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Parasites of Terrestrial Gastropods:

Prevalence of Nematodes and Trematodes, and Effects of a Commercially Available Nematode

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Preface

First of all, I would like to thank my two wonderful supervisors Nina Trandem and Solveig Haukeland for the many hours of guidance. I truly appreciate all you have done, and I could not have asked for better supervisors. Special thanks goes to each of you: Thank you, dear Solveig, for all the guidance with the practical work regarding the slugs and parasites, and for driving me back and forth to Eidsberg for my field collections. Thank you, dear Nina, for the guidance with the writing process and statistics, and for being available for questions, especially in the hectic time near the deadline.

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Furthermore, I am very happy that I chose to study at NMBU back in 2009 (then UMB) and stayed, as it must surely be one of the world's best universities, with interesting courses, inspiring lecturers, a beautiful campus and an unparalleled student social milieu. Finally, I tank my family and good friends, who have encouraged me through all my studies.

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Henrik Antzée-Hyllseth

Abstract

The invasive slug *Arion vulgaris* was first recorded in Norway in 1988, and has since become an agricultural pest and a serious nuisance for many garden owners. It may cause substantial damage to certain crops, herbs and ornamental plants. Furthermore, it is reported to hybridize with the native species *Arion ater*. Many traits are likely contributing to *A. vulgaris'* success, such as its ability to utilize a wide range of food sources, rapid reproduction, robustness when facing unfavourable conditions, and a lack of natural enemies.

The slug parasitic nematode *Phasmarhabditis hermaphrodita* has lately become much used in efforts to control the invasive species. A slug control product containing this nematode, Nemaslug®, produced by BASF Agricultural Specialities Ltd., is commercially available and used in many Norwegian gardens. However, scientific studies have shown that this nematode mainly kills the youngest *A. vulgaris*, but is effective for controlling another pest, *Deroceras reticulatum.* Several non-target gastropod species have also been reported to be susceptible.

The first objective of this study was to investigate if the success of the invasive slug is influenced by a lack of parasites. Its nematode and trematode prevalence and number was compared to that of three native gastropod species. A total of 61 *A. vulgaris*, 47 *Arion fasciatus,* 54 *Arion circumscriptus,* and 65 specimens of the snail *Arianta arbustorum* from two sites in south-eastern Norway were collected, dissected and searched for parasites. *A. vulgaris* turned out to have the highest prevalence of both parasite groups. With 44 % being nematode infected, its nematode prevalence was significantly higher than that of two of the native species. Its trematode prevalence of 74 % was significantly higher than all native species. It seems safe to conclude that parasite release is not a reason for the success of *A. vulgaris* in Norway.

The second objective was to test the effects of *P. hermaphrodita* on the two non-target species *A. fasciatus* and *A. arbustorum,* compared to *A. vulgaris,* one of the main target species for the commercial product. In a 20-day bioassay, five specimens of each of the three species were exposed to soil containing a high dose of nematodes (128/cm^2), five were exposed to a low dose (64/cm^2), and five were unexposed controls. Gastropods were weighed before and after, fed with 4x4 cm cabbage leaf squares, and finally dissected. This was followed by another identical bioassay. Mortality, infection, weight change and amount cabbage eaten was noted and compared. The non-target species *A. fasciatus* was most affected, with 100 % infection, 60 % mortality and near complete feeding inhibition. *A. vulgaris* also experienced 100 % infection, but only 20 % mortality and not much feeding inhibition. Only 35 % of *A. arbustorum* were infected and none died. The most severe effect on this species was a complete feeding inhibition in the high dose treatment. It also seemed to be actively avoiding the nematode infested soil by going into hibernation. Application of *P. hermaphrodita* in fields and gardens could potentially harm local populations of *A. fasciatus*, while *A. arbustorum* populations are not likely to be much affected.

Contents

1. Introduction

The Spanish slug (*Arion vulgaris*) (syn. *Arion lusitanicus*) is an invasive species in Norway and many other European countries (Hatteland et al. 2013; Kozłowski & Kozłowski 2011). The origin of the slug is a topic of much debate, which so far has resulted in two names being used for the same species. It was previously thought to originate in the Iberian Peninsula, while it is now commonly accepted to have originated in south-western France (von Proschwitz 2009a). However, a fresh study by Pfenninger et al. (2014) makes a strong case for it being native to Central Europe. In any case, it is certainly an introduced species in Norway, first recorded in 1988 (Hatteland et al. 2013; Von Proschwitz & Winge 1994). The slug has spread rapidly with unintended human help, through transport of especially soil for gardening, but also with all kinds of plant products like fruits, vegetables, flower bouquets, seedlings, potted plants etc. (Kozłowski & Kozłowski 2011). The introduction has often led to several problems, including damage to crops and gardens, competition with different native gastropod species and hybridization with the related species *Arion ater* (von Proschwitz 2009a) and *Arion rufus.* In their cross mating trials between *A. vulgaris* and *A. rufus*, Dreijers et al. (2013) found that mating occurred, and that the differences in their genitalia did not prevent sperm exchange. Furthermore, studying *A. vulgaris*' genetic dynamics, Engelke et al. (2011) found that in their only study site where *A. vulgaris* and *A. rufus* both occurred, "several specimens appeared to have an intermediate reproductive morphology". Supporting this, Roth et al. (2012) conducted cross mating experiments between *A. vulgaris* and *A. rufus* x *ater* hybrids and found evidence for a potential hybridization between the two, suggesting more or less a lack of cross mating barriers.

When it comes to crop damage, Kozłowski (2007) reported that it is most severe in the germination period, as *A. vulgaris* favours new, fresh plant parts such as seedlings, but also fruits are vulnerable. Among the types of crop and other plants most often consumed and damaged by *A. vulgaris*, are "lettuce, cabbage, red beet, radish, carrot, parsley and beans [...] sunflower, clover and potato [...] strawberry fruits [...] and some ornamental plants and herbs" (Kozłowski 2007). The slug may cause a lot of damage in gardens; Kozłowski and Kozłowski (2011) found a density of up to 65 individuals per square metre in gardens in Zawadka in southern Poland. In a study published in 2009, annual cost of the *A. vulgaris* control for private gardeners in Sweden was calculated to be between a low estimate of 45 million SEK and a high estimate of 450 million SEK (Gren et al. 2009).

1.1 Invasive success

Kozłowski (2007) lists the following traits that make this slug a successful invader and competitor in its new habitats: "large body size, rapid reproduction, high food and ecological tolerance, high survival rate under unfavourable conditions (lack of food, low temperatures), capability to hide in shelters and eating a variety of food, correlation between mass occurrence and phenology of cultivated plants and lack of natural enemies." *A. vulgaris* have

been reported to lay an average of 400 eggs, and generally have a univoltine lifecycle, although some survive a second overwintering (Kozłowski 2007). These may reach a weight of at least 22 g (pers. obs.). Knop and Reusser (2012) did a related study comparing the phenotypic plasticity of *A. vulgaris* and the native *Arion fuscus* in Switzerland. They found that the invading slug had higher survival and reproduction during summers with high temperatures and limited food sources, but lower survival during the winter. Slotsbo et al. (2012) compared cold tolerance in three *Arion* species (*A. ater, A. rufus and A. vulgaris*) in Denmark, but did not find any significant difference, further challenging the notion that *A. vulgaris* has a higher cold tolerance. Drought tolerance is another important factor that could be influencing its success. However, a study by Slotsbo et al. (2011) found that juveniles and eggs of *A. vulgaris* have a water loss rate and tolerance that is similar to other slug species. Gastropods generally have a relatively high water loss tolerance, and for *A. vulgaris*, they reported a mortality of 50 % when losing 72 % water for juveniles and when losing 81 % for eggs. Furthermore, they noted that drought generally limits foraging, causing a slowing of development and thereby delayed reproduction for slugs.

A. vulgaris is highly polyphagous and feeds on much of the same food sources as other gastropod species (e.g. *Deroceras reticulatum, Arion rufus*) (Kozłowski 2007). In a study from Germany, Kappes et al. (2012) found that *A. vulgaris* was attracted to certain acidic solutions and baits that most other gastropods avoid. This was interpreted as a sign that *A. vulgaris* is less conservative in testing new foods (Kappes et al. 2012).

Another explanation for invasive species' success that is often proposed is the enemy release hypothesis, which in short assumes that a lack of natural enemies in an invading species' new habitat greatly contributes to its success. In that regard, Ross et al. (2010) studied the role of parasite release for slugs introduced to the USA from Europe. Their results supported the hypothesis, with a nematode prevalence of 16.4 % in slugs collected in the UK, which was significantly higher than the 5.4 % in slugs collected in the USA. They also found nematodes in 93 % of UK collection sites, and only in 34 % of US sites.

1.2 Biological control

To control *A. vulgaris*, several methods are being used, including chemical and biological approaches, as well as manually collecting the slugs. Two common chemical compounds used for slug control are iron phosphate and metaldehyde, but lately the slug parasitic nematode *Phasmarhabditis hermaphrodita* has become widely used (Rae et al. 2009). Scientific studies do however show that *P. hermaphrodita* has a very limited to no effect on adult *A. vulgaris* (Grimm 2002; Rae et al. 2007; Speiser et al. 2001). Regarding juveniles, Speiser et al. (2001) found that feeding and survival were significantly reduced in individuals smaller than 0.15 gram with increasing dosage of the nematodes. They also reported that survival was reduced in individuals between 0.5 and 1 gram, but feeding was not affected in this weight class. This is supported by Grimm (2002) who found that *P. hermaphrodita* affected young *A. vulgaris* by reducing feeding by at least 50 % in individuals smaller than 0.5 grams and killing 47 % of

0.15 gram individuals. Individuals weighing 0.45 gram showed no significant mortality in the same study.

P. hermaphrodita is currently available as the commercial product Nemaslug® from BASF Agricultural Specialities Ltd. (nemaslug.no). The product is popular among private gardeners, and in Re municipality in Vestfold county, the local council hands out Nemaslug to the residents for free (Hansen 2013; re.kommune.no 2014). Some of the private gardeners claim that the nematodes also have a significant effect on adult *A. vulgaris* (pers. obs.). This could possibly be due to the alternative application methods commonly used, which is to dip slug carcasses in a nematode-water mix or mixing the nematodes into an oat porridge to be placed in gardens (pers. obs.). Another explanation could be that the nematodes are applied in "extremely" high doses.

