Nematode associates and susceptibility of a protected slug (*Geomalacus maculosus*) to four biocontrol nematodes

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Nematode associates and susceptibility of a protected slug (*Geomalacus maculosus*) to four biocontrol nematodes

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Nematode associates and susceptibility of a protected slug (*Geomalacus maculosus*) to four biocontrol nematodes

The impact of selected entomopathogenic nematodes and *Phasmarhabditis hermaphrodita* on the EU-protected slug *Geomalacus maculosus* and the sympatric *Lehmannia marginata* was investigated. There was no significant difference in mortality between slugs treated with nematodes and their controls. The presence of *P. hermaphrodita* in two *G. maculosus* cadavers may be the result of necromenic behaviour. This study constitutes the first record of *P. californica* in Europe.

Keywords: Biological control, Entomopathogenic nematodes, *Phasmarhabditis hermaphrodita*, risk assessment, non-target host.

Geomalacus maculosus Allman 1843 (Gastropoda: Arionidae) is protected under EU legislation due to its restricted worldwide distribution to western Ireland and north-western Iberia (Mc Donnell, O'Meara, Nelson, Marnell & Gormally, 2013). While it inhabits a range of open and deciduous woodland habitats in Ireland (Mc Donnell & Gormally, 2011), it has only recently been discovered in commercial conifer plantations (Kearney, 2010). Another slug species *Lehmannia marginata* Müller 1774, (Gastropoda: Limacidae) is commonly found in sympatry with *G. maculosus* (Reich, O'Meara, Mc Donnell & Gormally, 2012).

The development of novel biocontrol agents to control pest species continues to grow (Campos-Herrera, 2015) in both commercial forestry and agriculture. Studies are being undertaken in Britain and Ireland on the use of the rhabditoid entomopathogenic nematodes (EPNs) *Heterorhabditis downesi* Stock, Griffin and Burnell 2002, *Steinernema carpocapsae* Weiser 1955 and *Steinernema feltiae* Filipjev 1934 as potential biocontrol agents of the pine weevil *Hylobius abietis* Linnaeus 1758 (Coleoptera: Curculionidae) (Dillon, Ward, Downes & Griffin, 2006; Williams *et al.*, 2013). In addition, *Phasmarhabditis hermaphrodita* Schneider 1859 (Nematoda:

Rhabditida), a lethal slug parasite (Wilson, Glen & Georges, 1993), is currently retailed as Nemaslug® (produced by BASF) to farmers and crop growers throughout Europe (Rae, Verdun, Grewal, Robertson & Wilson, 2007).

No studies to date regarding the effect of EPNs or *P. hermaphrodita* on *G. maculosus* have been undertaken. Given the presence of *G. maculosus* in mature and clear-felled compartments of commercial conifer plantations and in domestic gardens adjacent to woodlands/forests, we investigated whether EPNs and *P. hermaphrodita* had any effect on the survival of the species. We also tested for possible effects of EPNs on the sympatric slug species *L. marginata*, heretofore untested.

Phasmarhabditis hermaphrodita (DMG0001) was supplied by BASF and stored at 9±1°C until use. *Heterorhabditis downesi* (K122), *S. carpocapsae* (All) and *S. feltiae* (4CFMO) were cultured *in vivo* on larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) and were stored at 9±1°C until use. Individuals of *G. maculosus* were collected (under licence nos. C158/2015 and C169/2015 issued by the National Parks and Wildlife Services, Ireland) from conifer plantations and clear-felled areas in Counties Galway and Kerry, Ireland. Individuals of *L. marginata* and *Deroceras reticulatum* Müller 1774 (Gastropoda: Agriolimacidae) were collected from woodlands, conifer plantations and gardens from Co. Galway, Ireland. Experiments were undertaken in the Applied Ecology Unit at the National University of Ireland-Galway. Statistical analyses were performed using MINITAB 17® (Minitab Inc., USA) and comparisons between mortality rates of treated groups and the corresponding control group were undertaken using a one-sided Fisher's exact test (P=0.001). All P values are given in Table 1.

