Survey, host preference and infectivity of *Phasmarhabditis californica* (Family: Rhabditidae) on pest slugs

by

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Abstract

Certain slug species are considered agricultural and horticultural pests worldwide. Nematodes offer a potential solution as biocontrol agents in controlling slug populations due to their natural associations with terrestrial gastropods. In some cases, they provide higher specificity and more efficient pest management outputs than many chemical or physical practices currently available. One of the most well-known biocontrol agents of slugs is a facultative parasite, *Phasmarhabditis hermaphrodita,* which has been widely established as a biocontrol agent after it was patented and commercialized as a molluscicide product (Nemaslug ® with the associated bacteria symbiont, *Moraxella osloensis*) in 1994 in the UK. However, Canada had no previous record of any *Phasmarhabditis* species until a recent discovery of a Canadian strain of *P. californica* collected from a local nursery in Edmonton, Alberta. This species was originally isolated from California and subsequently marketed by BASF as a biocontrol agent (Nemaslug® 2.0) against slugs in England, Scotland, and Wales in 2022. However, the immediate use of this species as a biocontrol agent is currently not available in Canada until a proper risk assessment of the biocontrol product Nemaslug 2.0 with the active organisms *P. californica* (with the bacteria symbiont, *Moraxella osloensis*) is made and its biology fully understood. First, I conducted an extensive survey to identify the diversity, distribution, and abundance of pest slug species and their associated nematodes in selected agricultural and horticultural sites in Alberta. I further investigated if any *Phasmarhabditis* species were present in the survey sites. I collected 1331 slugs belonging to nine species, with *Deroceras reticulatum* being the most common. Forty-five samples (3.38%) were positive for nematodes, the majority were identified to species level: *Alloionema appendiculatum, Caenorhabditis briggsae, Caenorhabditis elegans, Panagrolaimus subelongatus,* and *Mesorhabditis spiculigera*. I did not

isolate *P. californica* from any of the slugs collected from these survey sites, which included the original site where *P. californica* was discovered. However, four *D. reticulatum* slugs retrieved from a residential garden sample were infected with *P. californica,* thereby suggesting a possible fragmented distribution for this strain in the province. I then used an agar-based chemotaxis assay to evaluate the host preference of the laboratory-cultured Canadian strain of *P. californica* against four pest slug species, *D. reticulatum, A. rufus, A. fasciatus*, and *A. valentianus*. I showed that *P. californica* was strongly attracted to mucus of all slug species except for *D. reticulatum* for which I observed a weak attraction. In addition, I checked the host preference of a co-occurring nematode, *Pristionchus entomophagus,* a necromenic nematode on the same host species to check if they would have a similar host preference as *P. californica. P. entomophagus* showed a significant attraction to the mucus of *D. reticulatum* while being strongly repulsive to *A. rufus.* Given that these two nematode species have potential similarities in chemoattraction profiles towards *D. reticulatum,* I then investigated the efficacy of the infectivity of *P. californica* as a biocontrol agent in the presence of *P. entomophagus*. The ability to cause mortality in slugs infected by *P. californica* was the same in single and mixed infections, i.e., mortality rates remained the same despite its co-occurrence with *P. entomophagus.* Both in single and mixed infection treatments, the number of *P. californica* that entered the slug host also remained comparable and statistically non-significant. However, the number of progeny (F1) in mixed treatments was lower than that of the single treatments for *P. californica*. Interestingly, *P. entomophagus* was not affected by concomitant infection with *P. californica*. These discoveries on the local strains of *Phasmarhabditis* support the possibility of using *P. californica* as a biological control agent within Canada. Still, further investigation is

needed on the persistence and efficacy of *P. californica* in the presence of other nematode species in the soil community.

Preface

This thesis is an original work by Dayani Buddhika Maheshini Patuwatha Withanage.

Chapter 1 is the general introduction and objectives.

Chapter 2 is an adaptation of "Pestiferous slugs and their associated nematodes in agricultural fields, greenhouses, and nurseries in Alberta, Canada", an article published in the Journal of Helminthology (2023), co-authored by D.B.M. Patuwatha Withanage, D.K. Howe, C. Richart, R.J. Mc Donnell, D. Denver, and L.T. Luong. I conceptualized the study, conducted the survey, collected, and processed slug and nematode samples, analyzed the data, and wrote the manuscript. D.K. Howe and D. Denver assisted with the molecular identification of the nematodes and contributed to manuscript edits in the related method section. C. Richart and R.J. Mc Donnell assisted with the molecular identification of the slugs and contributed to manuscript edits in the related method section. L.T. Luong was the project supervisor and was involved in the manuscript composition.

Chapters 3 and 4 are original works done by Dayani Buddhika Maheshini Patuwatha Withanage. They have not previously been published.

Chapter 5 is the conclusions and future directions.

Dedication

To my beloved parents Susil and Malkanthi, and my dear brothers Nimesh and Tharusha.

Your unwavering love and support have been the driving force behind my academic journey. This thesis is dedicated to each of you, as a heartfelt expression of gratitude for the love and endless sacrifices you have made on my behalf.

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Chapter 1

Introduction

1.1. Background

1.1.1. Gastropods as pests

Gastropods (Phylum: Mollusca) including snails and slugs are a diverse group of aquatic and terrestrial organisms (Godan, 1983; South, 1992; Tan & Grewal, 2001). Of terrestrial gastropods, only a few are considered pests of agriculture and horticulture. The slugs are more resistant to physical disturbances such as planting and tillage than snails (Beeby & Richmond, 2007; Kunkel *et al.,* 2023). Additionally, their reduced dependence on calcium for shell development leads to higher energy reserves, which ultimately contributes to their greater pestiferous nature (Beeby & Richmond, 2007; Kunkel *et al.,* 2023). Being generalists in diet, slugs can cause severe damage to virtually all types of crops from specialty to field crops, especially at their seedling stages (Tulli *et al.,* 2009; Douglas & Tooker, 2012; Le Gall & Tooker, 2017; Kunkel *et al.,* 2023). This leads to severe economic losses for farmers due to crop establishment failure in spring and fall. Slugs often station in residue-rich stable habitats with frequent irrigation or precipitation, hence no-tillage systems are ideal for them to thrive. The damage caused by slugs is particularly prominent in regions with a temperate climate including Ireland, France, the Netherlands, the UK, North and South America, Australia, New Zealand, and South Africa (Speiser *et al.,* 2001; Wilson, 2007; Hynes, 2015; Ross, 2019; Barua *et al.,* 2021). Recently, the UK Agriculture and Horticulture Development Board postulated that the UK agriculture industry could experience losses of over GBP 100 million annually if failed to properly manage slugs (Barua *et al.,* 2021). Salisbury (2015) reported an annual USD60 million in losses to the grass seed industry in the Willamette Valley of Oregon, USA by *Deroceras reticulatum,* the most pestiferous slug species in the world. However, recent estimates suggest USD 100 million (Barua *et al.,* 2021) in losses in this USD 500 million industry. Based on these reports, it is noteworthy that these substantial economic impacts have undergone drastic changes within a relatively short timeframe (2015- 2021). *D. reticulatum* is known to cause severe damage to a wide variety of crops (Wilson, *et al*., 1993; Tan & Grewal, 2001). Often *D. reticulatum* occurs as the dominant gastropod pest species in crop fields, however, a sizeable minority of other slug species may also occur (Wilson, 2007). These may include slugs in the