P. hermaphrodita is much used in control of the pest slug *D. reticulatum*, for which it is highly effective with a mortality up to 98 % (Grimm 2002). Both *D. reticulatum* and the non-target species *A. ater* have been shown to avoid soil treated with *P. hermaphrodita* (Wilson et al. 1999). Rae et al. (2008) found that the slug *Milax gagates* was killed or showed reduced feeding when exposed to the nematode, while another slug, *Limax pseudoflavus*, encapsulated the intruding nematodes in its shell and was thereby not affected. In their study of *P. hermaphrodita*'s effect on the two aquatic non-target snail species *Lymnaea stagnalis* and *Physa fontinalis*, Morley and Morritt (2006) found that "survival of the *L. stagnalis* juveniles was significantly reduced by the presence of nematodes compared to controls". *P. fontinalis* was apparently not affected although infected, as there was no significant difference in the number of nematodes found in the two snail species when they were dissected at the end of the experiment (Morley & Morritt 2006). Other gastropods found to be affected by *P. hermaphrodita* were listed in a review by Rae et al. (2007), and include: *Deroceras panormitanum, Deroceras laeve, Arion silvaticus, Arion intermedius, Arion distinctus, Tandonia sowerbyi, Tandonia budapestensis, Leidyula floridana,* juvenile *Helix aspersa, Monacha cantiana, Cepaea hortensis, Theba pisana, Cochliella acuta* and *Cernuella virgata.*

1.3 Parasitology

Parasitic nematodes that are known to have terrestrial gastropods as main hosts are found in eight different families, and their life cycle, host species and adaptations are highly varied (Morand et al. 2004). One of the largest families, Rhabditidae, contains free-living species, insect commensals (the nematode benefits, while the host is unaffected), and parasites of a wide range of invertebrates (Morand et al. 2004). *P. hermaphrodita,* the main nematode in focus in this study, belongs to this family. Starting with the infective, or so-called dauer juvenile development stage, they are well adapted for between-host survival in harsh conditions for longer periods, without feeding and with an extra thick cuticle, they search for a suitable host (Weischer & Brown 2000). When they find one, e.g. a *D. reticulatum*, they enter through a pouch in the dorsal integument, just posterior to the mantle, which is connected to a sac containing the vestigial shell (Wilson et al. 1993). This shell sac is located between the mantle and mantle cavity containing the slug's heart and kidney. The nematodes

then release symbiotic bacteria, such as *Moraxella osloensis,* which multiplies and may eventually kill the slug (Morand et al. 2004). The nematodes develop into adults, feed on the bacteria and slug tissues, and reproduce until the slug is decayed and new dauer juveniles are formed, ready to search out a new host (Morand et al. 2004). The bacteria also seem to have an additional effect, besides being a food source and processing the gastropod host's tissues, as Foltan and Puza (2009) showed that scavenging carabid beetles avoided slug carcasses infected by *P. hermaphrodita,* even though they are not susceptible to the nematode.

The class Trematoda are divided into two orders; Monogenea and Digenea, with the first having only one host in their life cycle, and the latter having two or more and being most common (Barnes 1981). The digenean trematodes almost always have a gastropod, usually a snail, as their first and also often second intermediate hosts, and this relationship is thought to be very old and intimate, causing co-evolution and even co-speciation (Lockyer et al. 2004).

The trematode exploits the gastropod in several ways, as a source of food, for asexual reproduction, and as transport to its next host (Lockyer et al. 2004). Trematode infection has been shown to increase snails' growth rates, but also to severely reduce their reproductive ability and locomotion (Mouritsen & Thomas Jensen 1994). In some cases, the trematode even manipulate its host to facilitate its transfer to the next host. A very peculiar example is the *Leuchocloridium* spp., that accumulate in their snail host's tentacles, making them pulsate, often in vibrant colours, mimicking a moving caterpillar (Wesołowska & Wesołowski 2014). This in itself is not host manipulation, but the infected snails are also more likely to position themselves in elevated, exposed locations, making them easily spotted and eaten by the parasite's avian main hosts (Wesołowska & Wesołowski 2014). With several hosts and the ability to manipulate them, the life cycles of trematodes are often complex. Another well-known trematode species, *Dicrocoelium dendriticum,* or lancet fluke, which usually has an even-toed ungulate as main host, has an exceptional control over its ant second intermediate host (Pybus 2001) (fig. 1). Some of the trematode cercariae infecting the ant migrates to its brain and encysts, and then manipulate the ant to climb a straw and cling to it until it is eaten by the parasite's main host (Pybus 2001). Trematodes may cause serious diseases and even the death of their main hosts (Gryseels et al. 2006; Okulewicz & Sitko 2012; Pybus 2001).

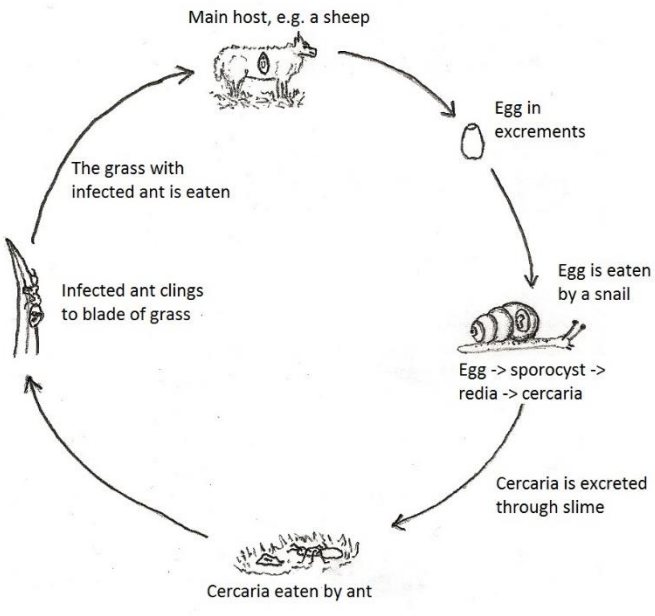

Figure 1: The life cycle of the lancet liver fluke (*D. dendriticum*), drawn after Benestad Hågvar (2010), p. 92.

1.4 Research objectives

The first objective of this study was to do a survey to compare the parasite prevalence in *A. vulgaris* with three gastropod species native to Norway (*Arion circumscriptus*, *A. fasciatus* and *Arianta arbustorum*) (table 1). The parasites investigated belong to the phylum Nematoda and the class Trematoda (phylum Platyhelminthes). A lower parasite prevalence was expected in *A. vulgaris*, due to its invasion success and possible parasite release.

The second objective was to test effects of the slug parasitic nematode *P. hermaphrodita* on Norwegian populations of the two non-target species *A. fasciatus* and *A. arbustorum*, compared to *A. vulgaris* in two bioassays. Neither *A. fasciatus* or *A. arbustorum* are mentioned in the review by Rae et al. (2007), and I have not found any studies investigating effects on these species. I expected *A. vulgaris* to be the most affected, as it is one of the two target species for the biocontrol product. Some effects on the related *A. fasciatus* were expected as well. The effects on *A. arbustorum* were expected to be very limited to none, as it is less related to the slugs for which the product is used to control.

Table 1: Names, simplified distribution and photograph of the four species studied. *Arion circumscriptus* photo: Anderson (2010). Other photos: Henrik Antzée-Hyllseth. References: 1: DAISIE (2011) 2: Welter Shultes (2013b) 3: Rowson et al. (2014) 4: Nilsson (1822) 5: Welter Shultes (2013a) 6: Falkner et al. (2013)

Names		Distribution	Photo
Latin	*Arion vulgaris / A. lusitanicus*	Usually considered native to South-Western Europe. Spread to most of Europe, except eastern parts. Observed in USA. (1)	
English	Spanish slug		
Norwegian	Brunskogsnegl		
Latin	*Arion fasciatus*	Most of Europe, except the southeast (2). Introduced to North America (3). First described in Sweden, 1822 (4).	
English	Orange-banded Arion		
Norwegian	Gulflankeskogsnegl		
Latin	*Arion circumscriptus*	North-West and Central Europe (5). Introduced to North America (3).	
English	Brown-banded Arion		
Norwegian	Gråflankeskogsnegl		
Latin	*Arianta arbustorum*	Most of Europe, excluding southern France and the Iberian Peninsula. Considered native to most European countries. (6)	
English	Copse snail		
Norwegian	Krattsnegl		

2. Materials and methods

2.1 Field collection sites

For the parasite survey, the four species of gastropods in table 1, of all weight classes found, were manually collected once per month from June to October 2013 in two localities in South-Eastern Norway; Eidsberg Municipality in Østfold County, and "Kirkejordet" in Ås Municipality, Akershus County (fig. 2). The gastropods were usually found by thoroughly searching the lower vegetation layer, including turning leaves, logs and rocks to look beneath them. Both sites included an edge between deciduous forest and fields, with most of the gastropods found between the edge and 20 metres into the forest. At the Ås site, the fields were mostly covered with 30 cm tall grass, and a part was tilled and used for a controlled semi-field trial of molluscicides. The forest floor was sparsely vegetated and relatively dry. The site was close to a cemetery and an open compost pile. At the Eidsberg site, the fields were mostly grain crops and some parts were strawberry crops. The forest floor was for the most part richly vegetated and rather moist due to small creeks of runoff from the fields. The plan was originally to collect 10 individuals of each species per month from each site, but due to difficulties finding sufficient numbers of the gastropods, this number had to be reduced in most cases. The species were identified morphologically in the field using "Snigel : fridstörare i örtagården : vetenskap och fakta" by Ted von Proschwitz (2009b) for comparisons. *A. circumscriptus* were separated from the similar *Arion silvaticus* in the laboratory by examination of their differences in the reproductive system.

Gastropods for the two bioassays were collected in July-August and September 2013 and only at the site in Ås. The species used were the same as for the natural occurrence of parasites survey, excluding *A. circumscriptus* because they were hard to find in sufficient numbers and due to their external similarity with *A. silvaticus*. Approximately 25 to 30 individuals of each species were collected for each of the two assays.