Experiment 1: Mortality rates of G. maculosus and L. marginata treated with EPNs. After a minimum of three weeks in isolation (Tandingan De Ley, Mc Donnell, Lopez,

Paine & De Ley, 2014) to exclude naturally infected or unhealthy individuals, slugs were placed in individual Petri dishes (5.5 cm diameter) with filter paper and a thin slice of carrot. The EPNs (500 infective juveniles (IJs)/slug, 25 times the application rate required to kill *G. mellonella*) contained in 1.5 ml of tap water were pipetted directly onto the mantle of each slug since EPNs generally enter the hosts through natural openings (Kaya & Gaugler, 1993) and in slugs EPNs are thought to enter through the pneumostome (Kaya & Mitani, 2000). For control slugs, 1.5 ml tap water was pipetted onto their mantle. The infection procedure followed Glen, Wilson, Brain and Stroud (2000) whereby slugs were kept in contact with the nematodes for the first three days, after which the slugs were transferred to individual nematode-free glass containers with moist tissue paper and carrot where they were kept for 18 days (21 days in total). For each slug species, there were three treatments (one for each nematode species) plus a control, each consisting of 15 repeats. To confirm that the EPNs used in the experiments were infective, three groups of *G. mellonella* larvae were infected with the three nematode species at a rate of 20 IJs/larva, with a fourth group receiving tap water only. All experiments/*G. mellonella* cultures were maintained at 20°C. Mortality of the slug species was recorded at two-day intervals throughout the experiment, while mortality of *G. mellonella* was recorded on Day 3 as the symbiotic bacteria of EPNs generally kill an infected host within 2-3 days (Grewal, 2012). *Galleria mellonella* cadavers were dissected and checked daily for nematodes. Slug cadavers were placed on White traps (White, 1927) and were checked every second day for nematodes. The nematodes recovered from the slugs were preserved in ethanol and identified by sequencing a fragment of the small subunit (SSU) or 18S, and/or D2-D3 domains of the large subunit (LSU) or 28S rRNA. About 700-800 base pairs from the 5' end of the 18S were amplified using primers SSU18A (5′-AAAGATTAAGCCATGCATG-3′) and SSU26R

(5′-CATTCTTGGCAAATGCTTTCG-3′) (Blaxter *et al.*, 1998); with the following PCR conditions: 2 min at 95°C, 35 cycles including, 15 s at 95°C, 15 s at 50°C, 2 min at 72°C, followed by 7 min at 72°C. D2-D3 domains of 28S were amplified and sequenced as described in Tandingan De Ley *et al*. (2014). DNA sequences were compared by BLAST with those published in GenBank.

Mortality rates of *G. mellonella* larvae treated with EPNs were significantly greater ($P \leq 0.001$ for each species) than the mortality rates of non-treated larvae indicating that the nematodes used in Experiment 1 were infective. This was further substantiated by the recovery of EPNs from all the *G. mellonella* cadavers. In contrast, there was no significant difference in mortality for *G. maculosus* or *L. marginata* between treated slugs and controls and none of the EPN species were recovered from cadavers of either slug species. Greater (unexplained but non–significant) mortality rates for *G. maculosus* treated with *H. downsei* were observed in the latter days of the experiment but this was not observed when the experiment was repeated (Figure 1). The results indicate, for the first time, that the survival of the two slug species tested is not affected by EPNs. This is supported by Wilson, Glen, Hughes, Pearce and Rodgers (1994) who demonstrated that the use of EPNs in biological control is unlikely to affect non-target mollusc species. Although Kaya and Mitani (2000) found that EPNs could infect (but not reproduce within) *D. reticulatum*, the absence of EPNs in the cadavers of *G. maculosus* and *L. marginata* suggest that the nematodes did not enter the slug species used in this study.

Experiment 2: Mortality rates of G. maculosus treated with P. hermaphrodita (Nemaslug®). Three groups of *G. maculosus* (15 individuals per group) were used. One group was kept as a control and the other two groups were treated with *P. hermaphrodita* at: (a) the commercially recommended application rate (30

nematodes/cm^2) (Glen & Wilson, 1997); and (b) five times the recommended application rate (150 nematodes/cm^2). Three groups (15 individuals per group) of *D. reticulatum*, also treated in the same manner, were used as positive controls since *D. reticulatum* is known to be vulnerable to *P. hermaphrodita* (Wilson *et al*., 1993). All experiments were undertaken at 16°C and nematodes were pipetted onto the slug mantle since it is believed that *P. hermaphrodita* uses the dorsal integumental pouch, posterior to the mantle, to enter the slug body (Wilson *et al*., 1993). Otherwise, procedures described in Experiment 1 relating to the maintenance and infections of slugs were the same.