genera *Arion*, *Milax, Ambigolimax,* and other slug species in the genus *Deroceras.* Fifteen species of slugs of European origin and three species of native slugs have been previously reported as pests in North America (Chíchester & Getz 1969; Prystupa, 1983). Among them, *D. reticulatum, D. laeve* (native and a few introduced populations in the US; Mc Donnell, 2009), *Limax maximus, Lehmania valentiana* (*Ambigolimax valentianus*)*, Arion hortensis, A. rufus,* and *A. intermedius* have been listed as major pests in North America with many of them having a wider distribution. *Limacus flavus*, *Lehmannia marginatus, Prophysaon andersonii* (native)*, Milax gagates, Arion circumscriptus, A. subfuscus, A. fasciatus* and *A. silvaticus* have been listed as less important agricultural pests in North America. The first report of a European slug in North America was from the coastal regions of New England, specifically in the state of Maine reported in 1822 (Chichester & Getz, 1969; Prystupa, 1983). It was suspected that these inadvertent introductions occurred either through the ballast of ships or through the frequent trade of plant commodities in nurseries and agricultural sites. (Chíchester & Getz 1969; Prystupa, 1983).

1.1.2. Pest slug control and nematodes as a tool for biocontrol

Pest slugs are economically important in agriculture and horticulture (Barker, 2002; Koslowski, 2012). Physical, chemical, and/or biological practices are undertaken to control these pest populations. The most popular method is the application of pesticides, especially in the form of granular baits with active ingredients including metaldehyde (IUPAC: 2,4,6,8-tetramethyl-1,3,5,7-tetroxocane) or iron phosphate (Abobakr *et al.,* 2021; Kunkel *et al.,* 2023). These products are expensive, yet a single application may cause only about 10-60% slug mortality (Mc Donnell *et al.,* 2020). There is a high risk to wildlife due to pesticide toxicity, especially to mammals such as canids and rodents (Kunkel *et al.,* 2023). There is also the potential for leaching of these pesticides into groundwater and streams. Even though the use of metaldehyde is permitted throughout the European Union, the UK Department for Environment, Food and Rural Affairs (DEFRA) announced its plans to ban metaldehyde from Spring 2020 due to the "unacceptable risks [posed …] to birds and mammals" (DEFRA, 2018; Gething *et al.,* 2020; Cutler and Rae, 2022). However, due to the lack of evidence on the impact of metaldehyde on non-target organisms, the proposal was subsequently overridden in the UK courts (Gething *et al.,* 2020) and the ban came into effect on April 1st 2022 (Department for Environment…[accessed

2023]). With public opposition toward the application of pesticides, physical and biological practices are getting more attention and appreciation (Barua *et al.,* 2021). Tillage is one of the physical methods that can reduce slug populations (Busscher, 2005; Kunkel *et al.,* 2023) since no-till fields provide residue-rich, low disturbance environments for slugs. However, tillage can cause soil erosion and runoff to nearby streams (Kunkel *et al.,* 2023). Hand-picking is practiced in small-scale fields but may not be feasible on a large scale (Cheney, 1987). A few bio-rational control measures including the use of essential oils such as garlic oil and spearmint oil, plant extracts, and caffeine have been found to be effective (Barua *et al.,* 2021). In parallel, promoting the natural enemies to the fields is also best practiced by farmers (Barua *et al.,* 2021). They include ground beetles, mites, spiders, rats, frogs, lizards, centipedes, millipedes, and nematodes. However, owing to limited efficacy in killing different slug species, only a few among these organisms have drawn research attention (Barua *et al.,* 2021). Nematodes among them hold much popularity. Nematodes associate with slugs in a variety of ways, i.e., phoretic, necromenic, or parasitic. Animal-parasitic nematodes have almost invariably originated from ancestral forms that were initially free-living and sustained themselves by feeding on bacteria and other microorganisms (Blaxter and Koutsovoulos, 2015, Schurkman *et al.*, 2021). Both phoresies and necromancy represent evolutionary pathways through which free-living nematodes can transform into parasitic forms. (Schurkman *et al.*, 2021). Among these parasitic nematodes, entomopathogenic nematodes (EPN) and malacopathogenic nematodes (MPN) are well studied. The EPNs have been tested on their efficacy in controlling pest insects that damage crops, but their utility as biocontrol agents against gastropods is still in debate as EPNs have not been well-studied in that context (Schurkman *et al.*, 2021). There are a few studies (Schurkman *et al*., 2021) that examined the effects of nematodes from the genera *Heterorhabditis* and *Steinernema* on various gastropod species, including *D. reticulatum*. Wilson *et al*. (1994) demonstrated that the specific nematodes *Steinernema feltiae* and *Heterohabditis* sp. did not effectively kill the slugs. Therefore, Wilson *et al*. (1994) concluded that the tested nematode species do not have the potential to serve as biocontrol agents against *D. reticulatum*. Several nematode families, i.e., Alloionematidae, Cosmocercidae, Mermithidae, and Rhabditidae, associate with slugs (Puža *et al.,* 2016; Kunkel *et al.,* 2023). An MPN in the family Rhabditidae that has received the most attention is *Phasmarhabditis hermaphrodita* which was rediscovered in 1987 and recognized for its biological control potential (Rae *et al.,* 2007). So far only two nematode species, *P.*

hermaphrodita, and *Phasmarhabditis californica,* have been commercialized as biocontrol agents against slugs. More research needs to be conducted to check their viability and safety as biocontrol tools (Kunkel *et al.,* 2023).

1.1.3. Malacopathogenic nematodes in the genus Phasmarhabditis

Phasmarhabditis is a genus of gastropod-parasitizing nematodes (Wilson & Grewal, 2005). They are facultative parasites and are capable of surviving on alternate resources such as decomposing organic matter and invertebrate feces (Tan & Grewal, 2001). This genus consists of eighteen nominal species including *P. hermaphrodita,* and *P. californica* (Ivanova *et al*., 2022; Rae, 2023). *P. hermaphrodita* is lethal to slugs in the families Agriolimacidae, Arionidae, Limacidae, Milacidae, and Veronicellidae (Tan & Grewal, 2001; Rae *et al*., 2007; Howlett, 2012; Mc Donnell *et al.,* 2020). They have reduced slug damage in field crops such as winter wheat and high-value crops such as strawberries (Glen *et al.,* 2000) and brassicas in Europe (Ester *et al.,* 2003). However, owing to its recent discovery compared to *P. hermaphrodita*, the native range, and distribution, as well as the current host range of *P. californica* is not yet well understood (Mc Donnell *et al.,* 2020). So far, it has been collected in California in 2016 (De Ley *et al.,* 2016), New Zealand (Wilson *et al.*, 2016), Europe (Carnaghi *et al*., 2017), and Canada (Brophy *et al*. 2020a; Brophy *et al*. 2020b). *P. californica* has been isolated from *D. reticulatum*, *D. laeve*, *A. valentianus*, and *A. hortensis* agg. (De Ley *et al.,* 2016), and the lethality of *P. californica* on *D. reticulatum* has been demonstrated multiple times (Mc Donnell *et al.,* 2020; De Ley *et al.,* 2020; Schurkman *et al.,* 2022).