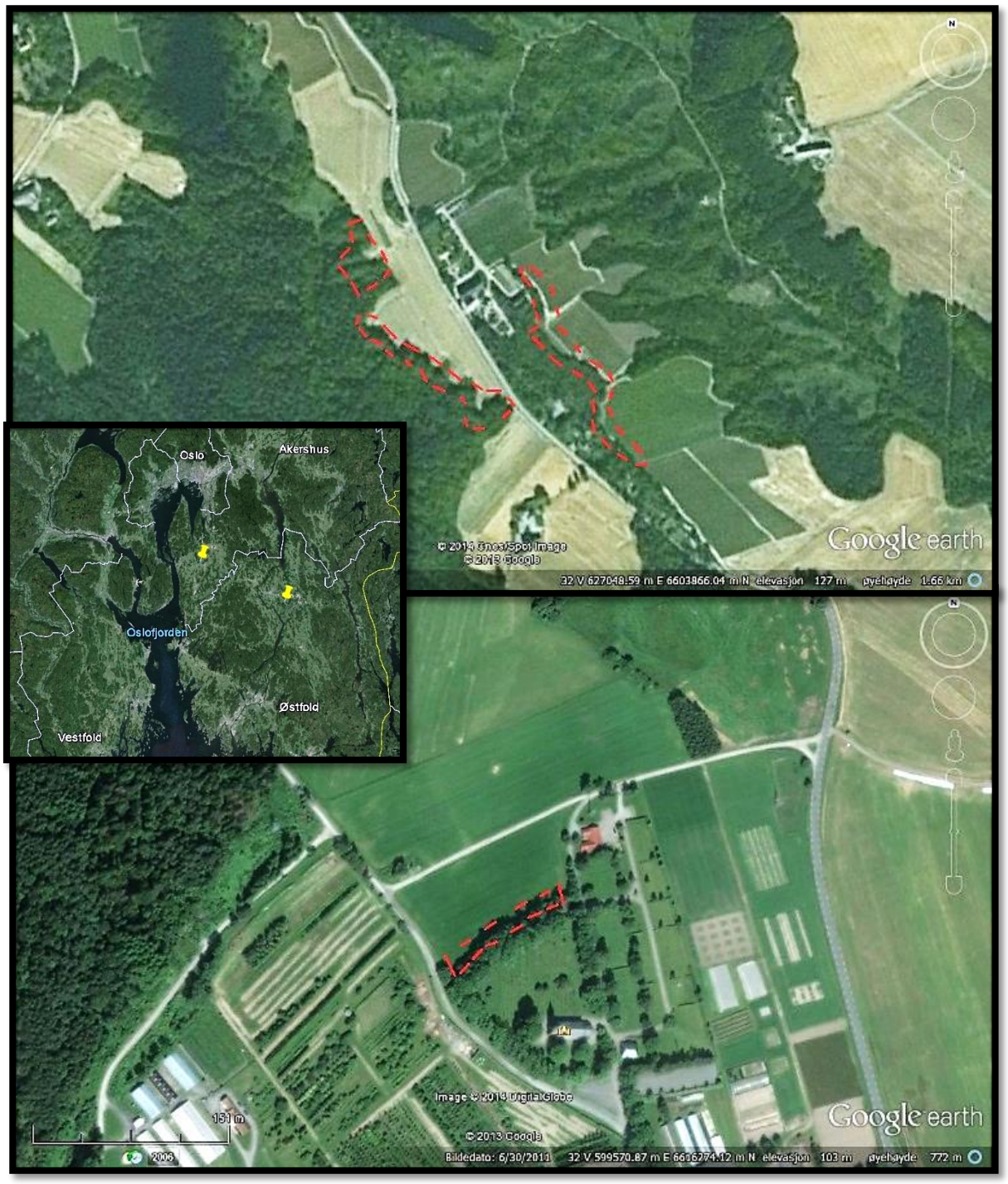

Figure 2: Satellite photos of the two field collection sites, provided by Google Earth. Red lines mark areas searched for gastropods. Top: Eidsberg. Bottom: Kirkejordet, Ås. Middle: Overview, Kirkejordet (west), Eidsberg (east).

2.2 Isolation of survey parasites

After each collection, the slugs and snails were stored at 4 °C in plastic boxes with moist paper, Chinese cabbage and thin slices of carrot. The gastropods were weighed before being immersed in water for 1-3 days to be "relaxed" before dissection. The dissections were conducted from the first field collection until the end of November. Dissection of snails were done by following an online protocol, developed by Center for Plant Health Science and Technology and the University of Florida (White-McLean 2011). For slug dissection details, see Appendix 1.

The gastropods' developmental stage were noted and categorized as juvenile, subadult or adult (development of the reproductive system). Furthermore, the amount of intestinal content were noted and categorized as empty (0) to full (4), and intestinal content digestion stage categorized as little (0) to completely (3). The position and number of nematodes and trematodes present in each gastropod were noted. Samples were collected and stored in 70 % alcohol for DNA-analyses.

2.3 Bioassays

The three gastropod species collected in the field were placed in four large, covered plastic boxes (h: 17 cm, w: 25 cm, l: 34 cm). *A. vulgaris* were put into two boxes, while *A. fasciatus* and *A. arbustorum* had one box each. The gastropods were kept in these boxes with moist paper, Chinese cabbage and thin carrot slices for approximately two weeks, with an approximate temperature of 15 °C and a relative humidity of 80 %. This quarantine served to avoid using sick and possibly already parasitized individuals in the assays by removing the dead, dying and those with any signs of infection.

Forty-five plastic boxes (h: 9 cm, w: 13 cm, l: 18 cm), half-covered by plastic lids, served as arenas for each of the two identical bioassays (90 boxes total). To avoid escaping, the top 3 centimetres of the inner box walls were coated with Neudorff® Antischneck-Gel®, a soap-like substance the gastropods will not touch. All boxes had a 2 cm layer of moist soil. Three treatments were used in the bioassays:

1: Control with 10 ml tap water added per box.

2: Low dose with approximately 15 000 (≈ 64/cm^2) nematodes (*P. hermaphrodita* from Nemaslug) added to the soil with 10 ml tap water per box

3: High dose with approximately 30 000 (≈ 128/cm^2) nematodes added to the soil with 10 ml tap water per box.

Of each of the three species, 15 seemingly healthy individuals of varying sizes were selected, weighed and moved to separate plastic boxes. There were then five untreated control boxes, five low dose, and five high dose per species (table 2). Gastropod weight classes were roughly

the same in each treatment for each of the species. The boxes were then placed in a room with light 10 hours a day (07:00 – 17:00), a relative humidity of 80 % and an approximate temperature of 15°C, for 20 days.

Table 2: Schematic overview of the bioassay experimental setup.

3 species	*A. vulgaris*			*A. fasciatus*			*A. arbustorum*		
3x3 treatments	Control	Low dose	High dose	Control	Low dose	High dose	Control	Low dose	High dose
5x3x3 specimens	1 1 1 1 1	1 1 1 1 1	1 1 1 1 1	1 1 1 1 1	1 1 1 1 1	1 1 1 1 1	1 1 1 1 1	1 1 1 1 1	1 1 1 1 1
x2									

To calculate food consumption and monitor the health of the gastropods, they were fed with 4x4 cm cabbage leaf squares. The approximate percentage eaten of a square was noted when it was replaced by a new square. The total number of squares eaten by each gastropod was then summarized at the end of the bioassay.

The gastropods were checked every second day throughout the period to change food and remove dead specimens. The dead were weighed immediately and dissected within the following day. At the end of the assay, all surviving gastropods were weighed and relaxed immediately, and dissected within a week. In each dissection, any nematodes or trematodes were roughly counted or calculated, their position inside or outside the gastropod noted and samples stored in 70 % alcohol for DNA-analysis. Visible marks or holes in the mantle, usually outside the kidney, were also noted if present.

Figure 3: Left: The bioassay setup with 45 half covered plastic boxes. Right: An *A. vulgaris* in its arena, feeding on a cabbage square (4 x 4 cm).

2.4 Morphological identification of parasites

The first *P. hermaphrodita* nematodes found were morphologically identified by Solveig Haukeland. After having been taught how to recognize them, I identified them on my own, but with her occasional verifications. The same was done with the nematode *Alloionema appendiculatum*, but the identification of these required less help, as they are easier to recognize due to their characteristic shape, size, colour, movements and location within their host. Other nematodes and all but one group of trematodes were simply categorized as "other nematodes" and "other trematodes". With much help from Solveig Haukeland, a few samples of a trematode occurring in vast numbers in some specimens of *A. arbustorum* were fixated on slides and sent by mail to Raúl Iglesias at the University of Vigo in Spain for identification.

2.5 Molecular identification of parasites

In an effort to identify some of the parasites categorised as "other", molecular identification was carried out as described below. A few samples of morphologically identified parasites were also done molecularly for verification.

Lysis and extraction

DNA extraction from 139 parasite individuals was performed according to the protocol "CCDB - DNA Extraction" (Ivanova et al. 2006a), developed by Ivanova et al. (2006b) at the Canadian Centre for DNA Barcoding (CCDB). The "insect version" of the protocol was applied. Nematodes and trematodes were lysed overnight in Insect Lysis Buffer containing 1.8 mg/ml proteinase K, and DNA was extracted from the lysate according to the protocol the following day. DNA was eluted in 50 µl ddH_2O, of which 5 µl was used as template in a standard barcoding PCR (see below). The proteinase K was supplied by Sigma-Aldrich® (catalogue number P2308).

Another DNA extraction method, provided by Jenna L. Ross, was used for an additional 80 parasites (appendix 2).

Polymerase chain reaction (PCR)

PCR amplification of the 658 base pair segment of cytochrome *c* oxidase I (COI) gene, commonly used for DNA barcoding, was conducted on the third day, carefully following a protocol provided by M.Sc. technician Even Sannes Riiser (appendix 3). The primers used were LCO1490 and HCO2198 (Vrijenhoek 1994).

Gel electrophoresis and DNA sequencing

To investigate if the DNA extraction and the PCR reaction were successful, gel electrophoresis was carried out, again following instructions given by Even Sannes Riiser. First, a 1 % agarose gel was made by mixing 50 ml TBE Buffer and 0.5 g agarose. This was then heated in a microwave oven and stirred until the solution was clear and no agarose could be seen. Next, one drop of the intercalating dye ethidium bromide (EtBr) (0.625 mg/ml) was mixed in. These ingredients were simply doubled or tripled to get the necessary amount of gel, depending on the number of samples. The liquid gel was poured into a gel tray and combs for making wells for the samples put in. Finally, the gel was left to set for 20 minutes while preparing the PCR product for the gel electrophoresis.

6 µl mQ H_2O, 4 µl of the PCR reaction and 2 µl6x gel loading buffer (GLB) was added to each well in a new 96-well PCR plate. The gel tray containing the solidified gel was placed in the electrophoresis apparatus, submerged in TBE Buffer, and the combs were removed. Finally, the 12 µl samples were loaded into the wells left in the gel by the combs, using a multichannel pipette. A 100 base pair DNA ladder was loaded into a well on either side of each of the well rows. The apparatus was then left to run for approximately 40 min. at 80 V (100 ml gel), or until the coloured loading dye had reached about 2 cm above the next row of wells or the bottom edge of the gel.

The gel was then removed from the apparatus and placed in a Bio-Rad Universal Hood II Gel Imager. The gel imager exposes the gel for UV light, causing any EtBr bound to DNA to fluoresce, thereby allowing the detection of PCR products. The observation of the amplified 658 bp COI segment confirmed the successful DNA extraction and PCR reaction (fig. 4).

If the correct PCR product was observed, the remaining PCR reaction was sent by mail to GATC Biotech in Germany for purification and sequencing. In the purification, polymerase, primers and nucleotides are removed (Sannes Riiser, pers. com.). The DNA sequences were received a few days later via e-mail, trimmed and assembled in the CLC Main Workbench software (CLC) by Even Sannes Riiser, and then used for searches in the DNA barcoding databases BOLD Systems (Ratnasingham & Hebert 2007) and NCBI BLAST (Altschul et al. 1990). These searches made it possible to identify the parasite to species, genus or family level, depending on the matching sequences found in the databases. All sequences were also compared to each other (aligned), using CLC Main Workbench in order to identify regions of similarity, regardless of whether the sequences gave hits in the databases or not.

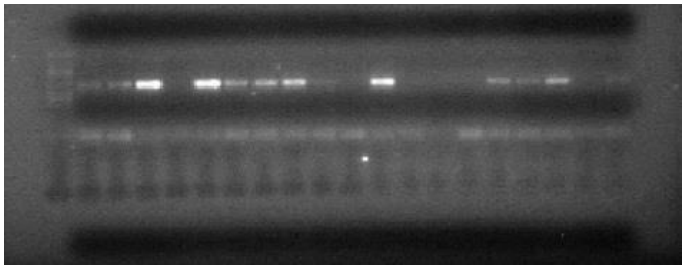

Figure 4: Image from the Bio-Rad Universal Hood II Gel Imager, showing the DNA ladder (on the left hand side) and the amplified 658 bp DNA fragments. Brightly coloured bands indicate successful extraction and PCR, and such samples were sequenced.

2.6 Statistics

Microsoft Excel 2013 was used for handling raw data, calculating means and making tables and most charts and diagrams. Statistical tests, analyses, and the making of box-plots was done using Minitab 17 Statistical Software. A 5 % significance level was used for all tests.

