* species is less than 90%. These results confirmed the isolated nematode species is conspecific to *O. myriophilus* [25]. The sequences obtained for the Slovenian *O. myriophilus* isolate were deposited in GenBank under accession numbers OP684306 (ITS) and OP684313 (SSU).

3.2. Laboratory Bioassay

Analyses of the pooled results showed that the mortality of the gastropods was influenced by all tested factors except the nematode species: the dose of nematodes ($F_{5,7199} = 52.8$; $p < 0.0001$); DAT ($F_{4,7199} = 189.1$; $p < 0.0001$); the gastropod species ($F_{2,7199} = 321.2$; $p < 0.0001$); temperature ($F_{1,7199} = 419.0$; $p < 0.0001$); replication ($F_{19,7199} = 2.2$; $p = 0.0021$); and interaction between the nematode species and temperature ($F_{1,7199} = 25.4$; $p < 0.0001$).

Analyses of the pooled results showed that gastropod feeding was influenced by the nematode species ($F_{1,7199} = 8.8$; $p = 0.0031$); the dose of nematodes ($F_{5,7199} = 140.9$; $p < 0.0001$); DAT ($F_{4,7199} = 421.9$; $p < 0.0001$); the gastropod species ($F_{2,7199} = 137.8$; $p < 0.0001$); temperature ($F_{1,7199} = 272.7$; $p < 0.0001$); replication ($F_{19,7199} = 2.1$; $p = 0.0037$); interaction between the nematode species and temperature ($F_{1,7199} = 15.7$; $p = 0.001$); and interaction between the nematode species and the gastropod species ($F_{2,7199} = 3.1$; $p = 0.0432$).

At a lower temperature of 15 °C, both nematode species caused higher mortality rates in the gastropods. On average, $18.9\% \pm 0.7\%$ of gastropods died at 15 °C compared to only $5.1\% \pm 0.4\%$ at 20 °C. *A. vulgaris* had the highest mortality rates caused by both nematode species ($23.4\% \pm 0.9\%$) compared to the other gastropods tested (*D. reticulatum* ($9.7\% \pm 0.6\%$); *C. virgata* ($2.9\% \pm 0.4\%$)). On the other hand, *C. virgata* had the greatest reduction in gastropod feeding caused by both species ($43.6\% \pm 1.0\%$) compared to *A. vulgaris* ($23.7\% \pm 0.9\%$) and *D. reticulatum* ($35.4\% \pm 1.0\%$). The reduction in gastropod feeding was more pronounced at lower temperatures (15 °C). In the nematode treatments, gastropod feeding was reduced on average to $42.6\% \pm 0.8\%$ at 15 °C compared to a reduction of $26.1\% \pm 0.7\%$ at 20 °C.

3.2.1. The Spanish Slug (*A. vulgaris*)

Analyses of the pooled results showed that the mortality of the Spanish slug was influenced by the dose of nematodes ($F_{5,2399} = 57.9$; $p < 0.0001$); DAT ($F_{4,2399} = 217.5$; $p < 0.0001$); temperature ($F_{1,2399} = 1033.4$; $p < 0.0001$); interaction between the nematode

species and the dose of nematodes ($F_{5,2399} = 2.4$; $p = 0.0324$); and interaction between the nematode species and temperature ($F_{1,2399} = 5.1$; $p = 0.0234$). The feeding of the Spanish slug was influenced by the dose of nematodes ($F_{5,2399} = 58.0$; $p < 0.0001$); DAT ($F_{4,2399} = 216.3$; $p < 0.0001$); temperature ($F_{1,2399} = 971.3$; $p < 0.0001$); and interaction between the nematode species and temperature ($F_{1,2399} = 8.0$; $p = 0.0046$).

No significant differences in mortality rates were observed among the tested nematode species for the Spanish slug. However, both *O. myriophilus* and *P. papillosa* were found to cause significantly higher mortality rates and reduced feeding of the slugs at lower temperatures (15 °C) than at higher temperatures (20 °C). At 15 °C, mortality rates for the Spanish slug were $43.5\% \pm 1.4\%$ compared to only $3.3\% \pm 0.5\%$ at 20 °C. Similarly, gastropod feeding was reduced by $43.5\% \pm 1.4\%$ at 15 °C compared to only $4.0\% \pm 0.4\%$ at 20 °C.

The survival of Spanish slugs was analyzed using the Kaplan–Meier survival curve and log rank test. The results showed that the overall survival time was significantly lower ($p < 0.0001$) for slugs treated with nematodes at 15 °C than at 20 °C ($p = 0.0165$ for *P. papillosa* and $p = 0.0218$ for *O. myriophilus*). The survival curves demonstrated that the most rapid rates of Spanish slug mortality occurred with 50 IJs of *P. papillosa* per slug (Figure 1B) and only 10 IJs of *O. myriophilus* per slug (Figure 1D) at 7 DAT. The slug population had a survival rate of 50% in *P. papillosa* treatments after 17 DAT and in *O. myriophilus* treatments after 19 DAT. The differences in survival curves among treatments with both nematode species at 15 °C were significant based on the Mantel–Cox log rank test ($p < 0.0001$). At 20 °C, the lowest dose of *O. myriophilus* (10 IJs per slug) caused the greatest mortality at 21 DAT. Both nematodes caused significant differences in Spanish slug mortality at 20 °C, but the slug survival probability was high.

