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Helminths associated with terrestrial slugs in Swedish agricultural fields

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Short Communication

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Abstract

Slugs are important agricultural pests causing yearly yield losses. However, parasitizing helminths potentially could affect the size of the slug population. Here, a survey of terrestrial slug-parasitic helminths (nematodes and trematodes) was conducted for the first time in Sweden. In total, 268 terrestrial slugs were collected from 27 agricultural field edges in three seasons over 2020 and 2021 and dissected for presence of helminth parasites. Slugs belonging to the genus *Arion* were molecularly identified by mitochondrial DNA cytochrome *c* oxidase subunit I (COI) while parasites were identified using ribosomal RNA (18S). Overall, 13% of the collected slugs had helminth parasites and the likelihood of a slug being parasitized was highest in autumn. Slugs identified as *Arion vulgaris* were more likely to be parasitized than native slug species. The prevalence of nematodes and trematodes were similar; the dominant species found were *Alloionema appendiculatum* and *Brachylaima thompsoni*, respectively. This is the first record of the presence of these two species in Sweden.

Introduction

Slugs are important economic pests of several agricultural and horticultural crops, and present a serious threat to agricultural production (Barker, 2002). Climate change, resulting in milder winters as well as warmer and wetter growing seasons in Sweden, is expected to benefit slug populations (Willis *et al.*, 2006). Historically, the grey field slug (*Deroceras reticulatum*) is the species of greatest economic importance in Europe and often causes damage of greater economic significance than insects (Speiser, 2001). In recent decades, however, the invasive Spanish slug (*Arion vulgaris*) has spread throughout Europe becoming an established pest in home gardens and agricultural crops (Zemanova *et al.*, 2016). Swedish farmers have reported increasing economic damage from slugs, but expensive molluscicides are economically infeasible as a mode of control. However, slug parasitic helminths (e.g. nematodes and trematodes) could provide natural biological control (Filipiak *et al.*, 2020).

Eight nematode families are parasites of terrestrial slugs as the definitive host (Ross *et al.*, 2017). Species in the genus *Phasmarhabditis* (Rhabditida: Rhabditidae) have shown the most promise for control (Mc Donnell *et al.*, 2020) and two of them have been formulated into commercial biological molluscicides. The use of *Phasmarhabditis hermaphrodita* was approved in 2008 in Sweden despite no confirmed natural presence in the country (Kemikalieinspektionen, 2013). Trematodes use gastropods as intermediate hosts but their effects on slug behaviour and survival are less clear (but see Foster, 1958; Moore, 2002; Antzée-Hyllseth *et al.*, 2020; Filipiak *et al.*, 2020).

The cost of parasite infection differs across slug species (Grimm, 2002; Antzée-Hyllseth *et al.*, 2020) and *A. vulgaris* could benefit from enemy release in its expanding invaded habitat. However, there is a knowledge gap about which slug species are attacked by parasites in Sweden and if attack rates vary seasonally. Parasite infection rates in marine systems display cyclic decreases of infested individuals due to winter mortality events (Le Cam & Viard, 2011). The winter period is expected to be a bottleneck for parasitism in terrestrial systems as well, but there is little knowledge regarding seasonal patterns of helminth parasites under current climate conditions (but see Foster, 1958; Vanderburgh & Anderson, 1987).

The aim of the present study was to quantify the occurrence of nematodes and trematodes in slugs from agricultural field edges. The objectives were to: (a) map the incidence of parasites in different slug species; (b) compare infection rates of native and invasive slug species; and (c) examine the seasonal variation in slug-parasitic infection rates.

Materials and methods

Study area

Slugs were collected from 27 agricultural field edges in south-western Sweden in the county of Västergötland, near Skara (N 58° 23,148′, E 13° 26,4839′). Most other studies investigating slug parasitic nematodes and trematodes have been done in forests, and those findings are not

relevant for expectations of parasitism rates in agricultural slug pests. Slugs were collected at three sampling time points: once when the adjacent fields were planted with winter wheat in summer 2020 (7–18 July); and twice when the fields were planted with winter oilseed rape, in autumn 2020 (25 August–5 September) and in spring 2021 (16–25 May). The monthly mean temperatures for the sampling periods were 14.6°C, 13.0°C and 10.4°C, respectively, while the monthly mean precipitations were 135 mm, 39 mm and 77 mm, respectively.