In general, the nematodes in the genus *Phasmarhabditis* are in symbiosis with a complex and variable assemblage of bacteria (*Moraxella osloensis* is used in commercial products) that assist in host mortality when inside slugs (Rae *et al.,* 2010). The third larval stage is infective to slugs (Wilson *et al.,* 1993; Tan and Grewal, 2001; Wilson & Rae, 2015; Cutler & Rae 2022; Kunkel *et al.,* 2023). The larvae enter the slug host via the dorsal integumental pouch, which is located immediately posterior to the mantle. Shortly after, they settle in the mantle cavity where they develop into self-fertilizing hermaphrodites (Wilson *et al.,* 1993; Tan & Grewal, 2001; Wilson & Rae, 2015; Cutler & Rae 2022; Kunkel *et al.,* 2023). The fluid accumulation due to bacterial infections makes a characteristic swelling in the mantle region of the host, and can also lead to shell ejection. Depending on the intensity of the infection and the temperature at which

they are proliferating, the host may die within 4-21 days (Wilson *et al.,* 1993; Tan & Grewal, 2001; Wilson & Rae, 2015; Cutler & Rae 2022; Kunkel *et al.,* 2023). The nematodes feed on the cadaver and once the resource is depleted, they produce infective juveniles and seek new hosts.

P. hermaphrodita and *P. californica* show promising mortality and feeding inhibition in many slug species upon infection (Glen *et al.,*2000; Stenberg *et al.,* 2021), and consequently have been developed into commercially available biocontrol products. *P. hermaphrodita* was developed as a water-dispersible, friable formulation of 12 million or 30 million nematodes that come in packs (Rae *et al.,* 2007). This product was launched under the trade name Nemaslug® and aimed at home gardeners by MicroBio Ltd, Littlehampton, UK (now BASF Agricultural Solutions,) in spring, 1994. This product is currently sold in 15 European countries including the UK, Ireland, France, the Netherlands, Belgium, Germany, Denmark, Norway, Finland, Poland, Spain, the Czech Republic, Italy, and Switzerland owing to their native inhabitancy in those regions (Rae *et al.,* 2007). *P. californica* ((strain P19D) was launched under the name Nemaslug®2.0 in 2022 by BASF Agriculture Solutions, UK (Stenberg *et al.,* 2021; Mc Donnell *et al*., 2023). The target species include common pest slugs in the order Stylommatophora, including *A. distinctus, A. vulgaris, A. hortensis, D. invadens,* and *D. reticulatum* (Stenberg *et al.*, 2021). *P. californica* was first discovered in horticultural areas in California, isolated from the cadavers of four invasive slug species, i.e., *D. reticulatum*, *D. laeve*, *A. valentianus,* and *A. hortensis* agg. *P. californica* closely resembles *P. hermaphrodita* by having approximately similar body lengths (De Ley *et al.,* 2016). However, they have a funnel-like short conical tail constricted at one-third of its length making it different from other species in the genus. Further, the presence of males of *P. californica* has not yet been reported in any of the studies, hence they are hermaphroditic.

1.2. Research objectives

Chapter 2 examines nine pestiferous slug species collected from a survey conducted in three agricultural sites and ten horticultural sites (greenhouses and nurseries) in Alberta. The main purpose of this study was to determine which pest slug species are at these sites in Alberta and to assess the slug-associated nematode species, especially *Phasmarhabditis* spp. This is a follow-up to a survey previously conducted by Brophy *et al*. (2020a, 2020b) in which a new Canadian strain of *P. californica* was first isolated from a single *Arion rufus* slug specimen collected from the exterior ground of a plant nursery in Edmonton, Alberta in 2019. In Canada,

the use of *P. hermaphrodita* as a biocontrol agent is not yet permitted as there are no previous records of this rhabditid nematode in the region. To date*, P. californica* is the only malacopathogenic nematode species found in Canada. However, since its discovery in Alberta, there have been no return surveys to confirm its presence in the province. Therefore, my second chapter is dedicated to investigating the slug-associated nematodes and confirming the presence of *P. californica* within the province.

Chapter 3 examines the host preference of *P. californica* (Family: Rhabditidae) and *Pristionchus entomophagus* (Family: Neodiplogasteridae) to the mucus of four slug species in chemotaxis assays. In contrast to *P. californica,* a malacopathogenic nematode (Mc Donnell *et al.,* 2020), *P. entomophagus* is a necromenic nematode that phoretically associates with beetles (Hong & Sommer, 2006). Having been isolated in association with slugs in a few other surveys and during my survey, I was interested in investigating the host preference of *P. entomophagus* to non-insect hosts, i.e., slugs. Further, this nematode is a close relative of *Pristionchus pacificus* which has previously been shown to have antagonistic effects on other nematodes such as *C. elegans.* Since they were found in a single model organism, *D. reticulatum,* these two nematodes (*P californica* and *P. entomophagus*) may be in direct competition over resources. Both species are microbivores and have remarkably similar lifecycles which further provided an incentive for this study.

Chapter 4 examines the effect of *P. entomophagus* on the infectivity of *D. reticulatum* by *P. californica.* In Chapter 3, these two species were found to have similar chemoattraction to a small number of slug species including *D. reticulatum,* the most pestiferous slug species in the world. Thus, I investigated the ability of *P. californica* to cause mortality in slugs in the presence of a second nematode, *P. entomophagus*. I recorded the mortality of slugs caused by *P. californica* both with and without *P. entomophagus*, counted the number of nematodes of *P. californica* entering the slugs to cause the infection, and finally, the progeny of *P. californica* to decide if *P. entomophagus* would have any impact.

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Chapter 2

Pestiferous slugs and their associated nematodes in agricultural fields, greenhouses, and nurseries in Alberta, Canada

2.1. Introduction

Certain introduced slug species are considered pests of agriculture, horticulture, and floriculture worldwide (Barker, 2002; Koslowski, 2012). Agricultural and horticultural sites, and residential gardens provide ideal conditions for slugs to thrive as they often provide moist, and shaded environments (Douglas & Tooker, 2012; Koslowski, 2012). When slug density is high, large-scale no-tillage crops such as wheat, barley, oats, rye, corn, canola, tobacco, soybean, alfalfa, and leguminous forages often suffer extensive damage (South, 1992; Douglas & Tooker, 2012). The damage is not limited to large-scale arable crops but may also include many other crops including strawberries, cabbages, leeks, potatoes, and carrots (Thomas, 2010). In severe cases, complete germination failure is possible due to grain hollowing even before the seeds germinate. Further, herbivory on the seedlings of arable plants can lead to a reduced crop stand and reduced yield. Pestiferous slug species are often inadvertently introduced to new areas through poor quarantine practices during the transport of agricultural and horticultural commodities, and human travel (Robinson, 1999; Cowie *et at.,* 2009, Howlett, 2012; Darrigran *et al.,* 2020; Schurkman *et al.,* 2022b). Rapid adaptability and spread in new settings in the absence of pressure from natural predators and competitors have led to some pest slug species establishing in many new parts of the world from the tropics to temperate regions (Howlett, 2012). Pestiferous slug genera in temperate climates include *Deroceras*, *Milax*, *Arion, and Limax*. For example, *Arion vulgaris* (Moquin-Tandon, 1885) and *D. reticulatum* are among the most pestiferous slug species in Europe (Howlett, 2012). Certain European slug species have also been introduced to temperate regions of New Zealand, Australia, and South Africa (Howlett, 2012).