Parasite prevalence survey

Two separate binary logistic regression (BLR) analyses were done to test the significance of species, developmental stage, collection site and species-site interaction, one on nematode prevalence, and the other on trematode prevalence. To compare the nematode prevalence of *A. vulgaris* to the other three species, three Fisher's exact tests (FET) (pairwise comparisons of proportions) were done, and three more were done for trematode prevalence comparisons. Six Kruskal-Wallis tests (non-parametric variance test using medians rather than means) were done to investigate the effect of species, stage and collection site on the number of parasites per infected gastropod: one test for each factor for the nematode numbers, and then the same for the trematodes.

Standard error for proportions (binomial data) were calculated:

$$\mathrm{SE} = \sqrt{\frac{\hat{p}\,\hat{q}}{n}}$$

, where $\hat{p}$ the proportion of events (e.g. nematode infection), $\hat{q}$ is 1 - $\hat{p}$ (e.g. no nematode infection), and n is the sample size (e.g. number of hosts dissected).

Bioassays

Data from the two bioassays were pooled for all tests, except the data from the first one for *A. fasciatus*, which was excluded from all tests due to a very high mortality in the control group.

One binary logistic regression was used to test the effect of species and assay number on infection by *P. hermaphrodita*, and another for the effect of species, treatment and assay number on natural parasitism. Two more were used to test the effect of the gastropods' start body weight on mortality of nematode exposed specimens (controls excluded), one for *A. vulgaris* and one for *A. fasciatus*. A chi-square test of association (CSTA) was done to test the overall effect of species on mortality. A total of seven Fisher's exact tests were done for various pairwise comparisons of proportion data. Three compared infection rate among the three species (treatments pooled), one compared the infection rate in high and low dose treatments for *A. arbustorum*, and the final three compared the mortality rates of the three species (high and low dose pooled, control excluded). To test the effect of species, treatment, and interaction between the two on the amount of cabbage eaten (standardized to cm^2 per gram start body weight), a general linear model (ANOVA) was used. Finally, a Spearman

rank-order correlation (SRho) was calculated for the gastropods' feeding versus percent weight change (treatments and species pooled).

Standard errors for proportions (binomial data) were calculated as described in the previous section. The standard errors for means of data with a normal distribution (weight change and cabbage eaten) were calculated:

$$\mathrm{SE} = {}^{s}/_{\sqrt{n}}$$, where *s* is the estimated population standard deviation, and n is the sample size.

The standard deviation was calculated:

$$s = \sqrt{\frac{\sum(X-\bar{X})^2}{n-1}}$$, where *x* is individual sample value, $\bar{x}$ is the sample mean, and n is the sample size.

3. Results

3.1 Parasite prevalence survey

The number and weights of the gastropods found in the field varied over time and collection site (table 3). As there was often few specimens to be found, all sizes were collected. The mean weight of *A. vulgaris* was much higher than that of the other species.

Table 3: The number and mean weight of gastropods collected and dissected during the survey (K: Kirkejordet, E: Eidsberg). There was no search for gastropods in Eidsberg in September.

	June		July		Aug.		Sep.		Oct.		**Total**	**Mean weight (gram)**	**Total number of gastropods**
	K	E	K	E	K	E	K	E	K	E			
A. vulgaris	10	10	10	8	6	5	5	-	5	2	**61**	**5.81**	**227**
A. fasciatus	10	7	5	5	5	0	5	-	6	4	**47**	**0.65**	
A. circumscriptus	10	10	4	7	1	2	4	-	6	10	**54**	**0.41**	
A. arbustorum	10	10	9	6	5	5	5	-	5	10	**65**	**1.62**	

Of the four species studied, *A. vulgaris* had the highest prevalence of both nematodes and trematodes, the two other slugs had intermediate prevalence, while *A. arbustorum* had the least (fig. 5).

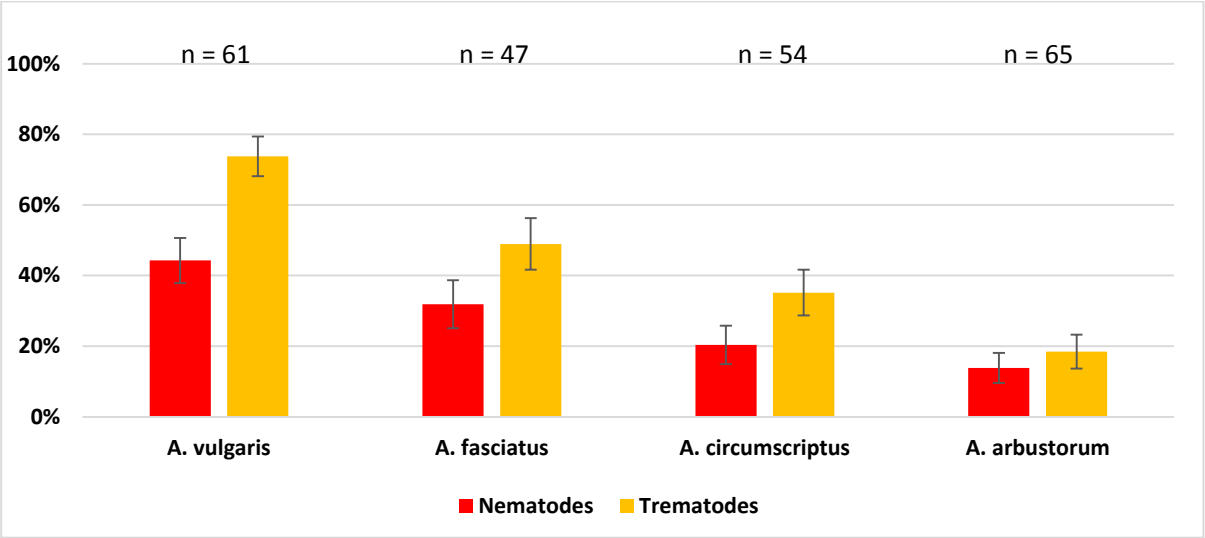

Figure 5: Total nematode and trematode prevalence (± standard error) in the four gastropod species collected.

As expected after a look at fig. 5, the nematode prevalence was significantly affected by species (df = 3, $\chi^2_{3, 217} = 12.81$, $P = 0.005$, BLR). Developmental stage also had a significant effect ($\chi^2_{2, 217} = 12.39$ $P = 0.002$, BLR), with adults having the highest prevalence (34 %). The snail *A. arbustorum* ($P < 0.0005$, FET) and the slug *A. circumscriptus* ($P = 0.009$, FET) had a significantly lower nematode prevalence than *A. vulgaris*. The site in which the gastropods were collected had no significant effect on the overall nematode prevalence ($\chi^2_{1, 217} = 2.60$, $P = 0.107$, BLR), and neither did the species-site interaction ($\chi^2_{3, 217} = 6.82$, $P = 0.078$) (fig. 6).

The trematode prevalence was also significantly affected by species ($\chi^2_{3, 217} = 36.70$, $P < 0.0005$, BLR) and stage ($\chi^2_{2, 217} = 22.21$, $P < 0.0005$, BLR), with the highest prevalence in adults (50 %). *A. vulgaris* had a significantly higher trematode prevalence than all other species (*A. fasciatus*: $P = 0.01$; *A. circumscriptus*: $P < 0.0005$; *A. arbustorum*: $P < 0.0005$ – FETs). Neither the collection site ($\chi^2_{1, 217} = 0.05$, $P = 0.831$, BLR) nor the species-site interaction ($\chi^2_{3, 217} = 5.35$, $P = 0.148$, BLR) had any significant effect (fig. 6).

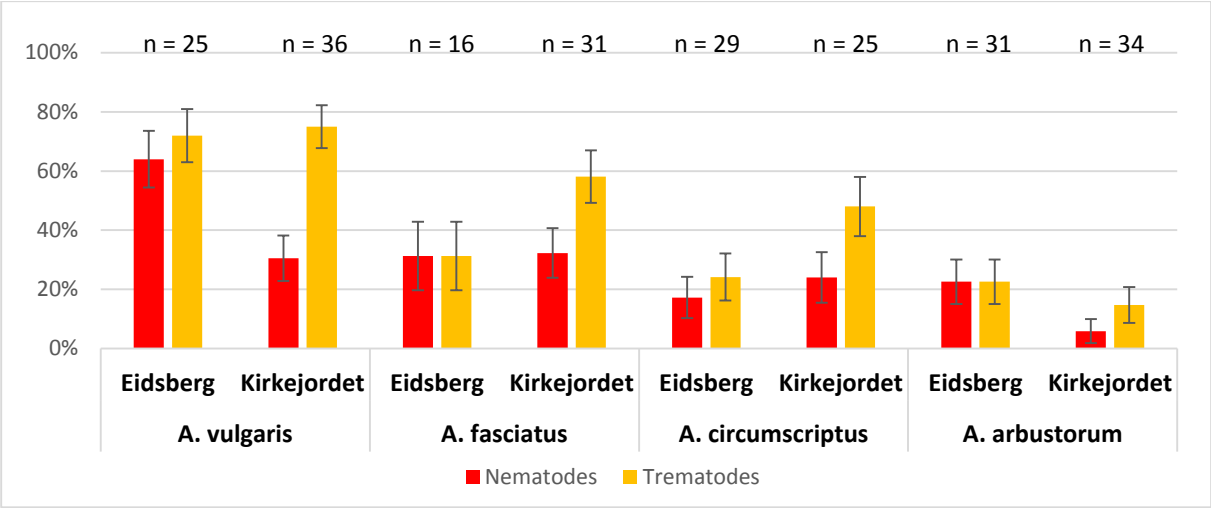

Figure 6: Nematode and trematode prevalence (± standard error) by species and site.

The number of parasite individuals per infected gastropod, analysed with Kruskal-Wallis tests, showed that the median number of nematodes was significantly affected by species (H = 12.86, df = 3, P = 0.005) and collection site (H = 3.97, df = 1, P = 0.046), but not by development stage (H = 0.46, df = 2, P = 0.8). The median number of trematodes were far from significantly affected by any of these factors (fig. 7). Three extreme specimens of *A. arbustorum* are however worth mentioning, as they contained 20000, 24000 and 74000 trematode individuals (rough estimates).

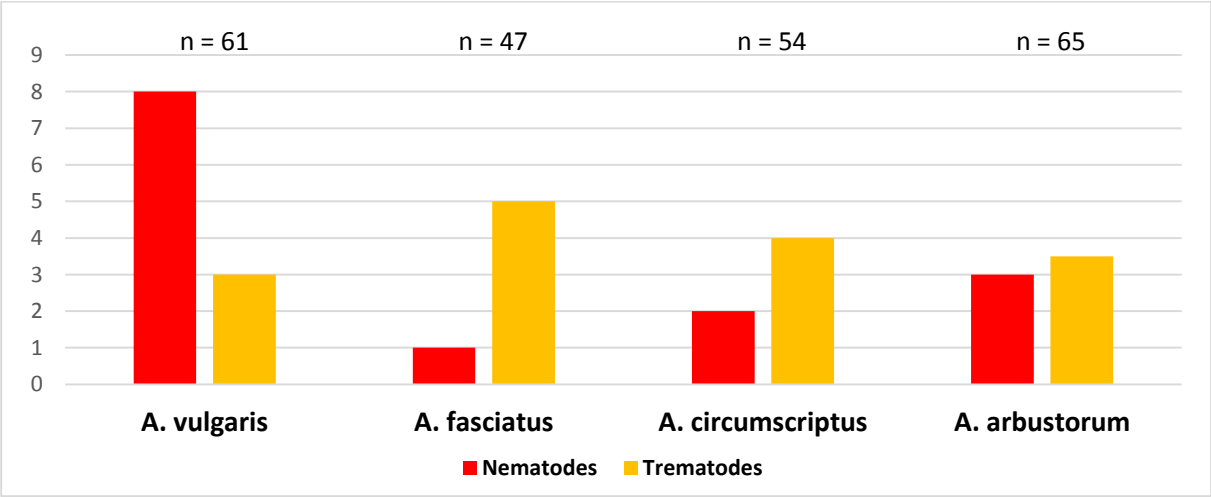

Figure 7: Median number of nematodes and trematodes per infected individual of each gastropod species.