Mortality rate of *D. reticulatum* treated with *P. hermaphrodita* (Nemaslug®) at the higher application rate was significantly greater ($P<0.001$) than that of the controls, although this was not the case at the recommended application rate, possibly due to the greater than expected mortality of the control group during the second half of the experiment. Nevertheless, *P. hermaphrodita* individuals were recovered from all treated *D. reticulatum* indicating that the nematodes used were infective. While *P. hermaphrodita* was also recovered from two individuals of *G. maculosus* which died during the experiment (Figure 1), there was no significant difference overall ($P>0.001$) in mortality between treated slugs and controls. It is possible that the *P. hermaphrodita* found in the two *G. maculosus* cadavers were the result of necromenic as opposed to parasitic behaviour by the nematodes i.e. the nematodes entered the living slugs and waited for the host to die before resuming their development (Wilson & Grewal, 2005). This possibility is further supported by the low mortality recorded in treated *G. maculosus* and the absence of *G. maculosus* mortalities until Day 8 of the experiment, at which stage *D. reticulatum* mortalities had already occurred using both the high and the recommended Nemaslug® application rates.

117 *Nematode species in association with G. maculosus in the wild*. Field-collected *G.*
118 *maculosus* individuals, which died during the quarantine period, were placed on White
119 traps and kept at 18±5°C (Iglesias & Speiser, 2001; Kaya & Mitani, 2000). Recovered
120 batches of emerging nematodes were divided into two parts. One was tested with
121 Koch's postulates to determine its pathogenicity (Dillman *et al.*, 2012) and the other
122 was preserved in 100% ethanol for identification by rRNA sequencing as previously
123 described. In total, four nematodes were identified: *Phasmarhabditis californica*,
124 *Pristionchus entomophagus, Pristionchus triformis* and *Rhabditophanes* sp. KR3021.
125 None of these species fulfilled Koch's postulates i.e. none of the nematode species
126 recovered was pathogenic to other *G. maculosus*. It is worth noting that this is the first
127 time *P. californica* has been isolated in a country other than the USA (Tandingan De
128 Ley *et al.*, 2016) and New Zealand (Wilson, Wilson, Aldeers & Tourna, 2016).
129 In conclusion, the results of this preliminary study indicate that the nematode
130 biocontrol agents tested are unlikely to impact significantly on *G. maculosus*
131 populations in the wild. Further work investigating the behaviour of *P. hermaphrodita*
132 in relation to *G. maculosus* is recommended .

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Disclosure statement

No financial interest or benefit has arisen from direct application of the research reported on here.

References

Blaxter, M. L., De Ley, P., Garey, J. R., Liu, L. X., Scheldeman, P., Vierstraete, A., Vanfleteren, J. R ., Mackey, L. Y., Dorris, M., Frisse, L. M., Vida, J. T. & Thomas, W. K. (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature* 392, 71-75.

Campos-Herrera, R. (Ed.). (2015). *Nematode pathogenesis of insects and other pests: Ecology and applied technologies for sustainable plant and crop protection.* Springer.

Dillman, A. R., Chaston, J. M., Adams, B. J., Ciche, T. A., Goodrich-Blair, H., Stock, S. P., & Sternberg, P. W. (2012). An entomopathogenic nematode by any other name. *PLoS Pathogens* 8, e1002527.

Dillon, A. B., Ward, D., Downes, M. J., & Griffin, C. T. (2006). Suppression of the large pine weevil *Hylobius abietis* (L.)(Coleoptera: Curculionidae) in pine stumps by entomopathogenic nematodes with different foraging strategies. *Biological Control* 38, 217-226.

Glen, D. M., & Wilson, M. J. (1997). Slug-parasitic nematodes as biocontrol agents for slugs. *Agro Food Industry Hi-Tech* 8, 23-27.

Glen, D. M., Wilson, M. J., Brain, P., & Stroud, G. (2000). Feeding activity and survival of slugs, *Deroceras reticulatum*, exposed to the rhabditid nematode, *Phasmarhabditis hermaphrodita*: a model of dose response. *Biological Control* 17, 73-81.

Grewal, P. S. (2012). Entomopathogenic nematodes as tools in integrated pest management. *Integrated pest management: Principles and practice*, 162-236.