The dominant method for controlling slugs worldwide is the application of agrochemicals e.g., methiocarb, metaldehyde, and iron phosphate (South, 1992). However, growing public opposition towards pesticides and appreciation of eco-friendly agricultural practices favor the use of biopesticides and biocontrol agents in controlling slug populations. These can provide higher specificity and sometimes are more efficient pest management tools than other practices

(Jaffuel *et al.,* 2019; Mc Donnell *et al.,* 2020). Nematodes, some of which are parasitic, offer a potential solution due to their natural associations with terrestrial gastropods (Wilson & Grewal, 2005). Previous surveys conducted in Germany, France, Slovenia, Bulgaria, the USA, Australia, Africa, and the UK found nematodes associated with slugs belonging to seven families, namely Agfidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogasteridae, Mermithidae and Rhabditidae (Ross *et al.,* 2016). The most well-known is a facultative parasite of slugs, *Phasmarhabditis* (Family Rhabditidae), a genus of bacterial-feeding soil-dwelling nematodes (Wilson & Grewal, 2005). *Phasmarhabditis* currently contains eighteen nominal species (Ivanova *et al*., 2022; Rae, 2023), four of which, *P. hermaphrodita*; *P. papillosa*; *P. neopapillosa*; and *P. californica* have been shown to be pathogenic to slugs (Wilson & Grewal, 2005; Holley, 2020; Ivanova & Spiridonov, 2021; Schurkman *et al.,* 2022a). Among them, *P. hermaphrodita* has been widely used as a biocontrol agent after it was commercialized in 1994 (Rae *et al*., 2007). The product was launched under the trade name Nemaslug® and is currently only commercially available in 15 European countries owing to the natural occurrence of *P. hermaphrodita* in those regions (Rae *et al.,* 2007; Laznik *et al*., 2020; Mc Donnell *et al.,* 2020). This nematode is lethal to many slug species (in the families Agriolimacidae, Arionidae, Milacidae, Limacidae, and Veronicellidae), particularly to *D. reticulatum* (Rae *et al*., 2007; Howlett, 2012). Since its commercialization, this nematode has been discovered in geographic areas other than Europe, such as Iran, Egypt, New Zealand, the USA, Norway, and China (Rae *et al.,* 2007; Howlett, 2012; De Ley *et al*., 2016). Similarly, *P. californica* was recently discovered in California and Oregon (De Ley *et al.,* 2016; Howe *et al*., 2020) and has recently been commercialized as Nemaslug 2.0 in the U.K. market (Stenberg *et al.,* 2021; Mc Donnell *et al*., 2023).

Canada had no previous records of *Phasmarhabditis* until a recent discovery of *P. californica,* which was reported from a single slug (*Arion rufus*) collected from the exterior grounds of a local nursery in Edmonton (Brophy *et al.,* 2020a; Brophy *et al.,* 2020b). However, isolating a Canadian strain of *P. californica* from a single slug specimen is not sufficient evidence to confirm that the nematode is established locally. Thus, the goal of my study was to complete a more comprehensive survey in agricultural and horticultural (commercial nurseries, and greenhouses) sites in Alberta, Canada for pest slug species and their associated nematodes. These results provide valuable information to agricultural practitioners in the region about pest

slug species that are of agricultural and horticultural importance (residential gardens were not considered economically important sites in this survey). My second objective was to confirm if the novel Canadian *P. californica* strain naturally occurs in agricultural and horticultural sites in Alberta, Canada.

2.2. Materials and method

2.2.1. Slug collection

Slugs were collected from field margins and adjacent vegetation stripes of three agricultural sites (crops include wheat, canola, and peas) in Alberta (Fig. 2.1) from August to September 2021, a period corresponding to peak slug activity (Brophy *et al.* 2020b). The chosen timeframe for slug collection was also influenced by the rainfall, which promoted the emergence of slugs. A total of ten commercial greenhouses and nurseries (crops include herbs, annuals, houseplants, perennials, trees, and shrubs) in Alberta were surveyed from June to September 2021 (Fig. 2.1). Surveys were conducted in potential slug microhabitats such as on the soil surface, leaf litter, crop foliage, near the roots, under nursery pots, rocks, and logs, and in the marginal vegetation; on certain occasions when the surface soil was dry, the slugs were collected 1-5 cm deep in the soil by digging. Each site was searched for a maximum of 30 minutes/person (2-4 people) and the slugs were handpicked and transferred into plastic containers with a perforated lid and lined with damp paper towels to prevent desiccation. Every site was visited at least twice during the survey period. Slugs collected from different sampling sites were put into separate containers to avoid any cross-contamination. Each slug species was photographed and identified using available taxonomic keys (Perez *et al.,* 2008; Grimm *et al.,* 2009; Mc Donnell *et al.*, 2009; http://idtools.org/id/mollusc/index.php). Molecular diagnosis for slugs was performed when I could not reliably make an identification using morphological traits, or if the samples contained immature slugs that were difficult to identify solely on external morphologies.

2.2.2. Slugs from residential gardens

In addition to the field-collected slugs, I occasionally received slugs from residential gardens in Alberta. Most of these samples were sterilized and used for slug colonies, except for 74 slugs (*D. reticulatum*) which were haphazardly chosen and placed on white traps to check for nematodes.

2.2.3. Nematode isolation

All slugs, except 55 (reserved for laboratory slug colonies) were rinsed thoroughly with distilled water to remove any external phoretic nematodes. The slugs were then decapitated to encourage the emergence of associated nematodes. The slug cadavers were kept in individual Petri dishes (6 cm diameter) lined with a damp paper towel, covered, and sealed with Parafilm™. The cadavers were incubated at 18°C, 80% RH, 12 h light; 12 h dark, 12°C, 60% RH for two weeks. After this time, cadavers were inspected under a stereomicroscope. Nematodes were collected into 95% ethanol or DESS (dimethyl sulphoxide, disodium EDTA, and saturated NaCl) for subsequent molecular identification (Yoder *et al.*, 2006; Brophy *et al.,* 2020b).