3.2 Bioassays

There was a highly significant effect of species ($\chi^2_{2, 71}$ = 13.44, P = 0.001, BLR) on the number of specimens infected by *P. hermaphrodita* (fig. 8), while the effect of assay number was far from significant ($\chi^2_{1, 71}$ = 0.08, P = 0.773, BLR). The slugs *A. vulgaris* and *A. fasciatus* were equally susceptible, with all treated individuals of both species being infected. The infection rate of *A. arbustorum* was significantly lower than that of *A. vulgaris* (P = 0.002, FET) and *A. fasciatus* (P = 0.008, FET), with only 7 of the 20 exposed specimens getting infected. None of the gastropods in the control treatments were infected or died. There were no significant differences in infection rate between high and low dose of nematodes (*A. arbustorum*: P = 1, FET).

The species also had a significant effect on mortality rate (χ^2 = 13.85, df = 2, P = 0.001, CSTA), with *A. fasciatus* having a significantly higher mortality than *A. vulgaris* (P = 0.045, FET) and *A. arbustorum* (P < 0.0005, FET) when exposed to the nematodes.

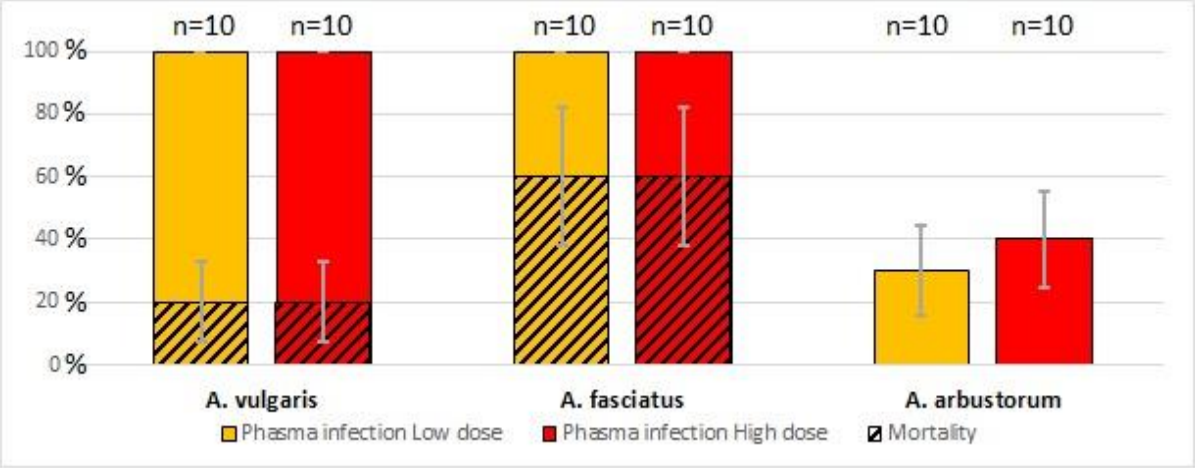

Figure 8: Percent gastropods infected by *P. hermaphrodita* (Phasma) in the bioassays (± standard error). Diagonal black lines show the percent dying (± standard error). The n indicates the number of specimens of each species in each treatment (two bioassays pooled, except for *A. fasciatus,* where first bioassay is not included). Controls are excluded as no slugs were infected or died in those, as found in dissection at the end of the bioassays.

Overall, species ($F_{2, 66}$ = 7.24, P = 0.001, ANOVA), treatment ($F_{2, 66}$ = 10.81, P < 0.0005, ANOVA) and the interaction of species and treatment ($F_{4, 66}$ = 2.82, P = 0.032, ANOVA) had significant effects on the amount eaten per specimen (standardized to cm^2 cabbage eaten per gram start body weight to account for size differences among species and individuals) (fig. 9).

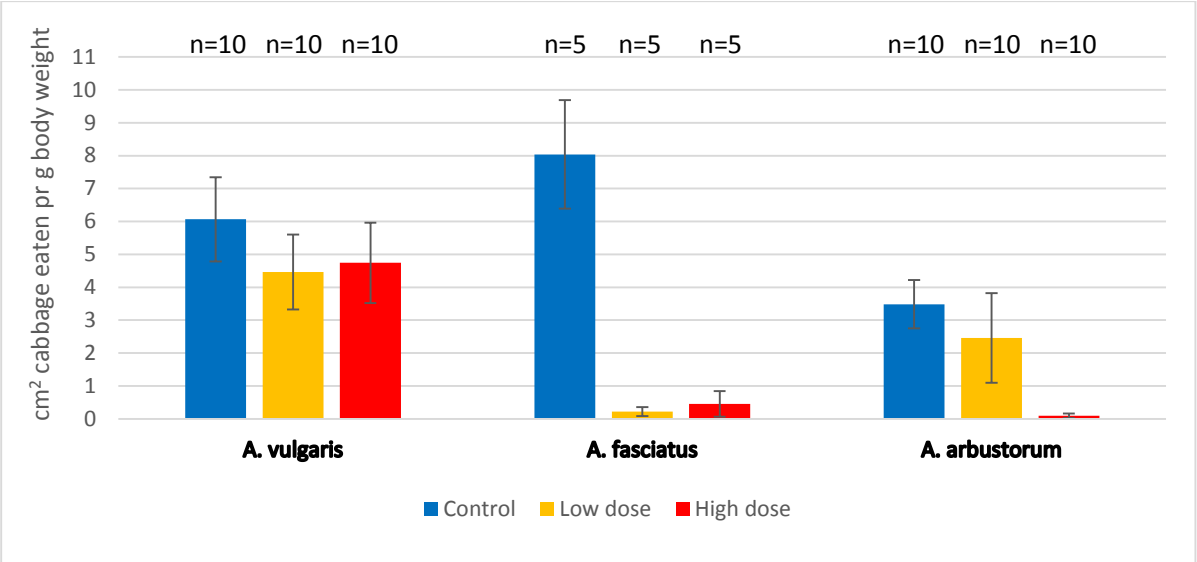

Figure 9: Amount of cabbage eaten during the bioassays (± standard error). Data from both 20-day bioassays are pooled (except for *A. fasciatus,* where first bioassay is not included). Results shown per gram body weight at the start of the bioassays.

The amount eaten by the gastropods had a significant positive correlation with their weight change during the bioassays (r_s = 0.5, P < 0.0005, n = 75, SRho), and *A. fasciatus* in the control treatment was the only group not experiencing weight loss (fig. 10).

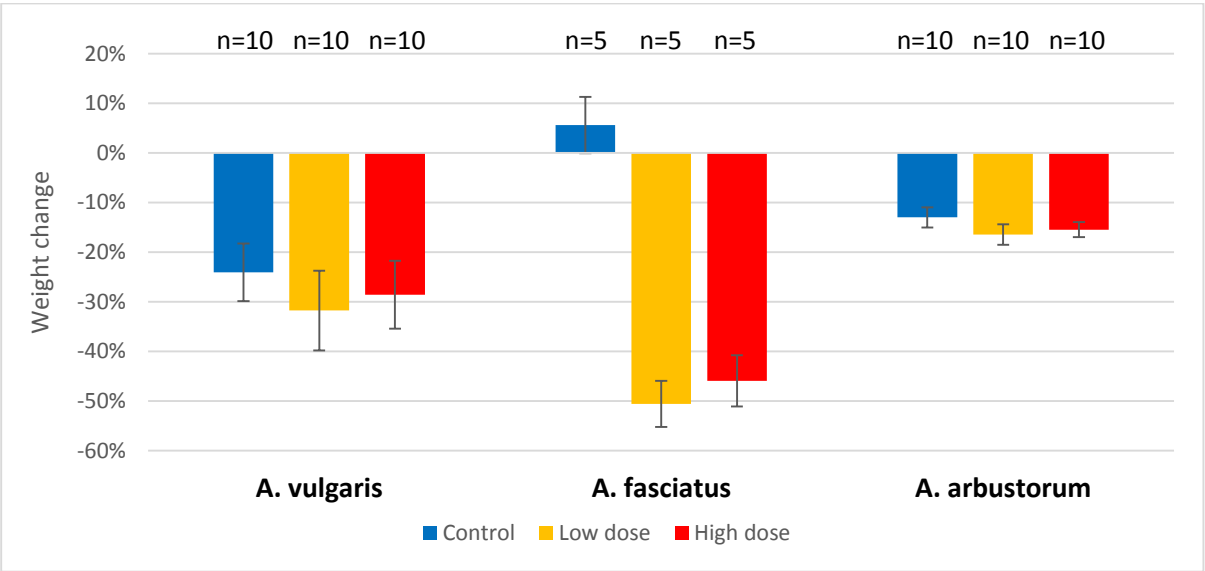

Figure 10: Percent weight change (± standard error) of the gastropods from start of the bioassays to death, or end of bioassays. Data from both assays are pooled (except for *A. fasciatus,* where first assay is not included).

No significant effect of start weight on mortality was found for nematode-exposed *A. vulgaris* ($\chi^2_{1, 18}$ = 2.54, *P* = 0.111, n = 20, BLR) or *A. fasciatus* ($\chi^2_{1, 8}$ = 2.66, *P* = 0.103, n = 10, BLR) (fig. 11).

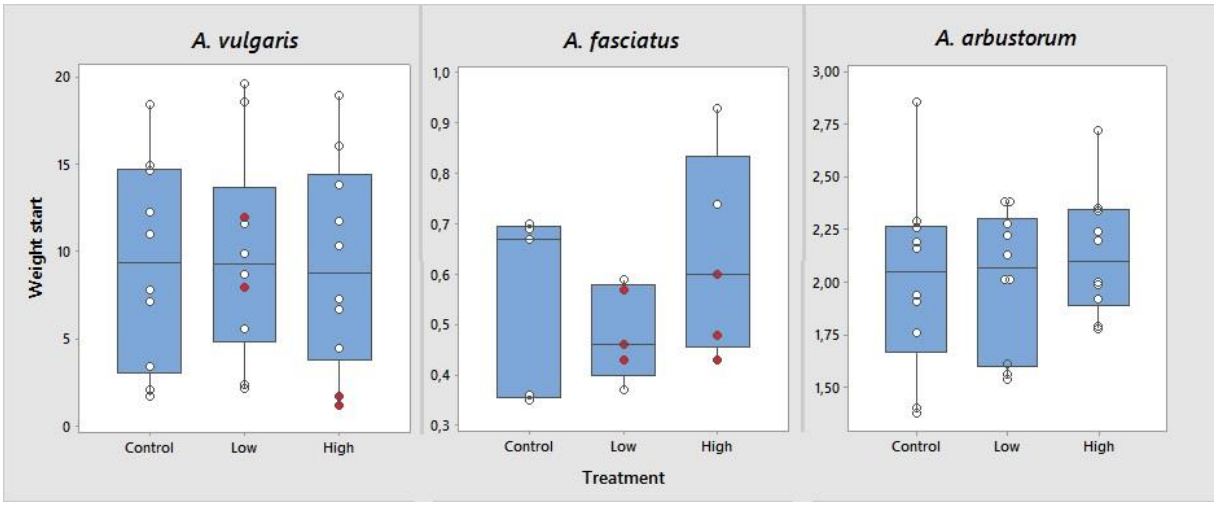

Figure 11: Box plots of start weight distribution (grams) of the gastropods used in the two bioassays (except the first batch *A. fasciatus*). Boxes represent middle 50 % of weights, "whiskers" the upper and lower 25 %, and horizontal line indicates median. Individual specimens are indicated by dots, red dots being those that died.