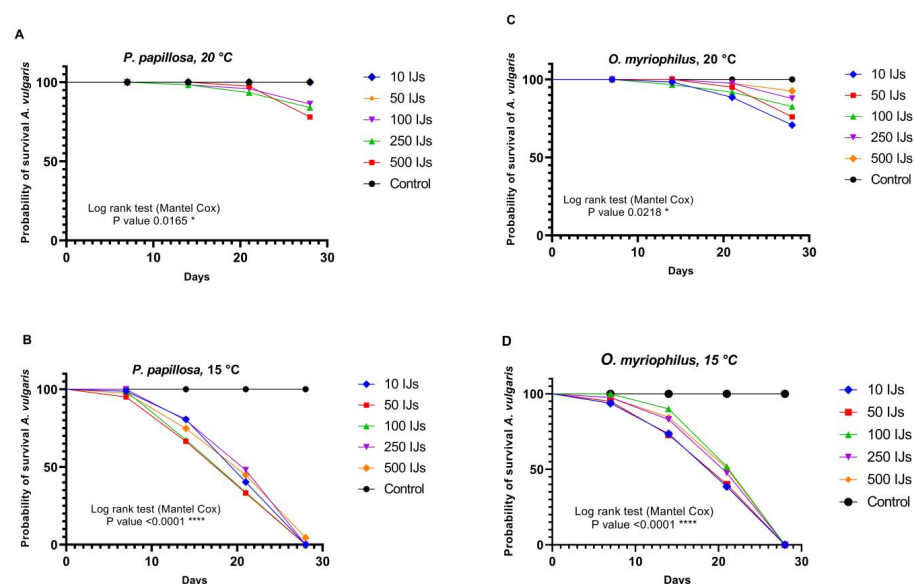


Figure 1. (A–D) Kaplan Meier curves showing the percent survival of the Spanish slug (*A. vulgaris*) after treatments with 0, 10, 50, 100, 250 or 500 nematodes per slug displaying a significant difference Mantel Cox log rank test: * indicates a p value of < 0.05 , and **** indicates a p value < 0.0001 compared to the slug only control). (A)—The slug survival after *P. papillosa* infection at 20 °C. The slug mortality caused by nematode infection is significantly different between 250 IJs dose and other treatments since 21 DAT. (B)—The slug survival after *P. papillosa* infection at 15 °C. The slug mortality caused by nematode infection is significantly different between 50 IJs and other treatments since 7 DAT. (C)—The slug survival after *O. myriophilus* infection at 20 °C. The slug mortality caused by nematode infection is significantly different between 100 IJs dose and other treatments, except 10 IJs dose since 14 DAT. (D)—The slug survival after *O. myriophilus* infection at 15 °C. The slug mortality caused by nematode infection is significantly different between all nematode treatments and control since 14 DAT.

The mortality of the Spanish slug did not differ significantly among the nematode species tested at 15 °C (*O. myriophilus* 41.7% $\pm$ 2.0%; *P. papillosa* 45.3% $\pm$ 2.0%), irrespective of other variables. However, the virulence of the nematodes was influenced by the duration of exposure, as the mortality rate increased with time: 8.8% $\pm$ 1.8% at 7 DAT, 51.7% $\pm$ 3.2% at 14 DAT, 74.6% $\pm$ 2.8% at 21 DAT, and 82.5% $\pm$ 2.5% at 28 DAT. Furthermore, the inhibition of gastropod feeding was significantly affected by the time of exposure to nematodes, with a reduction of feeding observed at 8.7% $\pm$ 1.8% after 7 DAT, 51.3% $\pm$ 3.2% at 14 DAT, 74.6% $\pm$ 2.8% at 21 DAT, and 82.5% $\pm$ 2.5% at 28 DAT.

Observations of slug mortality at 15 °C indicated that differences were present between doses of *O. myriophilus* at 7 and 14 days after treatment (Figure 1D). However, the dose of nematodes did not have a significant impact on slug mortality at 21 and 28 days after treatment. Similar results were observed with *P. papillosa* (Figure 1B). The most significant difference in gastropod feeding reduction between nematode doses for both species was observed at 7 and 14 days after treatment (Table 1). There was no significant influence on gastropod feeding at 21 and 28 days after treatment with varying doses of nematodes.

Table 1. Effect of parasitic nematodes on feeding inhibition of the Spanish slug at 15 °C.

15 °C Spanish Slug (<i>A. vulgaris</i>)				
Nematode Dose (IJs Gastropod ^{−1})	<i>Phasmarhabditis papillosa</i>			
	7 days	14 days	21 days	28 days
0	100 $\pm$ 0 Ac	100 $\pm$ 0 Ad	100 $\pm$ 0 Ac	100 $\pm$ 0 Ac
10	95 $\pm$ 5 Bbc	45 $\pm$ 11 Bc	0 $\pm$ 0 Aa	0 $\pm$ 0 Aa
50	75 $\pm$ 10 Ca	15 $\pm$ 8 Bab	0 $\pm$ 0 Aa	0 $\pm$ 0 Aa
100	95 $\pm$ 5 Bbc	5 $\pm$ 5 Aa	0 $\pm$ 0 Aa	0 $\pm$ 0 Aa
250	100 $\pm$ 0 C	40 $\pm$ 11 Bc	20 $\pm$ 9 Bb	0 $\pm$ 0 Aa
500	90 $\pm$ 7 Cab	30 $\pm$ 11 Bbc	20 $\pm$ 9 ABb	10 $\pm$ 7 Ab
Nematode dose (IJs gastropod ^{−1})	<i>Oscheius myriophilus</i>			
	7 days	14 days	21 days	28 days
0	100 $\pm$ 0 Ab	100 $\pm$ 0 Ad	100 $\pm$ 0 Ac	100 $\pm$ 0 Ab
10	80 $\pm$ 9 Ca	35 $\pm$ 11 Bab	5 $\pm$ 5 Aa	0 $\pm$ 0 Aa
50	80 $\pm$ 9 Da	30 $\pm$ 11 Ca	10 $\pm$ 7 Bab	0 $\pm$ 0 Aa
100	100 $\pm$ 0 Db	70 $\pm$ 11 Cc	15 $\pm$ 8 Bab	0 $\pm$ 0 Aa
250	90 $\pm$ 7 Da	55 $\pm$ 11 Cbc	15 $\pm$ 8 Bab	0 $\pm$ 0 Aa
500	90 $\pm$ 7 Da	60 $\pm$ 11 Cc	20 $\pm$ 9 Bb	0 $\pm$ 0 Aa

The presented data display the mean values $\pm$ S.E. The capital letters signify significant differences ($p < 0.05$) among various time intervals for the same nematode dose, whereas small letters indicate significant differences ($p < 0.05$) among different nematode doses during a specific time interval. The key denotes “feeding (100 $\pm$ 0)”, where all slugs consumed lettuce.