2.2.4. Molecular identification

2.2.4.1. Slugs

A piece of the tail tip was clipped from select slug specimens (n=24) and stored in 95% ethanol for molecular identification. QIAGEN DNeasy Blood and Tissue Kit (Germantown, MD, USA) was used to extract slug DNA from a ~2 mm × 2 mm piece of slug tissue. PCR cycling conditions followed Reich *et al.* (2015). Partial fragments of the mitochondrial cytochrome c oxidase subunit I (cox 1) gene were sequenced: Cox 1 primer set LCO-1490 (5′-GGTCA ACAAATCATA AAGATATTGG-3′) and HCO-2198 (5′-TAA ACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al.,* 1994). Geneious Prime® software (North America Biomatters Inc, San Diego, CA) was used to trim and edit the resulting sequences. A BLAST (Basic Local Alignment Search Tool) search was then performed on GenBank (Benson *et al*., 2013) to identify the species. In all cases, query coverage was >99.5%, the E value was 0, and the highest percentage identity was >99.0%. Sequences were deposited in GenBank under accession numbers OQ642082-OQ642105.

2.2.4.2.Nematodes

Nematodes were transferred from either 95% ethanol or DESS to a proteinase-K-based lysis buffer for DNA extraction (Williams *et al*., 1992). Primer sets 18A (5′-AAAGATTAAGCCATGCATG-3′) and 26R (5′-CATT CTTGGCAAATGCTTTCG-3′) were used in PCR amplification and direct-end sequencing of the 18S ribosomal DNA, as previously described (Blaxter *et al*., 1998; Denver *et al.*, 2003). The resulting 18S rRNA sequences were compared against GenBank's non-redundant (nr) database using blastn (NCBI Resource Coordinators). DNA sequences generated for this study were submitted to GenBank under accession numbers OQ645705 - OQ645739 (Table 2.1).

2.3. Results

A total of 1331 slugs were collected from select agricultural and horticultural sites in Alberta from June to September 2021. These comprised nine species: *Arion fasciatus*, *A. hortensis, A. rufus, A. subfuscus, Ambigolimax valentianus, Deroceras invadens, D. laeve, D. reticulatum*, and *Prophysaon andersonii.* Of the 551 slugs collected from the three agricultural sites, I identified four species: *A. fasciatus, D. invadens, D. laeve,* and *D. reticulatum* (the most abundant, Table 2.2). I collected a total of 780 slugs from ten greenhouses and nurseries, representing all nine slug species. The most prevalent slug species encountered in these sites were *D. laeve, A. valentianus, and D. reticulatum* (Table 2.2). Slug abundance rose gradually over the summer, peaking in August (Fig. 2.2).

Out of 1331 slugs collected during the survey, only 45 (3.38%) harbored nematodes (Table 2.3) but *Phasmarhabditis* was not isolated. The percentage of nematode-positive samples was high in June followed by a gradual decline until August but rose again in September (Fig 2.2). More nematode-positive samples were reported from nurseries and greenhouses (4.62%) than from agricultural (1.63%) sites (Table 2.3). Nematodes were isolated from four slug species: *A. rufus*, *A. valentianus*, *D. laeve, and D. reticulatum*, all of which were collected from two agricultural sites and four greenhouses/nurseries (Table 2.3). I identified 21 samples of nematodes to species level: *Alloionema appendiculatum, Caenorhabditis briggsae, Caenorhabditis elegans, Panagrolaimus subelongatus,* and *Mesorhabditis spiculigera.* Out of all the nematode species, *C. elegans* and *P. subelongatus* were common to two agricultural sites and greenhouses/nurseries; the most prevalent nematode species was *C. elegans* (35.56%). By comparison, *A. appendiculatum, M. spiculigera,* and *C. briggsae* were only found in greenhouses and nurseries. *Alloionema appendiculatum* was isolated from a single *A. rufus* slug cadaver, while *C. briggsae* and *M. spiculigera* were isolated from an *A. valentianus* and a *D. laeve* slug cadaver respectively (Table 2.3).

Out of 74 slug cadavers, 22 samples (29.7%) from residential gardens were associated with nematodes. Of all positives, four samples were *P. californica* (18.18%). The other nematode species identified include *C. elegans, Choriorhabditis cristata, Pristionchus entomophagus,* and *P. subelongatus.* Other (n=6) nematodes were only identified to the genus level: *Panagrolaimus* sp. and *Rhabditophanes* sp. (Table 2.3). The most common nematode species for both residential

gardens and the survey sites were *C. elegans* and *P. subelongatus,* while *C. cristata, P. californica, P. entomophagus,* and *Rhabditophanes* sp. were only found in residential gardens.

2.4. Discussion

This study aimed to conduct a more extensive survey of agricultural and horticultural areas following Brophy (2020a; 2020b), including nurseries where the nematode *P. californica* was previously isolated from a single slug. In total, 1331 slugs were collected with peak slug collection in August, similar to what was reported by Brophy *et al.* (2020b). The slugs belonged to nine species from four families: Arionidae, Limacidae, Agriolimacidae, and Anadenidae. All slug species except *P. andersonii* (native) and *D. laeve* (native), are of European origin and were likely introduced to Canada via various means, e.g., trade and commerce of agricultural and horticultural commodities and/or travel (Robinson, 1999; Grimm *et al*., 2009; Araiza-Gómez *et al.,* 2017). Collectively, *D. reticulatum* was the most abundant slug species recorded during the survey (49.9%), the majority coming from agriculture sites (72.9%). The most common slug species found in horticultural sites were *D. laeve* (41.5%), *A. valentianus* (26.9%), and *D. reticulatum* (23.1%) respectively. Brophy *et al*. (2020a; 2020b) reported 2406 slugs belonging to nine slug species collected from 82 sites including natural green spaces (e.g., parks and ravines), seasonal nurseries, and greenhouses, with the majority coming from residential gardens in and around Edmonton, Alberta. In comparison, I only collected about half the number of slugs during the current survey likely due to lower slug emergence under elevated temperatures and unseasonably dry conditions during the survey period in 2021 compared to the year 2019 [e.g. 2019 July Olds college AGDM: maximum air temp ranged from 27.8°C- 10.6°C, soil moisture at 005cm depth 39.4%-21%; 2021 July Olds college AGDM: maximum air temp ranged from 35.6°C- 17.6°C, soil moisture at 005cm depth 19.1%-16.1%., (ACIS, accessed February 2023). Further, unlike the previous survey by Brophy *et al*. (2020a; 2020b), this survey did not focus on slugs from residential gardens. I did not collect *Limax maximus*, despite its recovery during the previous survey (n=2) by Brophy (2020a; 2020b). The failure to recover any would most likely be due to the extremely low population size of this slug species in and around the nursery where they were initially found.

Retail nurseries play a significant role as a focal point of terrestrial slug dispersal as they facilitate the passive transportation of exotic and native slugs with plant material to and from the nurseries (Bergey *et al.,* 2014; Schurkman *et al.,* 2022b). This could presumably cause the slugs

to be transported over great distances, even provincial/ inter-state movements, with the primary destination being home gardens. Of the slug donations received from residential gardens, I identified two slug species, *D. reticulatum,* and *A. fasciatus*. The latter was only received from a single residential garden and is the first report of *A. fasciatus* in a residential garden in Edmonton, Alberta. The dispersal of this slug species could have been a result of the horticulture trade (we collected *A. fasciatus* in greenhouses and nurseries in this study), with slugs being transported as adults, juveniles, and/or eggs residing in plant material and associated soil. Pest slug species including *A. fasciatus* and *D. reticulatum* are most likely to thrive in the absence of their natural enemies and are hard to eradicate once established (Robinson & Hollingsworth, 2005; Kozlowski, 2012). Their presence often results in persistent plant damage in home gardens and significant economic losses to large-scale crop farmers in the form of yield losses, expenditure on labor, chemicals, and time required for pest management. Further, these pest gastropods are ecologically important as they replace the detritivorous gastropod species which mostly are non-pests and native to the region, resulting in habitat alteration and reduced biodiversity (Kozlowski, 2012). Therefore, I recommend regular monitoring and proper quarantine practices be in place, especially in retail nurseries.