During the dissections to register *P. hermaphrodita* infection in these bioassays, other parasites of Nematoda and Trematoda were also noted. Analysing the pooled occurrence of these with a binary logistic regression showed that the natural parasitism significantly differed among the gastropod species ($\chi^2_{2, 69}$ = 12.18, P = 0.002), but not among treatments ($\chi^2_{2, 69}$ = 0.96, *P* = 0.617) or between assays ($\chi^2_{1, 69}$ = 0.33, *P* = 0.566) (fig. 12).

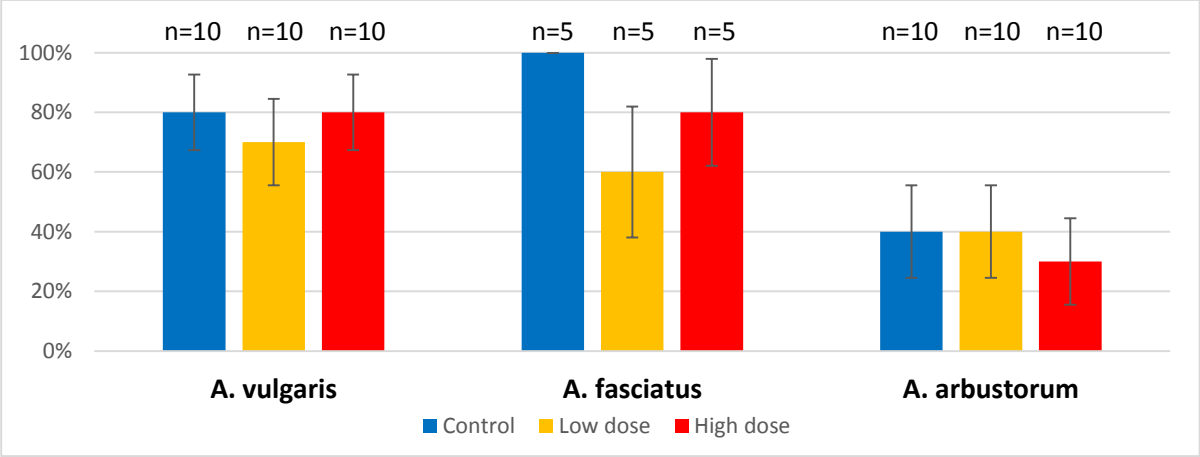

Figure 12: Percent (± standard error) of gastropods used in the bioassays that turned out to be naturally parasitized with nematodes (other than *P. hermaphrodita*) and/or trematodes. *A. fasciatus* from the first assay are not included.

3.3 Identification of parasites

The samples of the trematodes that appeared in extreme numbers in three *A. arbustorum* was identified as a *Brachylaima* sp. in the sporocyst and cercaria stage by Raúl Iglesias (pers. com.). They were located in the digestive gland, but the organ was largely replaced by the attached trematodes. *P. hermaphrodita* were identified in a few *A. vulgaris* specimens from the survey. A nematode species morphologically identified as *Alloionema appendiculatum* were found in *A. vulgaris* from Eidsberg.

Another group of organisms was also observed in some of the gastropods. These were tiny, transparent, concave creatures, swimming in a spiralling pattern using cilia. After consulting Solveig Haukeland and the book "Invertebrate zoology" (Barnes 1981), these were identified as Rotifera with some certainty, but they were not investigated further.

Of the 139 parasite samples chosen for the first DNA extraction method, 51 were successful and provided amplified COI-genes in the PCR, which were sent to be sequenced. The sequencing was successful for 36 of these. When searching the online barcoding databases for these sequences, only 18 matches were found. 14 matched either *P. hermaphrodita* or Rhabditidae sp., two matched the gastropod from which the parasite was isolated, one matched *Homo sapiens* (probably me), and the last one matched *Lipoptena cervi* (the deer ked), which was used as a positive control in the PCR, and must have contaminated another sample. The same sequence was found in several samples, making it possible to see that they were of the same species, even if they did not match any database sequence (table 4). The *P. hermaphrodita* isolated from survey gastropods had a different sequence than from those infected in the bioassays, but both sequences matched *P. hermaphrodita* in the database search. The single unknown nematode from *A. arbustorum* had a weak (90 %) match with *Angiostrongylus vasorum*, the French heartworm.

Table 4: Parasite species and groups found and identified morphologically and molecularly, from the survey and bioassays. Host species in which they were found is marked with an X, or a number representing the number of specimens directly identified (molecular ID).

		Host species			
Morphological ID		vulg	fasc	circ	arbu
	Brachylaima (Trematoda) sp.				**X**
	Other Trematoda spp.	**X**	**X**	**X**	**X**
	Alloionema appendiculatum (Nematoda: Alloionematidae)	**X**			
	Phasmarhabditis hermaphrodita (Nematoda: Rhabditidae)	**X**	**X**	**X**	**X**
	Other Nematoda spp.	**X**	**X**	**X**	**X**
Molecular ID					
	Phasmarhabditis hermaphrodita	**6**	**2**		**1**
	Rhabditidae spp. (Nematoda)	**2**	**1**	**2**	
	Unknown Nematoda sp.			**3**	**4**
	Unknown Nematoda spp.	**1**			
		1			
		1			
		1			
			1		
			1		
			1		
				1	
				1	
				1	
					1

Of the 80 parasite samples on which the DNA extraction protocol from Jenna Ross was used, 29 COI genes were successfully amplified and sent to sequencing, but the identification of these were not finished in time to be included.

4. Discussion

4.1 Parasite prevalence survey and identification

After dissection of the four gastropod species amounting to 227 individuals, the absolute highest prevalence of both nematodes and trematodes were found in the introduced slug *A. vulgaris.* With a total nematode prevalence of 44 %, being significantly higher than in two of the three other gastropod species studied, and a trematode prevalence of 74 %, significantly higher than that of all the other species, the expectation of finding the lowest prevalence in the introduced species could not have been much more wrong. That *A. arbustorum* had the lowest trematode prevalence was somewhat unexpected, when considering the especially close evolutionary relationship between trematodes and snails (Lockyer et al. 2004). The low nematode prevalence recorded for *A. arbustorum* is however not as surprising, as this is in accordance with Mengert (1953), who found that nematodes are more often parasitizing slugs than snails. Her explanation is that snails spend less time in contact with the soil, where they can be infected by nematodes.

Unfortunately, there does not seem to be many studies done on general parasite prevalences in gastropods, as most such studies I have found focus on the prevalence of only specific parasite species. However, Torchin et al. (2003) studied the parasite (nematode, trematode, cestode) prevalence of 26 host species of different taxa, including gastropods, and found that introduced species had significantly lower prevalences of parasites in their invasive than in their native range. But they also found that this difference was smaller for species such as the black rat (*Rattus rattus*), which in many cases has been introduced to the same area several times, increasing the possibility of bringing sufficient numbers of parasites from its native range for them to become established (Torchin et al. 2003). Additionally, they underlined that for parasite species with more than one host to become established, it is crucial that all hosts (or new suitable hosts) are present in the new range, or their life cycle will end with the host in which they arrived.

With regard to the parasite prevalence in the invasive *A. vulgaris* in Norway that I have recorded, there are some possible explanations. As it is very closely related to the native *A. ater*, being in the same subgenus, *Arion* (Anderson 2006), and reported to be hybridizing (von Proschwitz 2009a), it seems likely that many or most parasites of *A. ater* can also parasitize *A. vulgaris.* Parasites infecting *A. vulgaris* could also have been present in Norway from before its introduction, parasitizing its close relatives. Furthermore, with the rapid spread of this species, it may have been introduced to Norway several times since it was first recorded. In a study by Ross et al. (2010), no significant difference in the nematode prevalence of recently introduced slugs from mainland Europe and native slugs in the UK was found, but the European slugs introduced to the USA generally had significantly lower nematode prevalences. This could support the explanations regarding the presence of closely related native species and frequency of introduction events.

In order to strengthen results, it would of course help to have larger sample sizes of each species, but more collection sites around the country could also have been beneficial. Although the two collection sites used did not have significant effects on the prevalence, there is a chance that site would have an effect if for example a site at the west coast were included. It would also have been interesting to compare the parasite prevalences with those of *A. ater*, to have another large slug species and test the effect of adult size. With only one species that is so much larger than the others are, tests of weight effects would be very similar to testing the effects of being *A. vulgaris*. Ross et al. (2010) did however investigate the nematode prevalence of *A. ater* in the USA and UK, reporting prevalence of 40 % and 30 %, respectively, which is not much different from the 44 % in *A. vulgaris* from my study. Unfortunately, there was not enough time for one person to be collecting and dissecting more specimens or collecting at more sites, and *A. ater* is not very common in the areas searched for this survey.

The fact that trematode prevalence was higher than nematode prevalence for all four gastropod species makes an interesting pattern. This could have the explanation that nematodes more often than trematodes kill their gastropod host, and thus finding live specimens infected with trematodes is more common. Alternatively, it could simply mean that trematodes are a generally more common group of parasites in gastropods, which is more likely considering the co-evolutionary history of these two taxa.

The highest number of nematodes per infected gastropod was found in *A. vulgaris.* Again, it would be interesting to have compared these numbers with those of *A. ater*, to see if this to some degree could be explained by body size. It seems likely that larger, more robust species could sustain larger numbers of parasites. Another factor influencing the number of nematodes is that one species of nematodes, *A. appendiculatum*, were only found in *A. vulgaris.*

The trematode numbers were on the other hand more or less similar among all four species. There was however three extreme specimens of *A. arbustorum*, with 20000, 24000 and 74000 *Brachylaima* sp. sporocysts and cercariae, identified by Raúl Iglesias. Sporocyst being a reproducing stage, and cercaria the final development stage within the snail, reached after two reproducing generations (Barnes 1981). At least one species of this genus, *B. cribbi*, is known to parasitize humans (Butcher et al. 1996; Butcher et al. 1998; Butcher & Grove 2001).

Naturally occurring *P. hermaphrodita* were found in a few specimens of *A. vulgaris*, and two of them made it through the PCR, was successfully sequenced, and had a 98.52 % match in the barcoding databases. As the sequence of the *P. hermaphrodita* used in the bioassay had a 99.16 % match, it is plausible that the wild nematodes were from a native population, or descendants of commercial nematodes of a different strain, applied in the fields nearby some

time ago. It would certainly have been interesting to analyse their symbiotic bacteria, to see if they were only *M. osloensis,* as used in the commercial products, or a mix of bacteria species.