There were no significant differences in the mortality of the Spanish slug among the nematode species at 20 °C (*O. myriophilus* 4.3% $\pm$ 1.8%; *P. papillosa* 4.3% $\pm$ 1.8%) (Figure 1A,C). The time of exposure (DAT) was the most important factor influencing gastropod mortality, independent of other tested factors. The mortality of the Spanish slug increased gradually over time: 7 DAT 0.0% $\pm$ 0.0%, 14 DAT 2.1% $\pm$ 0.9%, 21 DAT 5.8% $\pm$ 1.5%, and 28 DAT 8.8% $\pm$ 1.8%. At 20 °C, the mortality and feeding reduction of the Spanish slug were slightly affected, and the nematode dose was not a significant factor (Table 2; Figure 1A,C).

3.2.2. The Grey Field Slug (*Deroceras reticulatum*)

Analyses of the pooled results showed that the mortality of the grey field slug was influenced by the nematode species ($F_{1,2399} = 8.1$; $p = 0.0045$); the dose of nematodes ($F_{5,2399} = 15.3$; $p < 0.0001$); DAT ($F_{4,2399} = 47.6$; $p < 0.0001$); temperature ($F_{1,2399} = 10.8$; $p = 0.0010$); interaction between the nematode species and the dose of nematodes ($F_{5,2399} = 6.3$;

$p < 0.0001$); and interaction between the nematode species and temperature ($F_{1, 2399} = 62.8$; $p < 0.0001$). The feeding of the grey field slug was influenced by the dose of nematodes ($F_{5,2399} = 73.4$; $p < 0.0001$) and DAT ($F_{4, 2399} = 122.8$; $p < 0.0001$).

Table 2. Effect of parasitic nematode feeding inhibition of the Spanish slug at 20 °C.

20 °C Spanish Slug (<i>A. vulgaris</i>)				
Nematode dose (IJs gastropod ⁻¹)	<i>Phasmarhabditis papillosa</i>			
	7 day	14 days	21 days	28 days
0	100 ± 0 Aa	100 ± 0 Aa	100 ± 0 Ab	100 ± 0 Ab
10	100 ± 0 Aa	100 ± 0 Aa	100 ± 0 Ab	100 ± 0 Ab
50	100 ± 0 Aa	100 ± 0 Aa	100 ± 0 Ab	100 ± 0 Ab
100	100 ± 0 Ca	95 ± 5 BCa	95 ± 5 BCab	85 ± 8 Aa
250	100 ± 0 Ba	95 ± 5 ABa	90 ± 7 Aa	90 ± 7 Aa
500	100 ± 0 Ba	100 ± 0 Ba	95 ± 5 ABab	85 ± 8 Aa
Nematode dose (IJs gastropod ⁻¹)	<i>Oscheius myriophilus</i>			
	7 days	14 days	21 days	28 days
0	100 ± 0 Aa	100 ± 0 Ac	100 ± 0 Ac	100 ± 0 Ab
10	100 ± 0 Ba	85 ± 8 Aab	85 ± 8 Aa	75 ± 10 Aa
50	100 ± 0 Ba	100 ± 0 Bc	90 ± 7 Aab	80 ± 9 Aa
100	100 ± 0 Ba	80 ± 9 Aa	90 ± 7 Aab	80 ± 9 Aa
250	100 ± 0 Ba	95 ± 5 ABbc	100 ± 0 Bc	90 ± 7 Aa
500	100 ± 0 Ba	95 ± 5 ABbc	95 ± 5 ABbc	90 ± 7 Aa

The presented data display the mean values ± S.E. The capital letters signify significant differences ($p < 0.05$) among various time intervals for the same nematode dose, whereas small letters indicate significant differences ($p < 0.05$) among different nematode doses during a specific time interval. The key denotes “feeding (100 ± 0)”, where all slugs consumed lettuce.

The mortality rates of the grey field slug caused by both nematode species were similar (*O. myriophilus* 11.3% ± 0.9%; *P. papillosa* 8.1% ± 0.8%). A higher mortality rate was observed at a higher temperature (20 °C). The mortality rate of the grey field slug at 20 °C was 11.5% ± 0.9% independent of other tested factors, while at 15 °C, only 7.8% ± 0.7% of the slugs died.

The Kaplan–Meier survival curve showed that the grey field slug had significantly lower survival time when treated with *P. papillosa* at both tested temperatures ($p < 0.0001$; Figure 2A,B). However, the survival rate of the slug was lower at 20 °C when treated with *O. myriophilus* (Figure 1C). The survival curves suggest that lower doses of *P. papillosa* were more effective at reducing slug survival rates at 15 °C from 14 DAT (Figure 2B). In *P. papillosa* treatments, 50% of the slug population had a survival rate of 26 DAT (15 °C) and 24 DAT (20 °C) in *O. myriophilus* treatments (Figure 2C). The differences in survival curves between treatments with both nematode species were highly significant according to the Mantel–Cox log rank test; however, the slug survival probability was high in treatments with *O. myriophilus* at 15 °C (Figure 2D) and *P. papillosa* at 20 °C (Figure 2A).

The effectiveness of both nematodes against the grey field slug varied at 15 °C, with *O. myriophilus* causing mortality in an average of 5.0% ± 0.9% of the tested slugs, while *P. papillosa* caused mortality in an average of 10.6% ± 1.3%. The mortality of the grey field slug was influenced by DAT, irrespective of other factors, with 0.4% ± 0.4% mortality at 7 DAT, 6.3% ± 1.6% mortality at 14 DAT, 13.3% ± 2.2% mortality at 21 DAT, and 19.2% ± 2.5% mortality at 28 DAT. Additionally, DAT significantly influenced the inhibition of the grey field slug feeding, with feeding inhibition being 39.6% ± 3.2% at 7 DAT, 51.3% ± 3.2% at 14 DAT, 41.3% ± 3.2% at 21 DAT, and 51.6% ± 3.2% at 28 DAT.

The mortality of the grey field slug was significantly affected by the dose of *O. myriophilus* at 21 and 28 DAT at 15 °C. However, the highest nematode dose (500 IJs per slug) also caused significant mortality at 14 DAT compared to other treatments (Figure 2D). On

the other hand, the dose of *P. papillosa* was an important factor in causing mortality of the grey field slug at 14 DAT (Figure 2B). The results of the feeding inhibition of the grey field slug at 15 °C can be found in Table 3.