Slug collection and identification

Each site was visited three times per sampling round and all slugs encountered in a field edge during the visits were collected (n = 268), leading to haphazard slug collection. The collected slugs were individually stored in containers with air holes for up to nine days at 5°C and provided with grass and moist soil. *Deroceras reticulatum* and *Limax maximus* were identified morphologically at collection, but slugs belonging to the genus *Arion* have high interspecific morphological similarity. As such, 204 *Arion* slugs were sent for molecular identification. Thirty-three *Arion* slugs were dissected and discarded before the decision was taken to do molecular identification. These are included in the results as *Arion* sp.

For the molecular identification of *Arion* slugs, a small portion of the foot fringe was placed in 95% ethanol and stored at 4°C. Genomic DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen®) following the manufacturer's instructions, and stored in elution buffer at −20°C. The extracted DNA was quantified by using 2 µl of DNA template/sample in full-spectrum microvolume UV-Vis (NanoDrop One; Thermo Scientific®).

A pair of primers of the mitochondrial DNA gene of the cytochrome *c* oxidase subunit I (COI) was used for the polymerase chain reaction (PCR); forward primer LCO1490 (5′-GGTCAAC AAATCATAAAGATATTGG-3′) and reverse primer HC02198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al.*, 1994). The reactions were performed in a total volume of 25 µl, containing 5 µl of genomic DNA template (≤1 µg/reaction), 1 × complete (contained 15 mM magnesium chloride) PCR Buffer (Qiagen®), 200 µM of each deoxynucleotide triphosphate (dNTP) (dNTP mix, PCR Grade; Qiagen®), 0.2 µM of each primer and 0.625 U of Taq DNA Polymerase (Qiagen®). Reactions were performed in T100™ Thermal Cycler (Bio-Rad®). The thermal cycle conditions consisted of an initial denaturation step (3 min at 94°C), 5 cycles of 60 s at 94°C, 90 s at 45°C and 90 s at 72°C, followed by 30 cycles of 30 s at 94°C, 60 s at 55°C and 60 s at 72°C, with a final elongation step of 10 min at 72°C (Zemanova *et al.*, 2016).

Purification of the PCR products and DNA sequencing were performed by Macrogen-Europe B.V. Sequences of the purified PCR templates were obtained in both directions. Macrogen sequencing service is provided by 3730XL DNA analyser (Applied Biosystems™, USA) and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, USA). Alignment of sequences was compared with Basic Local Alignment Search Tool (BLAST) sequences and a species identification cut-off level of 97% was used. Samples of *Arion* slugs that could not be identified to the species level were grouped with the other *Arion* sp.

Nematode and trematode identification

Slugs were dissected following Antzée-Hyllseth (2014); separating 113 nematodes and 123 trematodes from 34 parasitized slugs. Individual parasites were stored at 4°C in 95% ethanol and later picked into fresh 0.2 ml Eppendorf microtubes® containing 25 µl of 5% w/v autoclaved Chelex solution (Chelex100 Molecular Biology Grade Resin; Bio-Rad®) and 5 µl of Proteinase K solution (>600 mAU/ml; Qiagen®). Samples were incubated at 60°C for 30 min. The lysate was heated at 94°C for 10 min and stored at −20°C for 20 min and then centrifuged for 8 min at 6000 revolutions per minute. The supernatant was transferred into fresh 0.2 ml Eppendorf microtubes®, and stored at −20°C. Extracted genomic DNA from parasites was quantified using 2 µl of DNA template/sample in full-spectrum microvolume UV-Vis (NanoDrop One; Thermo Scientific®). All individual parasites had DNA concentrations between 50−215 ng/µl.