Iglesias, J., & Speiser, B. (2001). Consumption rate and susceptibility to parasitic nematodes and chemical molluscicides of the pest slugs *Arion hortensis* ss and *A. distinctus*. *Anzeiger für Schädlingskunde/Journal of Pest Science* 74, 159-166.

Kaya, H. K., & Gaugler, R. (1993). Entomopathogenic nematodes. *Annual Review of Entomology* 38, 181-206.

Kaya, H. K., & Mitani, D. R. (2000). Molluscicidal nematodes for the biological control of pest slugs. *Slosson Report*, 14.

Kearney, J. (2010). Kerry slug (*Geomalacus maculosus* Allman, 1843) recorded at Lettercraffroe, Co. Galway. *The Irish Naturalists' Journal* 31, 68-69.

Mc Donnell, R. J., & Gormally, M. J. (2011). Distribution and population dynamics of the Kerry Slug, *Geomalacus maculosus* (Arionidae). *Irish Wildlife Manuals*, No. 54. National Parks and Wildlife Service, Department of Arts, Heritage and the Gaeltacht, Dublin, Ireland.

Mc Donnell, R. J., O'Meara, K., Nelson, B., Marnell, F., & Gormally, M. J. (2013). Revised distribution and habitat associations for the protected slug, *Geomalacus maculosus* (Stylommatophora: Arionidea) in Ireland. *Basteria* 77, 33-37.

Rae, R., Verdun, C., Grewal, P. S., Robertson, J. F., & Wilson, M. J. (2007). Biological control of terrestrial molluscs using *Phasmarhabditis hermaphrodita*—progress and prospects. *Pest Management Science* 63, 1153-1164.

Reich, I., O'Meara, K., Mc Donnell, R. J., & Gormally, M. J. (2012). An assessment of the use of conifer plantations by the Kerry Slug (*Geomalacus maculosus*) with reference to the impact of forestry operations. *Irish Wildlife Manuals*, No. 64. National Parks and Wildlife Service, Department of Arts, Heritage and the Gaeltacht, Dublin, Ireland.

Tandingan De Ley, I., Mc Donnell, R., Lopez, S., Paine, T.D., & De Ley, P. (2014). *Phasmarhabditis hermaphrodita* (Nematoda: Rhabditidae), a potential biocontrol agent isolated for the first time from invasive slugs in North America. *Nematology* 16, 1129-1138.

Tandingan De Ley, I., Holovachov, O., Mc Donnell, R. J., Bert, W., Paine, T. D., & De Ley, P. (2016). Description of *Phasmarhabditis californica* n. sp. and first report of *P. papillosa* (Nematoda: Rhabditidae) from invasive slugs in the USA. *Nematology* 18, 175-193.

White, G. F. (1927). A method for obtaining infective nematode larvae from cultures. *Science* 66, 302-303.

Williams, C. D., Dillon, A. B., Harvey, C. D., Hennessy, R., Mc Namara, L., & Griffin, C. T. (2013). Control of a major pest of forestry, *Hylobius abietis*, with entomopathogenic nematodes and fungi using eradicant and prophylactic strategies. *Forest Ecology and Management* 305, 212-222.

Wilson, M. J., Glen, D. M., & George, S. K. (1993). The rhabditid nematode *Phasmarhabditis hermaphrodita* as a potential biological control agent for slugs. *Biocontrol Science and Technology* 3, 503-511.

Wilson, M. J., Glen, D. M., Hughes, L. A., Pearce, J. D., & Rodgers, P. B. (1994). Laboratory tests of the potential of entomopathogenic nematodes for the control of field slugs (*Deroceras reticulatum*). *Journal of Invertebrate Pathology* 64, 182-187.

Wilson, M. J., & Grewal, P. S. (2005). Biology, production and formulation of slug-parasitic nematodes. *PS GrewalRU EhlersD Shapiro-Ilan. Nematodes as biological control agents. Wallingford, UK: CABI Publishing*, 421-429.

Wilson, M.J., Wilson, D.J., Aldeers, L.T., & Tourna, M. (2016). Testing a new low-labour method for detecting the presence of *Phasmarhabditis* spp. in slugs in New Zealand. *Nematology* 18, 925-931.

Table 1. *P*-values (Fisher's exact test) comparing treatments and controls (positive controls are *G. mellonella* and *D. reticulatum* for the EPNs and *P. hermaphrodita* treatments respectively).