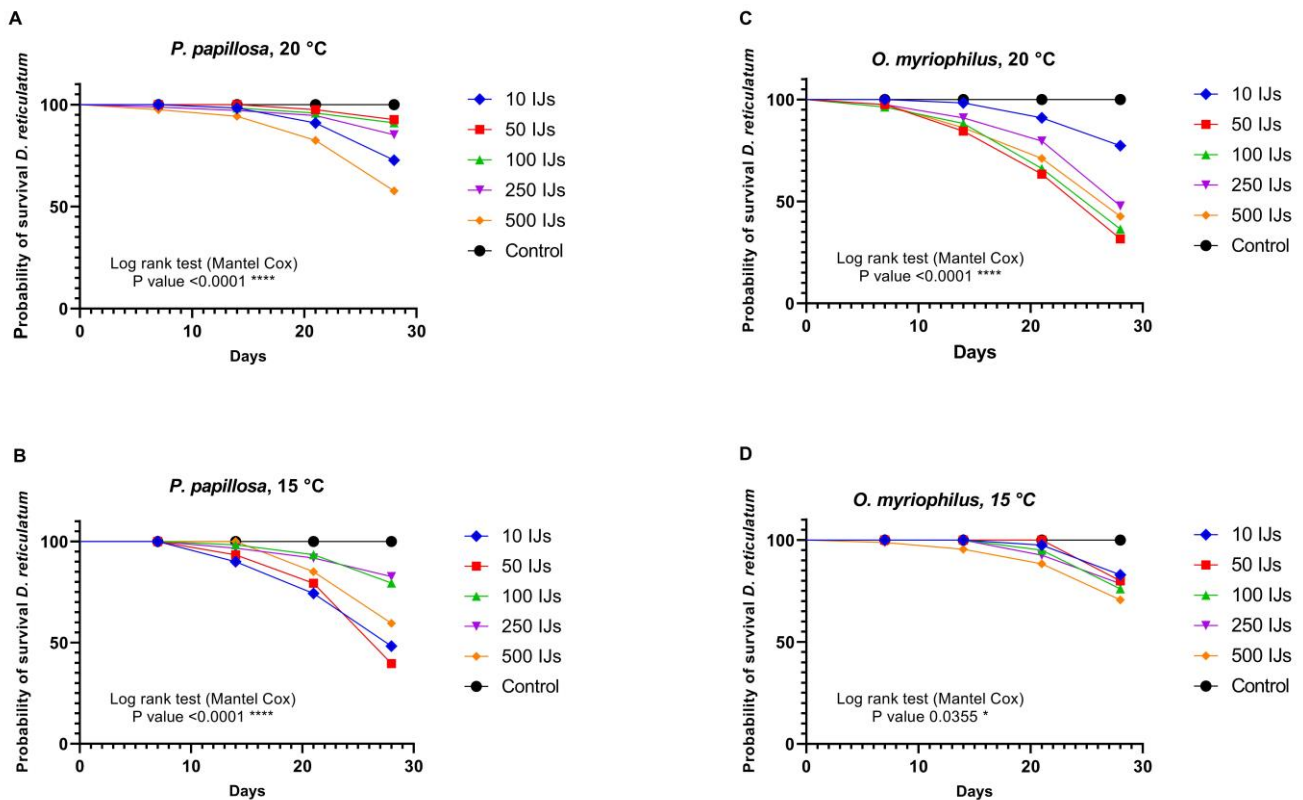


Figure 2. (A–D) Kaplan Meier curves showing the percent survival of the grey field slug (*D. reticulatum*) after treatments with 0, 10, 50, 100, 250 or 500 nematodes per slug displaying a significant difference Mantel Cox log rank test: * indicates a *p* value of <0.05, and **** indicates a *p* value < 0.0001 compared to the slug only control). (A)—The slug survival after *P. papillosa* infection at 20 °C. The slug mortality caused by nematode infection with dose 10 IJs and 500 IJs is significantly different between other treatments since 14 DAT. (B)—The slug survival after *P. papillosa* infection at 15 °C. The slug mortality caused by nematode infection is significantly different between all treatments and control, except 500 IJs dose since 14 DAT. (C)—The slug survival after *O. myriophylus* infection at 20 °C. The slug mortality caused by nematode infection is significantly different between all treatments and control, except 10 IJs since 7 DAT. (D)—The slug survival after *O. myriophylus* infection at 15 °C. The slug mortality caused by nematode infection is significantly different between 500 IJs and other treatments since 14 DAT.

At a temperature of 20 °C, the effectiveness of the two nematodes against the grey field slug varied. *O. myriophylus* resulted in a mortality rate of $17.5\% \pm 1.6\%$ among the tested slugs, whereas *P. papillosa* only caused a mortality rate of $5.5\% \pm 0.9\%$. The duration after treatment (DAT) was found to be a significant factor independent of the other factors tested. The mortality rate of the grey field slug was $5.0\% \pm 1.4\%$ at 7 DAT, $12.5\% \pm 2.1\%$ at 14 DAT, $18.3\% \pm 2.5\%$ at 21 DAT, and $21.7\% \pm 2.7\%$ at 28 DAT.

Table 4 and Figure 2A–D depict the impact of various factors on the mortality and feeding inhibition of the grey field slug at 20 °C. Significant differences in slug mortality were noted, with at least 50 IJs of *O. myriophylus* per slug, starting from 7 DAT (Figure 2C). From 21 DAT, the mortality of slugs was influenced by the dosage of *P. papillosa* (Figure 2A), with significant differences noted among nematode doses at all exposure times, albeit with low mortality rates. Table 4 presents the results of feeding inhibition caused by

O. myriophilus and *P. papillosa* nematodes at 20 °C, indicating that the nematode dosage affected feeding inhibition during all four weeks of the experiment.

Table 3. Effect of parasitic nematodes on feeding inhibition of the grey field slug at 15 °C.