The nematode that had a 90 % match to *A. vasorum* is probably not of that species, as a match of approximately 97 % or more is needed to justify species identification (Even Sannes Riiser pers. com.). It is however likely to be a rather closely related species, for which the DNA sequence was not stored in the barcoding databases.

4.2 Bioassays

In this study, the native, non-target species, *A. fasciatus* was more affected by the biological control product Nemaslug than the other two species tested. It was highly susceptible to infection, had the highest mortality rate (60 %) and close to 100 % feeding inhibition in both nematode exposed groups. Because of a high mortality in the control group of *A. fasciatus* in the first bioassay, the data for this species in that assay was left out of all calculations. With only half the amount of data (15 specimens total) for this species compared to the others (30), these results are weaker. The *A. fasciatus* first assay control group did not have any unusual or many parasites that could have caused the deaths, but a disease could have spread un-noticed in the quarantine. Two of the specimens that died started developing holes in the posterior dorsal integument, through which the digestive gland was visible, prior to death.

As *A. fasciatus* has not previously been tested for effects or susceptibility, the outcome was difficult to predict with any certainty. The related slug *Arion silvaticus*, in the same subgenus as *A. fasciatus* (*Carinarion*), was however tested and found to be infected and killed by *P. hermaphrodita* in a study by Wilson et al. (1993). However, two very closely related species of another Arionid subgenus (*Kobeltia*) have been shown to differ in susceptibility, with *Arion hortensis* being less susceptible than *Arion distinctus* (Iglesias & Speiser 2001). Similarly, a study by Grewal et al. (2003) confirmed that the nematode had an insignificant effect on *A. hortensis,* with less than a 5 % mortality for all treatments, including controls. Their study also showed that *Arion subfuscus* (subgenus *Mesarion*) was unsusceptible, with no mortality at all in any treatment. Therefore, the reported susceptibility of one species does not clearly justify an expectation of susceptibility of a related species.

The second most affected species in these bioassays was *A. vulgaris*, one of the main target species of the control product. Although it was highly susceptible to infection, the mortality of this species was not very high (20 % for both treated groups), compared to *A. fasciatus*. The nematode exposed specimens fed slightly less than controls, and the weight change was very limited. These results support the conclusions of similar studies (Grimm 2002; Rae et al. 2007; Speiser et al. 2001) to some degree, in that the effects were not very severe. They also indicate that *A. vulgaris* is robust and capable of handling stress well. The mortality observed for this slug in these assays does however challenge the notion that *P. hermaphrodita* only kills young *A. vulgaris,* as the four slugs killed weighed 1.16, 1.73, 7.97 and 11.99 g. For

example, Speiser et al. (2001) found a clear size-dependence, with no significant effects on specimens larger than 1 g, but significant feeding inhibition and mortality for specimens weighing up to 0.15 g, and significant mortality for specimens weighing between 0.5 and 1 g. Similarly, Grimm (2002) reported a mortality of just below 50 % for specimens weighing 0.15 g, while the largest slugs tested, weighing 0.45 g, had a mortality 8 %, which was not significantly different from that of the control.

Possible explanations for the mortality observed in my bioassays could include small arena sizes limiting the slugs' mobility, use of high nematode doses, or pre-parasitism. The arenas used by Grimm (2002) were boxes measuring 13.5 x 7.5 x 6 cm and Petri dishes with a diameter of 9 cm, while the nematode dose was 75000 per box (slugs were exposed to nematodes in the boxes for 4 days, then moved to un-treated Petri dishes for 10 days). Speiser et al. (2001) used boxes measuring 8 x 8 x 6 cm, and nematode doses from 2000 to 80000. Thus it would be fairly safe to say that my arenas were not too small (9 x 13 x 18 cm) or my doses too high (15000 and 30000). Of the four slugs that died, three were naturally parasitized, but none of which had any uncommon or particularly high numbers of other parasites. Of course, with a total of only 20 exposed specimens, the relatively high mortality for such big slugs could be due to chance.

The final species, the native, non-target *A. arbustorum*, was as expected the least affected, with only 35 % of nematode-exposed specimens getting infected and none dying. Interestingly, the treatments did however have a significant effect on its feeding, with complete feeding inhibition in the high dose group. As many of the exposed but un-infected specimens did not feed, this could indicate that the snails are capable of detecting the nematodes without being infected, and thus avoiding to cross the soil in order to reach the food. The exposed snails displayed a characteristic behaviour, retracting into the shell and sealing the shell mouth with dried mucus, usually attached to the box walls above the soil. The seal is known as an epiphragm, and is usually observed in hibernating snails (Kerney & Cameron 1979). Its main function seems to be reducing water loss when the snail is inactive for some time (Machin 1968). This was observed for most specimens at nearly every assessment, but only rarely in the control groups. It would seem that the exposed snails experienced their conditions as so unfavourable that they went into hibernation. In order to see if the snails were alive, they were picked up, gently poked with plastic forceps, breaking the epiphragm, and placed on the soil. They made a new epiphragm and moved back from the soil to the wall between every assessment. Other gastropods (*D. reticulatum* and *A. ater*) were reported to actively avoid soil containing *P. hermaphrodita* in a study by Wilson et al. (1999), so it is likely that *A. arbustorum* in my study could also detect the nematodes.

The closest relatives of *A. arbustorum* that have previously been tested for effect of *P. hermaphrodita* are *Cepaea hortensis* and *Cepaea nemoralis* (family Helicidae, the same as *A. arbustorum*), in a study by Wilson et al. (2000). They found that one of these species, *C. hortensis*, had a significant mortality when treated with their highest nematode dose of 75000

(arena size: 27 x 14 x 9 cm), while *C. nemoralis* did not differ significantly from the control. They did however not record feeding inhibition, infection rate or weight change.

The infection rates for *A. arbustorum* are somewhat uncertain, because the infected specimens had very low numbers of nematodes within the body. Out of the seven infected, three had only one nematode, and the highest number of nematodes registered in one specimen was four. Additionally, three of the infected specimens had nematodes only in the intestines, which could mean they were merely passing through. In the other four infected specimens, the nematodes were found in the body cavity or kidney, which proves that the nematodes must have penetrated tissues of the snail.

The small difference in weight change among the treatments for this species, despite the complete feeding inhibition in the high dose treatment, could possibly be explained by its shell. Because they all experienced a similar decrease in body weight, this could be the maximum possible decrease, with the shell and vital organs constituting the remaining weight.

A few previous studies have used laboratory-reared gastropods for their bioassays (Grimm 2002; Morley & Morritt 2006). This has the obvious advantage of avoiding use of naturally parasitized specimens and making quarantines prior to the bioassays redundant, which may affect the results. Most studies do however use field collected specimens, which are normally quarantined for approximately two weeks (Coupland 1995; Grewal et al. 2003; Grimm 2002; Tan & Grewal 2001). The quarantine has a clear benefit by allowing the researcher to avoid using specimens with signs of infection. However, it does also allow for diseases and parasites to spread, if individuals are kept together. This is especially relevant when studying cannibalistic species such as *A. vulgaris.* I would recommend that specimens be quarantined separately for optimal effect. Several sick and dying specimens were removed during the quarantines, but many of those used were also naturally parasitized. There was fortunately no significant difference in the distribution of naturally parasitized specimens among treatments or assays in this study, so one could assume this would not affect the outcome too much. However, as the identity of these parasites are unknown, some could be affecting the gastropods more than others, further weakening the results. To rear the gastropods in the laboratory would have been most optimal, but the time frame of this study did not allow it.

The fact that the control groups of both *A. vulgaris* and *A. arbustorum* decreased in weight is most likely an effect of the very unnatural experiment setup, and a semi-field trial might have been preferable. This could especially be beneficial for testing snails, if the shell weight makes the difference harder to detect. It would also be tempting to remove and weigh the shell, but a varying amount of mucus and tissues would then go with it.

5. Conclusions

There was a significant effect of species and developmental stage on both nematode and trematode prevalence in the gastropods. Not surprisingly, adults had the highest prevalence of the three stages. However, *A. vulgaris* had the highest prevalence of both parasite groups, which was the complete opposite of what was expected. With a nematode prevalence of 44 % for the 61 specimens searched, it was significantly more often parasitized than the native 54 *A. circumscriptus* and 65 *A. arbustorum* specimens. The trematode prevalence of *A. vulgaris* was 74 %, significantly higher than that of all three native species, also the 47 native *A. fasciatus*. It does therefore seem not to benefit from being an introduced species by having escaped from native range parasites.

A. vulgaris also had the highest number of nematodes when infected, with a median of eight individuals. Trematode numbers did on the other hand not differ significantly among the four species studied, although there were three extreme specimens of *A. arbustorum* containing ten thousands of trematodes, morphologically identified as a *Brachylaima* sp.

P. hermaphrodita and *A. appendiculata* were also morphologically identified. Additionally, five nematodes were molecularly identified as Rhabditidae spp. A selection of *P. hermaphrodita* were also successfully molecularly identified (both from survey and bioassay gastropods).

A. fasciatus was clearly the species most affected by exposure to the nematode *P. hermaphrodita*, used in the commercially available slug control product Nemaslug. All exposed specimens were infected, and 60 % of these died within the 20 days the bioassay lasted. Furthermore, both of the nematode doses caused near complete feeding inhibition. Use of *P. hermaphrodita* could potentially harm local populations of this species.

The second most affected was *A. vulgaris.* Like with *A. fasciatus,* all specimens that were exposed were infected, but the mortality was significantly lower, with only 20 % dying, independent of nematode dose. However, unlike *A. fasciatus,* the exposure to the nematodes caused no significant feeding inhibition for *A. vulgaris.* For *P. hermaphrodita* to be a useful control agent for this species, it would have to be applied when the slugs are newly hatched, as previous studies have shown significant effects only on specimens smaller than 1 gram.

A. arbustorum had an infection rate of only 35 %, with no significant difference between low and high dose nematode treatments. There was also no mortality in any of the treatments, but the snails experienced complete feeding inhibition when exposed to the highest dose of the nematodes. They also seemed to be avoiding soil containing nematodes. As the effects are generally not very severe for *A. arbustorum,* and it seems capable of avoiding the nematodes, use of the biocontrol product is not likely to have any negative implications for local populations of the species.

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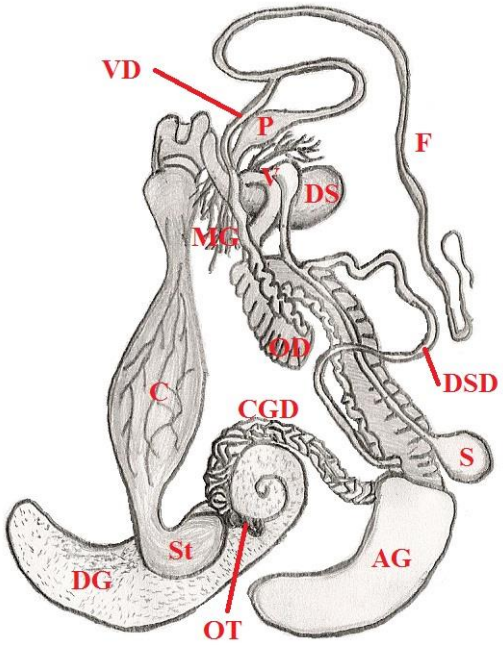
Appendix 1

Slug dissection protocol

The dissection protocol for slugs is mainly based on advice from my supervisor Solveig Haukeland. For snail dissection, the "Terrestrial Mollusc Tool" website developed by Center for Plant Health Science and Technology and the University of Florida was followed. A drawing of the anatomy of the snail (*Helix aspersa*) from Collins Field Guide to Land Snails of Britain and North-West Europe by Kerney and Cameron was also helpful. Once the snail was dissected and the various organs isolated, they were opened and searched in the same way as described in the slug dissection protocol.