Figure 1. Percentage mortality of (a) *G. maculosus* and (b) *L. marginata* exposed to EPNs (*H. downesi, S. carpocapsae* and *S. feltiae*). Note that the exposure experiment in (a) with *H. downesi* was repeated (tr.1 and tr.2) as the first results were unexpectedly high. Percentage mortality of (c) *G. maculosus* and (d) *D. reticulatum* exposed to *P. hermaphrodita*.

	Treatments			
	Entomopathogenic nematodes (EPNs)		***Phasmarhabditis hermaphrodita***	
	Comparison	*P*-value	Comparison	*P*-value
Geomalacus maculosus	Group treated with *H. downesi* vs. Control group	0.198	Group treated with 30 nematodes/cm^2 vs. Control group	0.5
	Group treated with *S. carpocapsae* vs. Control group	0.326	Group treated with 150 nematodes/cm^2 vs. Control group	0.5
	Group treated with *S. feltiae* vs. Control group	0.326	Group treated with 150 nematodes/cm^2 vs. Group treated with 30 nematodes/cm^2	1
Lehmannia marginata	Group treated with *H. downesi* vs. Control group	0.674	Not tested	-
	Group treated with *S. carpocapsae* vs. Control group	0.835	Not tested	-
	Group treated with *S. feltiae* vs. Control group	0.95	Not tested	-
Positive control	Group treated with *H. downesi* vs. Control group	<0.001	Group treated with 30 nematodes/cm^2 vs. Control group	0.072
	Group treated with *S. carpocapsae* vs. Control group	<0.001	Group treated with 150 nematodes/cm^2 vs. Control group	<0.001
	Group treated with *S. feltiae* vs. Control group	<0.001	Group treated with 150 nematodes/cm^2 vs. Group treated with 30 nematodes/cm^2	0.021

Table 1. *P*-values (Fisher's exact test) comparing treatments and controls (positive controls are *G. mellonella* and *D. reticulatum* for the EPNs and *P. hermaphrodita* treatments respectively).

338x190mm (96 x 96 DPI)

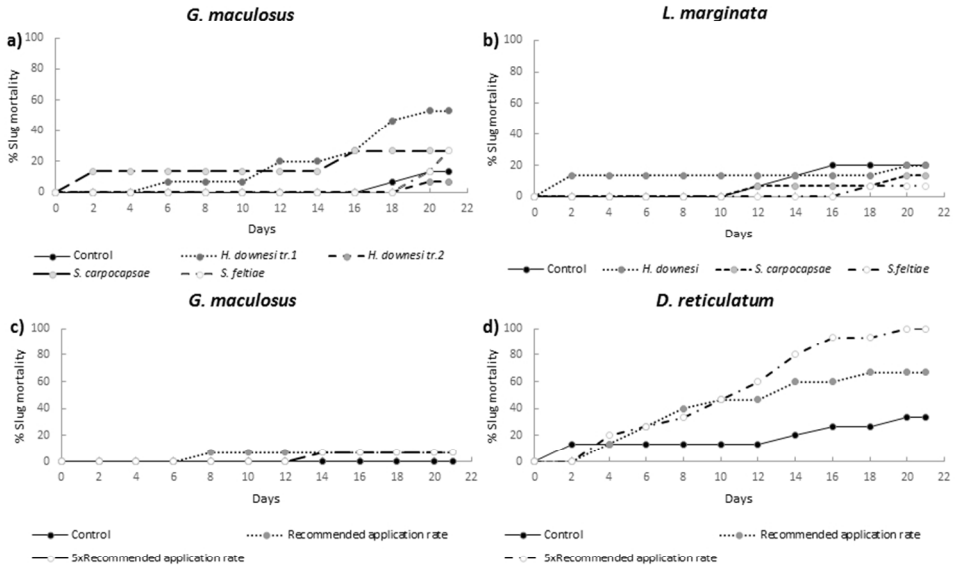

Figure 1. Percentage mortality of (a) *G. maculosus* and (b) *L. marginata* exposed to EPNs (*H. downesi*, *S. carpocapsae* and *S. feltiae*). Note that the exposure experiment in (a) with *H. downesi* was repeated (tr.1 and tr.2) as the first results were unexpectedly high. Percentage mortality of (c) *G. maculosus* and (d) *D. reticulatum* exposed to *P. hermaphrodita*.

254x190mm (96 x 96 DPI)