In my study, *D. reticulatum* was the predominant slug species collected in agricultural sites. Besides *D. reticulatum,* only three others, *A. fasciatus, D. invadens,* and *D. laeve* were recovered but in smaller numbers. Douglas & Tooker (2012) listed *D. reticulatum, D. laeve, A. subfuscus,* and *A. fasciatus* as the most common slug species to occur in field crops in the mid-Atlantic United States, and that most of those species were not commonly associated with damage except *D. reticulatum*. In the Pacific Northwest region of the United States, *D. reticulatum* is thought to cause an estimated $60 million annual loss to the grass seed industry (Mc Donnell *et al.,* 2020). During the current study, *D. laeve* was reported primarily from wheat field margins in Innisfail, and around 5cm deep in the soil in one of the post-harvest fields in St. Albert. *A. fasciatus* and *D. invadens* were recovered mainly from wheat field margins in Innisfail and canola field margins in Olds, respectively. They were mostly associated with the marginal vegetation with no visible direct damage to the cash crops. However, damage (active feeding resulting in irregular holes and tears in foliage) was obvious with *D. reticulatum* in wheat fields in Innisfail. Marginal vegetation strips appear to provide ideal conditions for slugs, where these animals retain well-moist and are in close proximity to crop fields. This observation aligns with

those by Frank (1998), who found that *D. reticulatum* occurred in large numbers in both grass strips and adjacent rape fields in Belp, Switzerland. Further, it was reported that the marginal rape plants were more vulnerable to slug damage (defoliation), especially by *D. reticulatum.* A global survey recently revealed that slugs, and *Deroceras* species, in particular, are serious pests of rapeseed/canola (*Brassica napus*) production (Zheng *et al.,* 2020). Canada is one of the major canola producers in the world, yet little is known about the extent of slug damage. This highlights the need for more systematic surveys to assess the abundance and distribution of pest gastropods and their economic impact on crops across the country. Slug damage is often identified by the presence of slime trails on crop plants or the soil, however, certain symptoms could be similar to those of other organisms such as wireworms (e.g., tunneling through tubers such as potatoes) (Keiser *et al.,* 2012), and black cutworms (e.g., cut the seedling stages of most cruciferous plants) (Chandel *et al.,* 2022). This might result in an overestimation or underestimation of crop damage by pest slugs. Thus, I also recommend robust quantitative assessments of damage caused by slugs to a variety of crops grown under different conditions (e.g., till versus no-till) using different management strategies.

Nematodes were recovered from four slug species (*A. rufus, A. valentianus, D. reticulatum,* and *D. laeve*) collected from agricultural sites, greenhouses, and nurseries. I isolated nematodes belonging to four families: Alloionematidae, Rhabditidae, Panagrolaimidae, and Neodiplogasteridae. Twenty-one samples of nematodes were identified to five species; the rest remained unidentified due to mix-population of nematodes. Nurseries that have a more frequent exchange of plant material are more likely to support a higher diversity of slug-associated nematodes than agriculture fields with a single crop or a few crops cultivated with a less frequent exchange of plants. Additionally, in Alberta, field crops which are mainly cultivated from seeds have a lower likelihood of slug and nematode spread compared to vegetable or other horticulture production, which frequently involves the use of transplants along with the soil. This was evident in my study by the higher prevalence of slug-associated nematodes in greenhouses and nurseries (4.62%) compared to the agricultural fields (1.63%). In greenhouses and nurseries, the nematodes were isolated from *A. rufus, A. valentianus,* and *D. laeve* (table 2.3). Of the slugs collected, *A. rufus* only harbored *Alloionema appendiculatum*, a known parasite of *Arion* spp., including *Arion vulgaris,* a serious pest in central Europe (Nermut *et al.,* 2019). Despite *A. appendiculatum* causing high snail mortality in heliculture (Nermut *et al.,* 2019), research has

shown that this nematode had no success causing mortality in *A. vulgaris* under laboratory conditions, suggesting that this nematode has limited potential as a biocontrol agent against *A. vulgaris* (Nermut *et al.,* 2019). Surprisingly, *D. reticulatum*, the third most abundant slug species in greenhouses and nurseries, had no associated nematodes. However, among the agriculture sites, *D. reticulatum* was the only slug species to harbor nematodes (1.86%). I did not recover *P. californica* from any of the slugs in the agricultural or horticultural sites I surveyed. However, *P. californica* (accession number KM510210) was isolated from half of the *D. reticulatum* slug cadavers (4/8) from one of the residential garden samples in Lethbridge, Alberta. This nematode may have a patchy distribution in the province, and as such more extensive surveys are needed to determine its true range. Previously, *P. californica* has been isolated from greenhouses and nurseries in different parts of North America (Schurkman *et.al*., 2022b), including Alberta, Canada (Brophy *et al*., 2020a; Brophy *et al*., 2020b), but never from residential gardens. Therefore, this is the first report of *P. californica* being isolated from slugs in a residential garden in North America.

My results along with Brophy *et al.* (2020a; 2020b) demonstrate that the Canadian strain of *P. californica* can utilize both *D. reticulatum* and *A. rufus* as their hosts. I further suggest the possibility of the presence/abundance of *P. californica* in 2021 compared to 2019 be the impact of the climatic variables (unseasonably dry conditions in 2021) and/or nursery substrate origins. Some *Phasmarhabditis* species, including *P. hermaphrodita, P. californica,* and *P. papillosa* cause mortality in *D. reticulatum* (Mc Donnell *et al*., 2020). Further, *P. californica* has a cosmopolitan distribution and has been isolated in Wales (Andrus & Rae, 2019a; Andrus & Rae, 2019b), Ireland (Carnaghi *et al*., 2017), the USA (De Ley *et al*., 2016; Mc Donnell *et al*., 2020), New Zealand (Wilson *et al.,* 2016) and Canada (Brophy *et al*. 2020a; Brophy *et al*. 2020b). Given that *D. reticulatum* also has a global distribution and has been reported as a pest in Europe, North America, Australia, and New Zealand (Howlett, 2012), there could be an opportunity to utilize *P. californica* as an alternative biocontrol agent against *D. reticulatum* in the absence of *P. hermaphrodita*. In fact, this species was commercialized (Nemaslug 2.0) for the UK market in 2022 (Stenberg *et al.,* 2021; Mc Donnell *et al.*, 2023). However, different strains of *P. californica* show striking differences in host preference to slug cues (Cutler & Rae, 2021). Therefore, I recommend further research on the host preference, infectivity, and pathogenicity of

the Canadian strain of *P. californica* to determine its efficacy as a potential biocontrol agent of pest slugs.