15 °C				
Grey Field Slug (<i>D. reticulatum</i>)				
Nematode dose (IJs gastropod ^{−1})	<i>Phasmarhabditis papillosa</i>			
	7 days	14 days	21 days	28 days
0	100 ± 0 Ac	100 ± 0 Ac	100 ± 0 Ac	100 ± 0 Ac
10	45 ± 11 Aab	30 ± 11 Aa	30 ± 11 Aa	35 ± 11 Aab
50	60 ± 11 Ab	50 ± 12 Aab	45 ± 11 Aab	40 ± 11 Aab
100	30 ± 11 Aa	35 ± 11 Aab	40 ± 11 Aa	55 ± 11 Aab
250	65 ± 11 Bb	55 ± 11 Bb	65 ± 11 Bb	20 ± 9 Aa
500	40 ± 11 Aab	40 ± 11 Aab	40 ± 11 Aa	35 ± 11 Aab
Nematode dose (IJs gastropod ^{−1})	<i>Oscheius myriophilus</i>			
	7 days	14 days	21 days	28 days
0	100 ± 0 Ac	100 ± 0 Ac	100 ± 0 Ad	100 ± 0 Ab
10	55 ± 11 Aa	45 ± 11 Ab	55 ± 11 Aab	40 ± 11 Aa
50	45 ± 11 Aa	40 ± 11 Aab	75 ± 10 Bbc	30 ± 11 Aa
100	60 ± 11 Bab	35 ± 11 Aab	40 ± 11 ABa	45 ± 11 ABa
250	80 ± 9 Cb	20 ± 9 Aa	80 ± 9 Cc	45 ± 11 Ba
500	45 ± 11 Aa	35 ± 11 Aab	40 ± 11 Aa	35 ± 11 Aa

The presented data display the mean values ± S.E. The capital letters signify significant differences ($p < 0.05$) among various time intervals for the same nematode dose, whereas small letters indicate significant differences ($p < 0.05$) among different nematode doses during a specific time interval. The key denotes “feeding (100 ± 0)”, where all slugs consumed lettuce.

Table 4. Effect of parasitic nematodes on feeding inhibition of the grey field slug at 20 °C.

20 °C				
Grey Field Slug (<i>D. reticulatum</i>)				
Nematode dose (IJs gastropod ^{−1})	<i>Phasmarhabditis papillosa</i>			
	7 days	14 days	21 days	28 days
0	100 ± 0 Ab	100 ± 0 Ad	100 ± 0 Ac	100 ± 0 Ab
10	55 ± 11 Ba	65 ± 11 Bc	65 ± 11 Bb	25 ± 10 Aa
50	60 ± 11 Ba	60 ± 11 Bbc	35 ± 11 Aa	25 ± 10 Aa
100	65 ± 11 Ba	55 ± 11 Bab	55 ± 11 Bab	30 ± 11 Aa
250	70 ± 11 Ba	35 ± 11 Aa	50 ± 12 Bab	30 ± 11 Aa
500	65 ± 11 Ba	40 ± 11 Aab	45 ± 11 ABab	35 ± 11 Aa
Nematode dose (IJs gastropod ^{−1})	<i>Oscheius myriophilus</i>			
	7 days	14 days	21 days	28 days
0	100 ± 0 Ac	100 ± 0 Ad	100 ± 0 Ab	100 ± 0 Ab
10	75 ± 10 Bb	70 ± 11 Bc	60 ± 11 ABa	40 ± 11 Aa
50	70 ± 11 Bb	40 ± 11 Aa	45 ± 11 Aa	50 ± 12 ABa
100	55 ± 11 Bb	55 ± 11 Bab	40 ± 11 ABa	30 ± 11 Aa
250	55 ± 11 Ab	65 ± 11 Abc	55 ± 11 Aa	45 ± 11 Aa
500	25 ± 10 Aa	45 ± 11 Aab	45 ± 11 Aa	35 ± 11 Aa

The presented data display the mean values ± S.E. The capital letters signify significant differences ($p < 0.05$) among various time intervals for the same nematode dose, whereas small letters indicate significant differences ($p < 0.05$) among different nematode doses during a specific time interval. The key denotes “feeding (100 ± 0)”, where all slugs consumed lettuce.

3.2.3. The Vineyard Snail (*Cernuella virgata*)

Analyses of the pooled results showed that the mortality of the vineyard snail was influenced by the dose of nematodes ($F_{5, 2399} = 4.7$; $p = 0.0003$); DAT ($F_{4, 2399} = 7.2$;

$p < 0.0001$); temperature ($F_{1, 2399} = 52.0$; $p < 0.0001$); and interaction between the nematode species and temperature ($F_{1, 2399} = 5.1$; $p = 0.0252$). The feeding of the vineyard snail was influenced by the dose of nematodes ($F_{5, 2399} = 33.8$; $p < 0.0001$); DAT ($F_{4, 2399} = 187.6$; $p < 0.0001$); temperature ($F_{1, 2399} = 6.2$; $p = 0.0129$); the nematode species ($F_{1, 2399} = 12.0$; $p = 0.0005$); and interaction between the nematode species and temperature ($F_{1, 2399} = 14.8$; $p < 0.0001$).

At 20 °C, the overall survival time of vineyard snails treated with both nematodes was slightly impacted, as depicted by the Kaplan–Meier survival curve (Figure 3A,C). Conversely, at 15 °C, the survival chances of vineyard snails treated with *O. myriophylus* rapidly declined with increasing DAT (Figure 3D). The survival curves indicate that lower doses of *P. papillosa* were effective in reducing the snail survival rates at 15 °C, starting from 14 DAT (Figure 3B). The differences in survival curves between treatments with both nematode species were only significant in treatments with *O. myriophylus* at 15 °C according to the Mantel–Cox log rank test ($p = 0.0015$); however, snail survival at the end of the experiment was still high.