A pair of universal primers of the 18S rRNA gene was used for the PCR; forward primer G18S4 (5′-GCTTGTCTCAAAG ATTAAGCC-3′) and reverse primer 26R (5′-CATTCTTGGCAA ATGCTTTCG-3′) (Blaxter *et al.*, 1998). PCRs were performed as described in the case of COI. The thermal cycle conditions consisted of an initial denaturation step (5 min at 94°C), 35 cycles of 60 s at 94°C, 90 s at 52°C, and 60 s at 72°C, with a final elongation step of 10 min at 72°C (Ross *et al.*, 2010a). Purification of PCR products and DNA sequencing of parasites were performed by Macrogen-Europe B.V., as described for the slug identification. If neither the forward nor reverse reads returned high confidence species identifications, specimens were labelled as unidentified trematodes or nematodes based on the microscopic classification at the time of dissection.

Statistical analysis

The likelihood of being parasitized was analysed using binomial generalized linear models with parasitization as the response variable and season and parasite type (nematode vs. trematode) as predictor variables. The likelihood of being parasitized for native vs. non-native slugs was tested using a binomial generalized linear model. *Arion vulgaris* were classified as non-native (n = 139), while the native group consisted of all other identified *Arion* species as well as *D. reticulatum* and *L. maximus* (n = 74). The *Arion* slugs that were only identified to genus were excluded from this analysis (n = 55).

The variation across season of parasite load, that is, the number of parasites within a parasitized slug (n = 34), was analysed using a Gaussian linear model. Tukey's honestly significant difference was calculated using the multcomp package to assess pairwise differences in the likelihood of parasitization and in parasite load across the three seasons (Hothorn *et al.*, 2008). All analyses were performed in R version 4.2.1 (R Core Team, 2022).

Results and discussion

Slug species

In total, 268 slugs were collected during the three sampling periods: 86 in summer; 105 in autumn; and 77 in spring (table 1). Slugs were found in all field edges at least once. In summer, slugs were found in 17 of the field edges and in autumn and spring in 19 of the fields. The majority of the collected slugs belonged to the genus *Arion* (n = 237), with *A. vulgaris* by far the most abundant (table 1). The diversity of *Arion* species found was in line with what could be expected based on known occurrences of *Arion* slugs in Sweden and their habitat preferences, but other mitochondrial DNA (ND1 or 16S) or nuclear

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Table 1. Number of slugs belonging to different slug species, collected in summer and autumn 2020 and in spring 2021, and how many of these that were unparasitized (U) or parasitized by either nematodes (N) or trematodes (T).

Slug species	Summer			Autumn			Spring		
	U	N	T	U	N	T	U	N	T
Arion ater	1	2	0	1	0	0	9	0	0
Arion circumscriptus	1	0	0	1	0	0	3	0	0
Arion distinctus	0	0	0	0	0	0	4	0	0
Arion fasciatus	1	0	0	0	0	0	2	0	0
Arion rufus	8	0	0	3	0	0	6	1	0
Arion vulgaris	14	0	0	65	9	14	35	2	0
Arion sp.	42	1	1	5	1	2	3	0	0
Deroceras reticulatum	14	0	0	1	0	0	8	1	0
Limax maximus	1	0	0	3	0	0	3	0	0

The number of *Arion* sp. is higher in summer because some slugs were dissected without molecular identification.

DNA (ITS-1) markers could better clarify cryptic and hybrid species (Rowson *et al.*, 2014; Zemanova *et al.*, 2016).

Parasite infection

Overall, 13% of the slugs had helminth parasites with 17 slugs being parasitized by nematodes and 17 by trematodes (table 1). No slugs were simultaneously parasitized by both nematodes and trematodes, and parasitized slugs originated from only four fields. This 6% nematode infection level is comparable to other studies in agricultural land (e.g. Ross *et al.*, 2010b, 2012), but the 6% trematode infection level was substantially lower than previous studies where slugs were sampled from other habitats (Antzée-Hyllseth *et al.*, 2020; Filipiak *et al.*, 2020).