Other than *P. californica, P. entomophagus* was also isolated from residential slug samples during the current study. *Pristionchus* species show a species-specific necromenic association with beetles (Brown *et al.,* 2011). However, they can hitchhike on other insects to reach their final hosts. Interestingly, I observed a non-insect host interaction of *Pristionchus* sp. during this study which we found in association with *D. reticulatum* (n=4). This aligns with the observations by Brophy *et al.* (2020b) who also found this taxon associated with *D. reticulatum.* This nematode is capable of producing dauer larvae under low food availability and infesting hosts (Brown *et al.,* 2011). However, these necromenic nematodes cause no mortality to the host but wait until it dies to feed on the bacteria and resume development (Sommer & McGaughran, 2013; Ishaq *et al.,* 2021). I suggest that the dauers I observed on slugs had a phoretic association, and when grown on *D. reticulatum* slug cadavers under laboratory conditions, *P. entomophagus* successfully propagated suggesting that dead slugs are also a suitable growth substrate for this nematode taxon.

The introduction of a 'potentially exotic' nematode species as a biocontrol agent is restricted in many countries by legislation, and as such the importation and release regulations of those organisms are limited, often with the exception of those proven to be indigenous (Ehlers, 2005; Wilson *et al.,* 2016). Therefore, it is important to extend future survey efforts into the rest of Alberta, especially in and around the Lethbridge region. This is because we discovered *P. californica* in slug samples from residential gardens in Lethbridge, but we did not conduct a survey at the horticulture centers where the plants were originally purchased. It may be also worthwhile to investigate other provinces in Canada to confirm the existence and distribution of *Phasmarhabditis* species in gastropods. Further, there should be a thorough investigation of the infectivity and pathogenicity of this nematode on potential slug hosts to determine if this strain could be used as a biocontrol agent in Canada. Keyte *et al.,* (2022) reported a higher prevalence of *P. californica* and *P. hermaphrodita* in snail hosts than in slugs. *P. californica* has been isolated from snail species such as *Theba pisana* (Tandingan De Ley *et al*., 2020) *Discus rotundatus* (Keyte *et al.* 2022), and *Oxychilus draparnaudi* in Germany and UK (Grannell *et al.,* 2021; Keyte *et al.,* 2022). Therefore, future studies on nematodes associated with both terrestrial snails and slugs may reveal a wider distribution of *Phasmarhabditis* spp. in Canada. Relatively

few studies have been conducted to determine the distribution and environmental impact of the introduced pest gastropods in Canada. There are even fewer studies on pest slugs in agriculture and horticulture habitats, which are of great economic importance. Here I report seven introduced and two native slug species in Alberta which are of agricultural and horticultural significance and the nematodes associated with those slugs. However, the economic damage and ecological impact of those slug species are poorly understood. *P. californica* has the potential to be used as a biological control agent in Canada due to its ability to cause mortality in pest slugs. Further studies including impacts on native non-target species are necessary to better understand the biological and ecological aspects of this nematode before an informed decision can be made on the use of this nematode as a slug control tool.

2.5. Acknowledgement

Weather data provided by Agriculture and Irrigation, Alberta Climate Information Service (ACIS) https://acis.alberta.ca (February 2023).

2.6. Conflicts of interest

RMcD declares that he is a co-inventor on a patent application entitled Mollusk-killing Biopesticide (U.S. application Serial No. 62/236,674).

2.7. Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. 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.

Table 2.1: GenBank accession numbers for the slug-associated nematodes from various collection localities in Alberta and dates across 2021 (Location types: agricultural sites, Agri; horticultural sites, GH/N; residential garden, Residential)

No	Seq Hit	Host	Location type	Accession number
1	*Alloionema appendiculatum*	*Arion rufus*	GH/N	OQ645707
2	*Caenorhabditis briggsae*	*Ambigolimax valentianus*	GH/N	OQ645729
3	*Caenorhabditis elegans*	*Deroceras reticulatum*	Agri	OQ645708
4	*Caenorhabditis elegans*	*Deroceras reticulatum*	Residential	OQ645709
5	*Caenorhabditis elegans*	*Deroceras laeve*	GH/N	OQ645710
6	*Caenorhabditis elegans*	*Ambigolimax valentianus*	GH/N	OQ645715
7	*Caenorhabditis elegans*	*Ambigolimax valentianus*	GH/N	OQ645716
8	*Caenorhabditis elegans*	*Ambigolimax valentianus*	GH/N	OQ645717
9	*Caenorhabditis elegans*	*Deroceras laeve*	GH/N	OQ645718
10	*Caenorhabditis elegans*	*Deroceras laeve*	GH/N	OQ645719
11	*Caenorhabditis elegans*	*Ambigolimax valentianus*	GH/N	OQ645722
12	*Caenorhabditis elegans*	*Ambigolimax valentianus*	GH/N	OQ645724
13	*Caenorhabditis elegans*	*Ambigolimax valentianus*	GH/N	OQ645725
14	*Caenorhabditis elegans*	*Ambigolimax valentianus*	GH/N	OQ645727
15	*Caenorhabditis elegans*	*Ambigolimax valentianus*	GH/N	OQ645730

16	*Caenorhabditis elegans*	*Ambigolimax valentianus*	GH/N	OQ645731
17	*Caenorhabditis elegans*	*Ambigolimax valentianus*	GH/N	OQ645732
18	*Caenorhabditis elegans*	*Ambigolimax valentianus*	GH/N	OQ645735
19	*Caenorhabditis elegans*	*Ambigolimax valentianus*	GH/N	OQ645738
20	*Choriorhabditis cristata*	*Deroceras reticulatum*	Residential	OQ645705
21	*Mesorhabditis spiculigera*	*Deroceras laeve*	GH/N	OQ645737
22	*Panagrolaimus sp.*	*Deroceras laeve*	GH/N	OQ645711
23	*Panagrolaimus subelongatus*	*Deroceras reticulatum*	Agri	OQ645706
24	*Panagrolaimus subelongatus*	*Deroceras reticulatum*	Residential	OQ645713
25	*Panagrolaimus subelongatus*	*Deroceras reticulatum*	Residential	OQ645721
26	*Panagrolaimus subelongatus*	*Deroceras laeve*	GH/N	OQ645728
27	*Phasmarhabditis californica*	*Deroceras reticulatum*	Residential	OQ645733
28	*Phasmarhabditis californica*	*Deroceras reticulatum*	Residential	OQ645734
29	*Phasmarhabditis californica*	*Deroceras reticulatum*	Residential	OQ645736
30	*Phasmarhabditis californica*	*Deroceras reticulatum*	Residential	OQ645739
31	*Pristionchus entomophagus*	*Deroceras reticulatum*	Residential	OQ645714

32	*Pristionchus entomophagus*	*Deroceras reticulatum*	Residential	OQ645720
33	*Pristionchus entomophagus*	*Deroceras reticulatum*	Residential	OQ645723
34	*Pristionchus entomophagus*	*Deroceras reticulatum*	Residential	OQ645726
35	*Rhabditophanes sp. 1*	*Deroceras reticulatum*	Residential	OQ645712