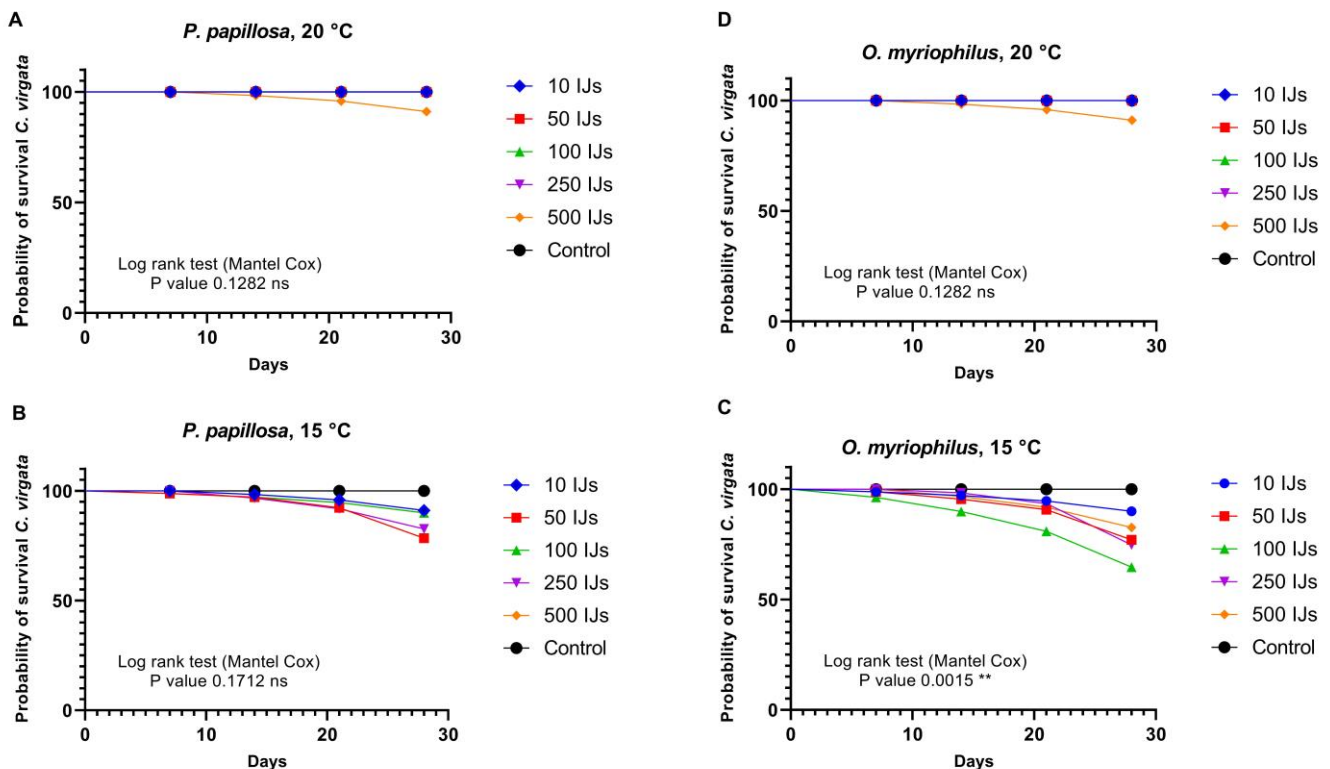


Figure 3. (A–D) Kaplan Meier curves showing the percent survival of the vineyard snail (*C. virgata*) after treatments with 0, 10, 50, 100, 250 or 500 nematodes per snail (displaying a significant difference, Mantel Cox log rank test: ** indicates a p value of <0.01 compared to the slug only control). (A)—The snail survival after *P. papillosa* infection at 20 °C. The snail mortality caused by nematode infection was slightly affected by the highest nematode dose, but the mean differences among the treatments were not significant. (B)—The snail survival after *P. papillosa* infection at 15 °C. The snail mortality caused by nematode infection was not significantly different between the nematode’s doses. (C)—The snail survival after *O. myriophylus* infection at 20 °C. The snail mortality caused by nematode infection slightly affected by the highest nematode dose, but the mean differences among the treatments were not significant. (D)—The snail survival after *O. myriophylus* infection at 15 °C. The snail mortality caused by nematode infection with 100 IJs per snail was significantly different treatment between and 10 and 250 IJs since 14 DAT.

Irrespective of other tested factors, no significant differences were observed in the mortality of the vineyard snail between the nematode species (*O. myriophylus* $3.5\% \pm 1.5\%$;

P. papillosa $2.3\% \pm 0.4\%$). Both nematodes exhibited higher efficacy against the vineyard snail at a lower temperature ($15\text{ }^{\circ}\text{C}$). At a temperature of $15\text{ }^{\circ}\text{C}$, regardless of the other factors tested, the average mortality rate of the vineyard snail was $5.3\% \pm 0.6\%$, whereas the mortality rate was $0.5\% \pm 0.2\%$ at $20\text{ }^{\circ}\text{C}$.

At $15\text{ }^{\circ}\text{C}$, *O. myriophilus* caused snail mortality of $6.7\% \pm 1.0\%$, while *P. papillosa* caused mortality of $4.0\% \pm 0.8\%$. The duration of exposure to nematodes greatly influenced the inhibition of vineyard snail feeding. After 7 days, feeding of the vineyard snail was reduced by $49.2\% \pm 3.2\%$, at 14 DAT $53.8\% \pm 3.2\%$, at 21 DAT $69.2\% \pm 3.0\%$, and at 28 DAT $51.7\% \pm 3.2\%$. Significant differences in snail mortality caused by *O. myriophilus* between the doses of 100, 250, and 500 IJs per snail and the control were observed at 7 DAT. The dose of *P. papillosa* did not significantly influence the mortality of the vineyard snail at $15\text{ }^{\circ}\text{C}$. However, statistically significant differences in the reduction of vineyard snail feeding were observed among the doses of both nematode species at all exposure times (Table 5) at $15\text{ }^{\circ}\text{C}$.

Table 5. Effect of parasitic nematodes on feeding inhibition of the vineyard snail at $15\text{ }^{\circ}\text{C}$.