The likelihood of a slug being parasitized was over six times higher (infection levels of 25%) in the autumn ($P < 0.005$), than in the spring or summer (infection levels of 5%) (table 1). However, parasite load was highest in the spring (estimated 10.5 parasites per infected slug, $P < 0.05$), though this was driven by two slugs with high nematode load and a low overall infection rate (total 54 nematodes in four slugs) (fig. 1). Our data indicate that the likelihood of trematode infection and parasite load are both higher in autumn than summer (six trematodes in one slug in the summer compared to 117 trematodes in 16 slugs in autumn), but due to the small sample size it could not be analysed (fig. 1).

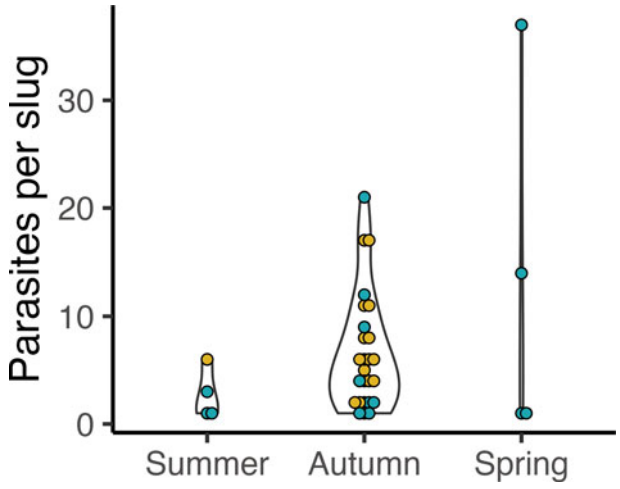

Fig. 1. A violin plot showing the distribution of parasitized slugs and their individual parasite loads in the different seasons. Slugs parasitized with nematodes are shown as blue points and slugs parasitized with trematodes as yellow points.

Arion vulgaris had a higher probability (0.18) to be parasitized compared to the native slug species (0.054, $P = 0.016$), in line with previous studies (Antzée-Hyllseth *et al.*, 2020). Interspecific variation in mortality due to nematode infection could be contributing to this pattern (Grimm, 2002). Our results show that *A. vulgaris* have high parasite levels in Sweden, and that release from parasites is not a probable explanation for its invasive success here.

Parasite species

Our DNA analysis of parasites identified just two dominant species, one nematode and one trematode, though high-quality reads were only successful for 39% and 54% of samples, respectively. Parasites from a single slug never returned a high-quality molecular identification for more than one species. All high-quality matches for nematode reads were identified as *Alloionema appendiculatum* ($n = 44$). *Alloionema appendiculatum* was identified in four slug species (*Arion ater*, *Arion rufus*, *A. vulgaris* and *D. reticulatum*) as well as the unidentified *Arion* sp. This is the first confirmed presence of this nematode in Sweden, though it has a broad European geographical distribution (Filipiak *et al.*, 2020). We did not find *Phasmarhabditis hermaphrodita*, the nematode approved as a commercial biological molluscicide. Additional surveys in other habitats closer to residential areas, where it has been released for use in gardens, are needed to confirm whether this nematode species is established in Sweden.

Only *A. vulgaris* and *Arion* sp. were infected with trematodes. The dominant trematode species was identified as *Brachylaima thompsoni* ($n = 65$), which previously has been found in the United States (Barger & Hnida, 2008) but not in Europe. *Brachylaima mesostoma* has been reported in Europe (Filipiak *et al.*, 2020), but three low-quality matches to this species were below our identification cut-off level of 97%. The 18S rDNA locus is less variable and sequences of this locus are identical in some closely related species, so a misidentification at the species

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level is possible (Heneberg *et al.*, 2016). A second trematode species was identified as *Trichobilharzia regenti* with a single high-quality match from the autumn in *A. vulgaris*.

This is the first study attempting to identify slug-parasitic helminths in Sweden. Other studies have assessed slug parasites, but there is a dearth of studies looking in agricultural areas. This is important for assessing the natural infection of agricultural slug pests.

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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