Carnaghi, M, Rae, R, Tandingan De Ley, I, Johnston, E, Kindermann, G, Mc Donnell, R, O'Hanlon, A, Reich, I, Sheahan, J, Williams, CD and Gormally, MJ

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Journal:	*Biocontrol Science & Technology*
Manuscript ID	CBST-2016-0345.R1
Manuscript Type:	Short Communication
Date Submitted by the Author:	n/a
Complete List of Authors:	Carnaghi, Manuela; National University of Ireland Galway, School of Natural Science Rae, Robbie; Liverpool John Moores University De Ley, Irma Tandingan; Univ Calif Riverside, Nematology; Univ Calif Riverside, Entomology Johnston, Erin; National University of Ireland Galway, School of Natural Science Kindermann, Gesche ; National University of Ireland Galway, School of Natural Science Mc Donnell, Rory; Oregon State University, Department of Crop and Soil Science O'Hanlon, Aidan; National University of Ireland Galway, School of Natural Science Reich, Inga; National University of Ireland Galway, School of Natural Science Sheahan, Jerome; National University of Ireland - Galway, School of Mathematics, Statistics and Applied Mathematics Williams, Dr. Chris; Liverpool John Moores University, School of Natural Sciences & Psychology Gormally, Mike; National University of Ireland, Galway, Applied Ecology Unit, Centre for Environmental Science, School of Natural Sciences
Keywords:	Biological control, Entomopathogenic nematodes, Phasmarhabditis hermaphrodita, risk assessment, non-target host

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

Manuela Carnaghi[1*], Robbie Rae[2], Irma Tandingan De Ley[3], Erin Johnston[1], Gesche Kindermann[1], Rory Mc Donnell[4], Aidan O'Hanlon[1], Inga Reich[1], Jerome Sheahan[5], Christopher D. Williams[2], Michael J. Gormally[1]

[1]*Applied Ecology Unit, Centre for Environmental Science, National University of Ireland Galway, Ireland.* [2]*School of Natural Sciences & Psychology, Liverpool John Moores University, UK.* [3]*Department of Nematology, University of California, Riverside, CA 92521, USA.* [4]*Department of Crop and Soil Science, Oregon State University, Corvallis OR 97331, USA.* [4]*School of Mathematics, Statistics and Applied Mathematics, National University of Ireland Galway, Ireland.*

*Corresponding author: manuela.carnaghi@gmail.com

[1] Postal address: Applied Ecology Unit, School of Natural Science, National University of Ireland Galway, Ireland. Emails: manuela.carnaghi@gmail.com (Manuela Carnaghi), erin.johnston90@gmail.com (Erin Johnston), gesche.kindermann@nuigalway.ie (Gesche Kindermann), A.OHANLON4@nuigalway.ie (Aidan O'Hanlon), ingaimperio@gmail.com (Inga Reich), mike.gormally@nuigalway.ie (Michael J. Gormally).

[2] Postal address: Liverpool John Moores University, School of Natural Sciences and Psychology, Liverpool, L33AF, UK. (Robbie Rae) and (Christopher D. Williams). Emails: R.G.Rae@ljmu.ac.uk (Robbie Rae), chris.david.williams@gmail.com (Christopher D. Williams).

[3] Postal address: Department of Nematology, University of California, Riverside, CA 92521, USA. Email: irma.deley@ucr.edu.

[4] Postal address: Department of Crop and Soil Science, Oregon State University, Corvallis OR 97331, USA. Email: rory.mcdonnell@oregonstate.edu.

[5] Postal address: School of Mathematics, Statistics and Applied Mathematics, National University of Ireland Galway, Ireland. Email: jerome.sheahan@nuigalway.ie.

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

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

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

Nematode species in association with G. maculosus in the wild. Field-collected *G. maculosus* individuals, which died during the quarantine period, were placed on White traps and kept at 18±5°C (Iglesias & Speiser, 2001; Kaya & Mitani, 2000). Recovered batches of emerging nematodes were divided into two parts. One was tested with Koch's postulates to determine its pathogenicity (Dillman *et al.*, 2012) and the other was preserved in 100% ethanol for identification by rRNA sequencing as previously described. In total, four nematodes were identified: *Phasmarhabditis californica*, *Pristionchus entomophagus, Pristionchus triformis* and *Rhabditophanes* sp. KR3021. None of these species fulfilled Koch's postulates i.e. none of the nematode species recovered was pathogenic to other *G. maculosus*. It is worth noting that this is the first time *P. californica* has been isolated in a country other than the USA (Tandingan De Ley *et al.*, 2016) and New Zealand (Wilson, Wilson, Aldeers & Tourna, 2016).

In conclusion, the results of this preliminary study indicate that the nematode biocontrol agents tested are unlikely to impact significantly on *G. maculosus* populations in the wild. Further work investigating the behaviour of *P. hermaphrodita* in relation to *G. maculosus* is recommended .

Acknowledgments

This work was partially supported by the funding of Erasmus + programme and by internal funding at the Applied Ecology Unit, National University of Ireland Galway. Thanks are due to the National Parks and Wildlife Services (NPWS) – Ireland, for issuing the Licences of Capture/Kill Protected Wild Animals for Educational or Scientific Purpose. We would also like to thank Mike Coughlan, Maurice Martyn, Katrina Lacey and Ann Smyth, Senior Technical Officers in the School of Natural Sciences (Microbiology), National University of Ireland Galway. Thanks are also due to John Carey and Allison Bistline-East for their assistance.

Disclosure statement

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

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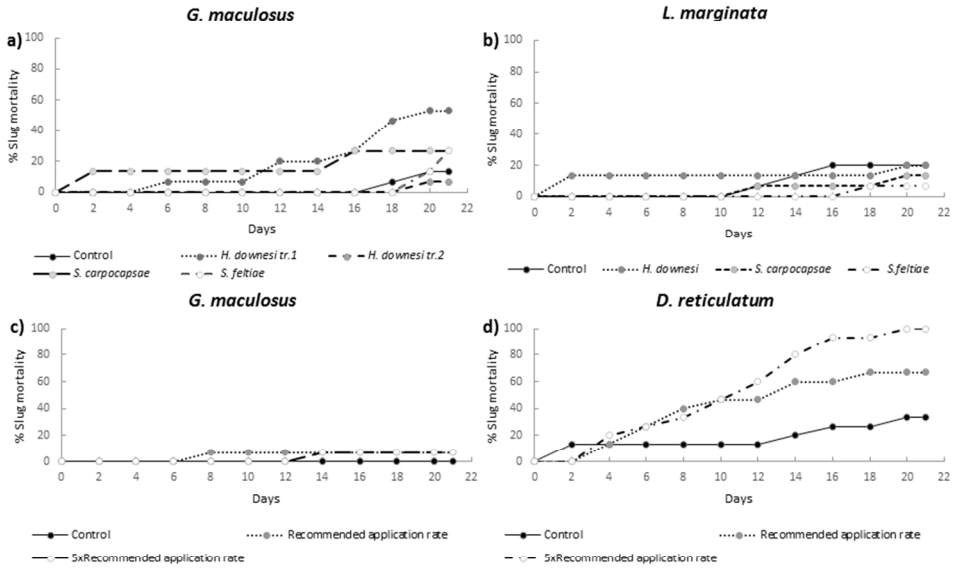
Table 1. *P*-values (Fisher's exact test) comparing treatments and controls (positive controls are *G. mellonella* and *D. reticulatum* for the EPNs and *P. hermaphrodita* treatments respectively).

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)



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254x190mm (96 x 96 DPI)