$15\text{ }^{\circ}\text{C}$				
Vineyard Snail (<i>C. virgata</i>)				
Nematode dose (IJs gastropod ^{−1})	<i>Phasmarhabditis papillosa</i>			
	7 days	14 days	21 days	28 days
0	100 ± 0 Ad	100 ± 0 Ac	100 ± 0 Ac	100 ± 0 Ac
10	75 ± 10 Cc	40 ± 11 Bab	30 ± 11 Ab	30 ± 11 Aab
50	50 ± 12 Bb	30 ± 11 ABab	20 ± 9 Aab	25 ± 10 Aab
100	20 ± 9 Aa	45 ± 11 Bab	10 ± 7 Aa	15 ± 8 Aa
250	20 ± 9 Aa	55 ± 11 Bb	10 ± 7 Aa	20 ± 9 Aab
500	25 ± 10 Aa	25 ± 10 Aa	30 ± 11 Ab	40 ± 11 Ab
Nematode dose (IJs gastropod ^{−1})	<i>Oscheius myriophilus</i>			
	7 days	14 days	21 days	28 days
0	100 ± 0 Ad	100 ± 0 Ac	100 ± 0 Ab	100 ± 0 Ab
10	75 ± 9 Bc	30 ± 11 Aa	40 ± 11 Aa	45 ± 11 Aa
50	70 ± 11 Bc	45 ± 11 Aab	45 ± 11 Aa	60 ± 11 ABa
100	35 ± 11 Aa	60 ± 11 Bb	35 ± 11 Aa	65 ± 11 Ba
250	40 ± 11 ABab	45 ± 11 BCab	20 ± 9 Aa	65 ± 11 Ca
500	60 ± 11 Bbc	45 ± 11 ABab	30 ± 11 Aa	65 ± 11 Ba

The presented data display the mean values ± S.E. The capital letters signify significant differences ($p < 0.05$) among various time intervals for the same nematode dose, whereas small letters indicate significant differences ($p < 0.05$) among different nematode doses during a specific time interval. The key denotes “feeding (100 ± 0)”, where all slugs consumed lettuce.

The mortality of the vineyard snail did not differ between the two tested nematode species at $20\text{ }^{\circ}\text{C}$, with both *O. myriophilus* and *P. papillosa* causing low mortality rates of $0.3\% \pm 0.2\%$ and $0.7\% \pm 0.3\%$, respectively. Additionally, the dose of nematodes did not have a significant impact on vineyard snail mortality during the 4-week experiment. However, significant differences in the reduction of vineyard snail feeding at $20\text{ }^{\circ}\text{C}$ were observed among the different doses of both nematode species at all exposure times, as presented in Table 6.

Table 6. Effect of parasitic nematodes on feeding inhibition of the vineyard snail at 20 °C.

20 °C Vineyard Snail (<i>C. virgata</i>)				
Nematode dose (IJs gastropod ^{−1})	<i>Phasmarhabditis papillosa</i>			
	7 days	14 days	21 days	28 days
0	100 ± 0 Ab	100 ± 0 Ad	100 ± 0 Ac	100 ± 0 Ac
10	75 ± 10 Aa	25 ± 10 Aab	30 ± 11 Aab	30 ± 11 Ab
50	55 ± 11 Ba	60 ± 11 Bc	30 ± 11 Aab	45 ± 11 ABb
100	60 ± 11 Ba	45 ± 11 Bbc	45 ± 11 Bb	10 ± 7 Aa
250	95 ± 5 Bb	20 ± 9 Aa	15 ± 8 Aa	30 ± 11 Ab
500	100 ± 0 Bb	35 ± 11 Aab	20 ± 9 Aa	30 ± 11 Ab
Nematode dose (IJs gastropod ^{−1})	<i>Oscheius myriophilus</i>			
	7 days	14 days	21 days	28 days
0	100 ± 0 Ac	100 ± 0 Ab	100 ± 0 Ac	100 ± 0 Ab
10	65 ± 11 Ca	40 ± 11 ABa	45 ± 11 ABCb	25 ± 10 Aa
50	70 ± 11 Ca	50 ± 12 BCa	25 ± 10 Aab	30 ± 11 ABa
100	65 ± 11 Ba	45 ± 11 Ba	20 ± 9 Aa	15 ± 8 Aa
250	90 ± 7 Bb	35 ± 11 Aa	25 ± 10 Aab	30 ± 11 Aa
500	55 ± 11 Ca	45 ± 11 Ba	10 ± 7 Aa	20 ± 9 Aa

The presented data display the mean values ± S.E. The capital letters signify significant differences ($p < 0.05$) among various time intervals for the same nematode dose, whereas small letters indicate significant differences ($p < 0.05$) among different nematode doses during a specific time interval. The key denotes “feeding (100 ± 0)”, where all slugs consumed lettuce.

4. Discussion

Parasitic nematodes are known to infect a wide range of organisms, including gastropods [14,15]. The evaluation of the best gastropod parasitic nematode for biocontrol involves assays to ensure that the nematodes are effective, safe, and economically viable. The promising nematode candidates for use in biocontrol against gastropods possess traits, such as host-specificity, high virulence, reduction of gastropod feeding, low impact on nontarget organisms, fast reproduction rates and short generation times, and environmental tolerance. Other desirable nematode traits include persistence in the environment, compatibility with other control methods, and efficient and cost-effective large-scale production [14,15].

In general, the mortality of gastropods can vary widely depending on the species of nematode, the species and age of the gastropod, and the environmental conditions [8,15,17]. It is important to note that gastropod mortality should not be used as a sole criteria in determining the potential of nematodes as biocontrol agents. Nematode host specificity, reduction in gastropod feeding, environmental safety, economic viability, and other traits are equally important [8,15]. For instance, nematodes could cause low mortality rates of gastropods but may still be effective if the nematode causes sublethal effects, such as reduced feeding, or affects the gastropod life cycle.

This publication represents the first report of *O. myriophilus* in Slovenia and the first record of this nematode species in the gastropod host, *A. vulgaris*, the Spanish slug, as well as its virulence against gastropods. *O. myriophilus* has been confirmed in various host species, mainly invertebrates, and has shown potential as an entomopathogenic nematode [24–27]. Recent studies identified *Serratia nematodiphila* and *Serratia marcescens* [24] as associated bacteria. In Mexico, *S. marcescens* isolated from *O. myriophilus* caused 100% mortality of *Galleria mellonella* larvae, demonstrating its potential as a biocontrol agent [24]. Previous studies have classified *Oscheius* species as entomopathogens, but their classification as such has been contested [24,31,32]. Our results suggest that *O. myriophilus* should be reconsidered only as parasitic, and further investigation is necessary to confirm this hypothesis. This species is essential for experimental evolution studies of the process of symbiosis and parasitism.