The internal anatomy of a snail (*Helix aspersa*), drawn after Kerney and Cameron (1979) page 18.

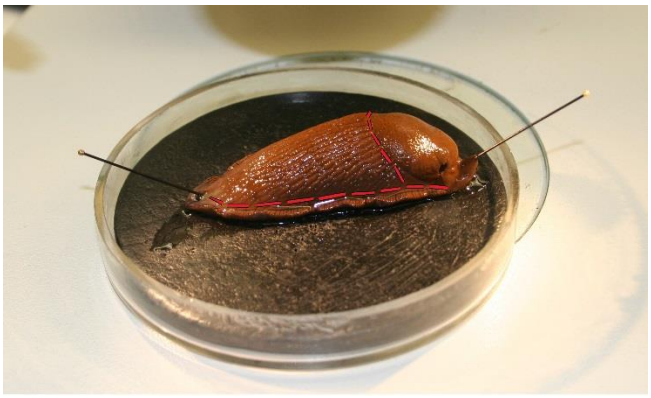
VD: Vas deferens **P**: Penis **F**: Flagellum **V**: Vagina **DS**: Dart-sac **MG**: Mucus glands **OD**: Oviduct **C**: Crop **CGD**: Common genital duct **DSD**: Diverticulum of spermatheca duct **S**: Spermatheca **DG**: Digestive gland **St**: Stomach **OT**: Ovotestis **AG**: Albumen gland

Equipment

Tools and equipment used in the dissection process include:

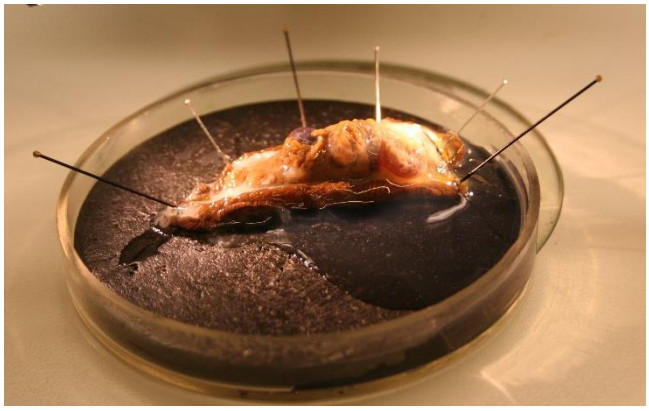
- fine surgical scissors
- forceps
- small pliers
- steel picks
- very fine picks (made by fitting a 2 cm cactus needle to a matchstick)
- pins
- a petri dish with a wax disc
- petri dishes with water
- a small beaker for waste
- a square watch glass
- microscope slides
- a lighter
- Eppendorf tubes
- 70 % alcohol
- 4 % formalin
- tap water
- an electronic balance
- a Leica stereo microscope

Slug dissection protocol (Arionidae sp.)



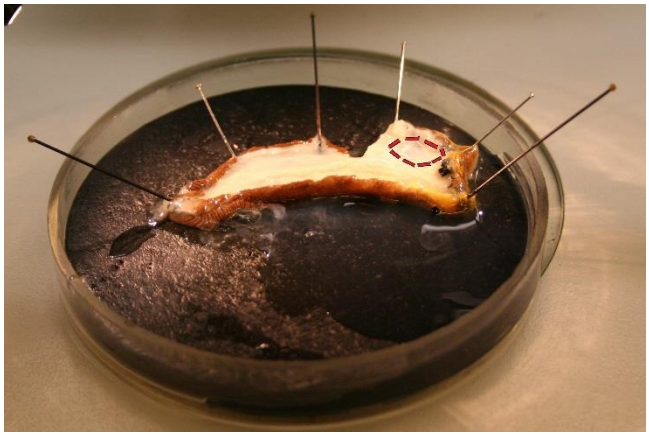
Step 1:

The relaxed slug is placed on the wax disc in a petri dish, oriented so the head points to the right and the pneumostome toward you. Pin the slug down at front and back. Make sure the pins go through the pedal musculature, and not just the mantle or foot fringe, as these will tear. Using the surgical scissors, make an incision beneath the pneumostome, between the mantle and foot fringe. Cut along the fringe all the way back to the needle and along the rear end of the mantle (see the red lines in the photo). Be careful not to cut too deep, as the intestines should be intact.



Step 2:

Fold the mantle and posterior/dorsal integument over from the incisions and pin down with needles, so the internal organs are exposed.



Step 3:

Remove the digestive system by cutting the mouth and cloaca loose from the integument. Remove the reproductive system by cutting around the genital pore. Finally, remove the kidney and heart by cutting around it through the membrane holding it. The vestigial shell should now be visible. Place all the removed organs to one or more Petri dishes with water. Search the vestigial shell, sole, mantle and posterior/dorsal integument for parasites. The remains may be cut to strips to look within the pedal musculature.

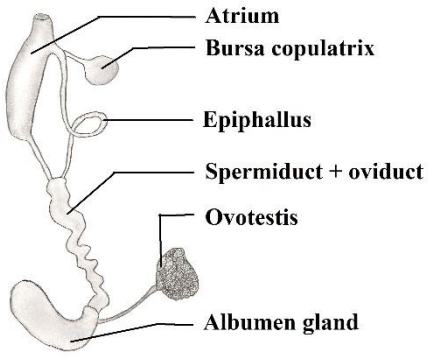
Step 4:

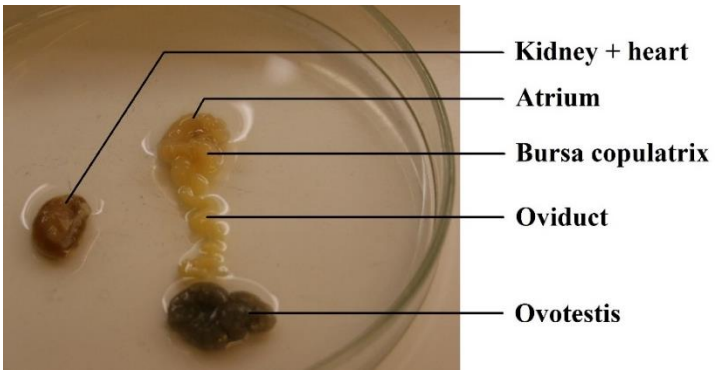
Separate the entire bowel (cloaca to radula) from the digestive gland by carefully cutting the connections between the two parts while gently pulling the bowel. Look through the digestive gland by tearing it apart with forceps. Using the scissors, cut along and unfold the bowel from radula to cloaca, and look for parasites within. I suggest using an underneath light source when looking for the parasites.

Nematodes can be isolated using the steel picks or the cactus picks if the nematodes are too small for the steel picks. Trematodes are usually easier to isolate using forceps.

Step 5:

Like with the digestive system, cut along and unfold the atrium, spermiduct/ oviduct and the bursa copulatrix. Albumen gland and ovotestis are most easily searched by tearing them apart with forceps. The heart and kidney are searched in the same way.





Step 6:

The parasites can be stored in 70 % alcohol in Eppendorf tubes for DNA analysis and their location within the gastropod can be noted. Nematodes can be fixed with 4 % formalin for later morphological identification. Before adding the nematodes to the formalin, they should be placed in a drop of water on a microscope slide and "heat relaxed" with a lighter until they are in a straightened position.

Appendix 2

DNA extraction protocol provided by Jenna L. Ross

1) Make up 5% Chelex mix. I usually do this by autoclaving 9.5g of tap water in a glass universal bottle. Once this is cool, I then add 0.5g of Chelex beads*. I then mix by shaking and then autoclave again. Autoclave inside a jar to avoid spillages.
2) Prepare the 0.5ml PCR tubes by autoclaving and drying.
3) For each sample, prepare a PCR tube with 25µl of 5% Chelex (mix before dispensing- this can be done by shaking gently) and 5µl of Proteinase K. Mix by pipetting. You should now have 30µl in your PCR tube.
4) Add one nematode into each PCR tube containing the Chelex/proteinase K mix. Use a sterile pick (wash with 100% ethanol first and between each sample). Then check under the microscope that the nematode is suspended in the Chelex/proteinase K mix.
5) Once all samples are prepared, inoculate at 60C for 30 minutes, then 94C for 10 minutes and finally store at -20C until frozen.
6) Once samples are all frozen (usually after 40 minutes), centrifuge for 5 minutes at 14000 RPM.*
7) Remove supernatant (i.e. the surface liquid- this is your extracted DNA!) and add to a fresh PCR tube. Label and store at -20C.

* Chelex beads: Chelex 100 Resin (Bio-Rad)

* Frozen samples were centrifuged at 6000 RPM

Appendix 3

PCR amplification protocol provided by Even Sannes Riiser

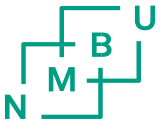
1) Place an empty 96-well PCR plate, 2-3 ml mQ H_2O and empty 1.5 ml Eppendorf tubes (for Mm) into a PCR-cabinet and UV-irradiate them for 10 minutes.
2) Working inside the PCR cabinet, make the Master mix (Mm) by mixing 14.2 µl mQ H_2O, 2.5 µl 10X PCR Buffer (Applied Biosciences®), 2 µl dNTPs (2.5 mM), 0.5 µl primer LCO1490 (10 µM), 0.5 µl Primer HCO2198 (10 µM) and 0.3 µl Taq-polymerase (Applied Biosciences®). Measures are for one well – if full 96-well plate, multiply measures by 98 (accounting for pipetting errors).
    a. Remember to shortly vortex the primers, buffer and dNTPs before adding
    b. Add compounds in the order above (Taq-polymerase at the very end, and keep cold)
    c. Finally, vortex the Mm
3) Add 20 µl Mm to each well (using an electronic step-pipette)
4) Add 5 µl template from the DNA extraction
    a. Use a multichannel pipette
    b. Remember to use new pipette tips every time
    c. Remember two wells for positive control (use template known to give PCR product) and two for no template control (NTC) (5 µl mQ H_2O instead of 5 µl template)
    d. Seal the plate with cap strips
5) Centrifuge plate at 1000 g for 30 seconds
6) Put the plate in the PCR machine* and start the programme*

*PCR machine:

Bio-Rad T100™ Thermal Cycler

*Programme steps:

1: 94°C, 3 min. **2**: 94°C, 30 sec. **3**: 45°C, 30 sec. **4**: 72°C, 1 min. **5**: Go to step 2, 5 times. **6**: 94°C, 30 sec. **7**: 51°C, 1 min. **8**: 72°C, 1 min. **9**: Go to step 6, 34 times. **10**: 72°, 10 min. … **11**: 4°C, ∞.



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