Traditionally, chemical bait pellets are used to control slug populations. However, an alternative method is biological control using the gastropod-parasitic nematode *P. hermaphrodita*, which has been found to be effective against several species of slugs and snails [14,15,33]. Despite this, various studies have reported low efficacy of *P. hermaphrodita* against certain Arionidae species [15], including *A. vulgaris* [33]. Some studies suggest that the nematode is only effective against younger stages of *A. vulgaris* [34]. Additionally, *A. hortensis* and *A. distinctus* have shown low susceptibility to *P. hermaphrodita* [15]. However, our study found that Slovenian strains of *P. papillosa* and *O. myriophilus* were capable of causing mortality in *A. vulgaris*, even when the specimens weighed over 1 g (the average weight in our study was over 5 g). Both nematodes were less effective against *C. virgata* and *D. reticulatum*. Other studies have shown that *P. papillosa* is lethal to *D. panormitanum* and has potential for commercial use against invasive gastropods in high-value crops [11]. Furthermore, *P. papillosa* has been found to be highly effective against *D. reticulatum*, causing over 60% mortality after only 4 days [17]. In a related study, *P. hermaphrodita*, *P. californica*, and *P. papillosa* were found to be equally as lethal as the recommended molluscicide Sluggo Plus[®] against white garden snails [18]. Our investigation found that, even at extremely low doses (from 0.15 to 7.9 nematodes/cm²), *P. papillosa* and *O. myriophilus* were effective against *A. vulgaris* at 15 °C and could reduce the costs of nematode application while still providing significant plant protection comparable to chemical molluscicides.

Using nematodes as a substitute for chemical molluscicides can be limited due to their higher cost [15]. However, studies by the authors of [8] showed that certain slug species, such as *D. reticulatum* and *A. ater*, avoid areas treated with nematodes (*P. hermaphrodita*) at rates of 38 IJs/cm² and 120 IJs/cm², suggesting a potential deterrent effect. Additionally, hosts may quickly identify high populations of pathogenic nematodes and either avoid them or activate defensive behaviors (e.g., rubbing against surfaces) on the first day after application. Cutler et al. [35] also reported that *P. hermaphrodita* could attract gastropods. In our study, the better effect of the low dose of *O. myriophilus* and *P. papillosa* could be attributed to intraspecific competition among nematodes [36]. However, the optimal number of nematodes for host infection and mortality varies among nematode and host species [37,38]. Moreover, Kruitbos et al. [39] demonstrated that nematode behavior is substrate-specific and varies by species. Therefore, to support our findings, future tests should be conducted on more appropriate substrates than the moistened filter paper used in our investigation [40].

Temperature and moisture are critical factors for both gastropods and nematodes. Slugs require a lot of water for locomotion, as they consume water through mucus production and lose it when breathing. They are highly susceptible to drying conditions [2]. *Phasmarhabditis* nematodes are intolerant of moderately high temperatures [8] and are very sensitive to desiccation and exposure to ultraviolet light when applied to the soil surface [15]. Our investigation results indicate that both nematodes (*P. papillosa* and *O. myriophilus*) perform better at lower temperatures (15 °C) than at higher temperatures (20 °C). Although the thermal ecology of *Phasmarhabditis* and *Oscheius* nematodes is not well understood, Wilson and Rae [8] demonstrated that nematodes are less tolerant to increasing temperatures from 14 to 24 °C than the host slug *D. reticulatum*. Our findings also support previous studies by the authors of [8] indicating that the optimal temperature range for population growth of *Phasmarhabditis* nematodes is between 12 and 18 °C.

Our investigation found that using *P. papillosa* and *O. myriophilus* resulted in a significant decrease in mollusk herbivory. This aligns with previous studies that tested *P. hermaphrodita* against *A. vulgaris* slugs and observed a decrease in herbivory [10,15]. However, it is important to note that information on the effectiveness of *P. papillosa* against other slug/snail species is lacking [11]. While Dörler et al. [10] suggested that avoidance behavior could explain the reduction in herbivory, this was not applicable in our experiment as the slugs were confined to the experimental arena and unable to seek food elsewhere.

Developing biological control products using natural enemies of pests rather than chemical pesticides offers numerous advantages. One of the main benefits is greater

specificity, which reduces the risk of harming nontarget organisms [8]. This approach also helps to decrease the use of toxic agrochemicals, thus promoting ecologically safe food production [41]. Additionally, the development of new synthetic pesticides has become increasingly challenging and costly compared to biopesticides [42]. The existence of organic growers who avoid synthetic pesticides has created a niche market for biopesticides based on natural enemies, such as *P. papillosa* and *O. myriophyllus*. Furthermore, strict residue limits imposed by regulatory bodies, the food industry, and supermarkets have driven the use of biopesticides [9]. Biopesticides are also a necessary tool in integrated pest management programs to prevent pest resistance [8]. However, in the case of gastropod pests, the lack of studies on the efficacy of other beneficial organisms and the reluctance of major agrochemical producers to develop new active ingredients for a fragmented market are key drivers for the development of new nematode-based biomolluscicides.

5. Conclusions

In summary, this report provides the first record of the nematode species *O. myriophyllus* from the gastropod host, *A. vulgaris*, also known as the Spanish slug. The results suggest that *O. myriophyllus* has virulence against gastropods and may have potential as a biocontrol agent against insect species. Furthermore, the study shows that the Slovenian strains of *P. papillosa* and *O. myriophyllus* are capable of causing mortality of the Spanish slug, even when the slug specimens were over 1 g, demonstrating their potential as an alternative to chemical molluscicides. However, further investigation is needed to confirm the hypothesis that *O. myriophyllus* should be classified only as parasitic, rather than entomopathogenic. Overall, this study highlights the potential of *O. myriophyllus* and *P. papillosa* as model organisms for experimental evolution studies of the process of symbiosis and parasitism.

Author Contributions: Ž.L. and I.M. designed the project; Ž.L., S.T., T.T., S.Á., T.L. and I.M. conducted the experiment and analyzed the data. Ž.L. and I.M. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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