Table 2.2: Abundance of the slug species collected in 2021 from Alberta agriculture sites and adjacent vegetation (AG), greenhouses, and nurseries (GH/N)

Slug species	AG	GH/N	Total
Family: Arionidae			
Arion fasciatus (Nilsson, 1823)	41	2	43
Arion hortensis (Ferrusac, 1819)		15	15
Arion rufus (Linnaeus, 1758)		4	4
Arion subfuscus (Draparnaud, 1805)		13	13
Family: Limacidae			
Ambigolimax valentianus (Ferussac, 1821)		210	210
Family: Agriolimacidae			
Deroceras invadens (Reise *et al*., 2011)	2	21	23
Deroceras laeve (Müller, 1774)	24	324	348
Deroceras reticulatum (Müller,1774)	484	180	664
Family: Anadenidae			
Prophysaon andersonii (Cooper, 1872)		11	11
Total	**551**	**780**	**1331**

[The following number of slugs were used to initiate laboratory slug colonies: *Arion fasciatus* (34)*, A. hortensis* (4)*, A. rufus* (3)*, A. subfuscus* (3)*, Ambigolimax valentianus* (3), *Deroceras laeve* (4) and *Prophysaon andersonii* (4)].

Table 2.3: Slug-associated nematode species and their prevalence in agricultural sites (AG), horticultural sites (N/GH), and residential gardens (RE). *A. rufus* (AR); *A. valentianus* (AV); *D. reticulatum* (DR) and *D. laeve* (DL)

Nematode	AG	N/GH	RE	Total	Prevalence of nematodes	
					AG/N/GH	RE
Family: Alloionematidae						
Alloionema appendiculatum		1(AR)		1	2.22%	
Rhabditophanes sp.			1(DR)	1		4.55%
Family: Rhabditidae						
Caenorhabditis briggsae		1(AV)		1	2.22%	
Caenorhabditis elegans	1(DR)	12(AV), 3(DL)	1(DR)	17	35.56%	4.55%
Choriorhabditis cristata			1(DR)	1		4.55%
Mesorhabditis spiculigera		1(DL)		1	2.22%	
Phasmarhabditis californica			4(DR)	4		18.18%
Family: Panagrolaimidae						
Panagrolaimus sp.			1(DR)	1		4.55%
Panagrolaimus subelongatus	1(DR)	1(DL)	2(DR)	4	4.44%	9.09%
Family: Neodiplogasteridae						
Pristionchus entomophagus			4(DR)	4		18.18%
A mix of nematodes/ Unidentified	7(DR)	17(AV, DL)	8(DR)	32	53.33%	36.36%
Total	9	36	22	**67**		

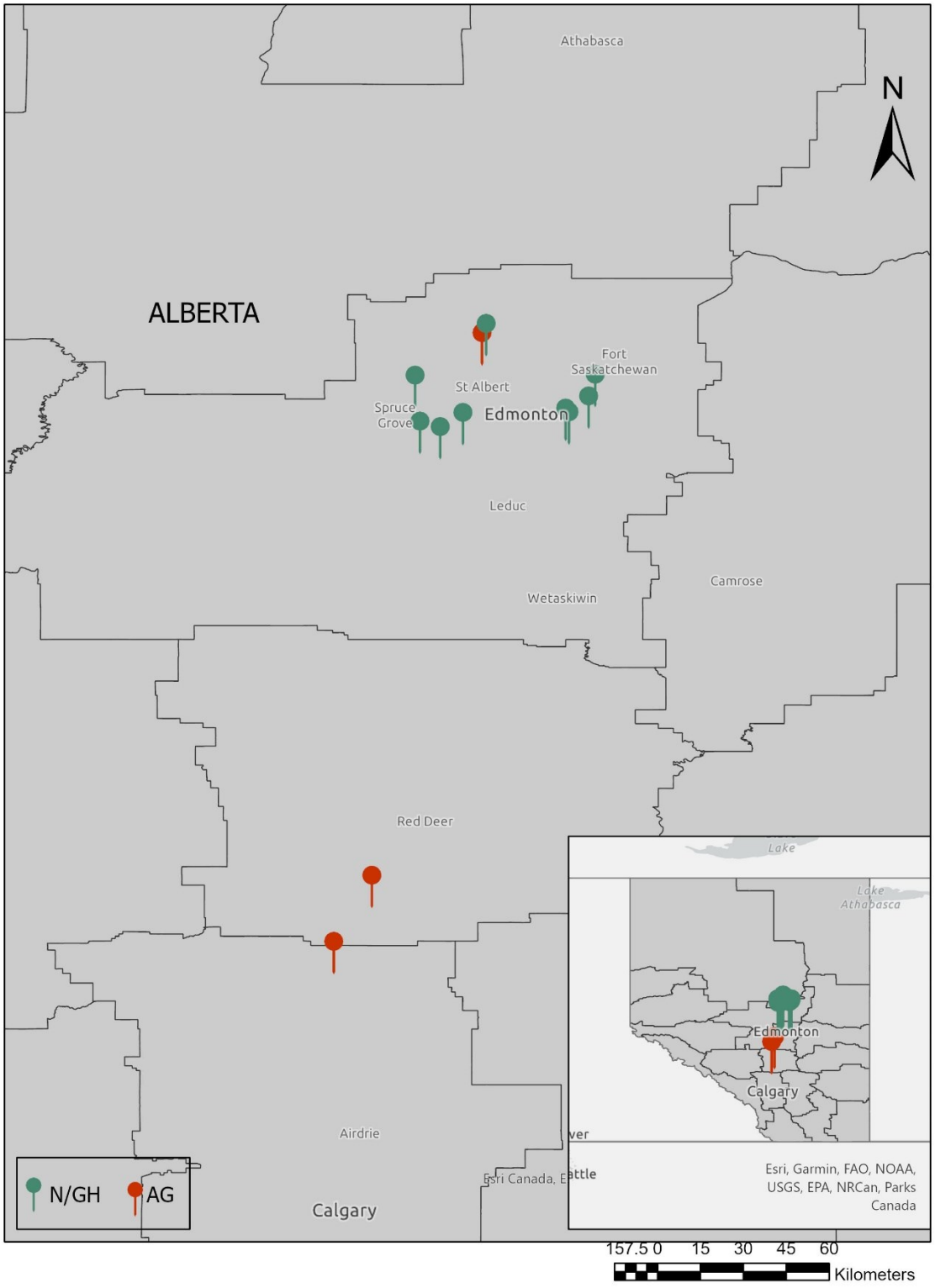

Fig. 2.1: Slug survey locations (2021). Slugs were collected from ten horticultural sites: i.e., nurseries and greenhouses (N/GH; green pins) and three agricultural sites (AG; red pins)

in Alberta (main map). ArcGIS Pro software was used for visualization. (Esri, USGS | Sources: NRCan, Esri Canada, and Canadian Community Maps contributors. | Esri Canada | Northwest Territories, State of Alaska, Esri Canada, Esri, HERE, Garmin, FAO, NOAA, USGS, EPA, NPS, NRCan, Parks Canada

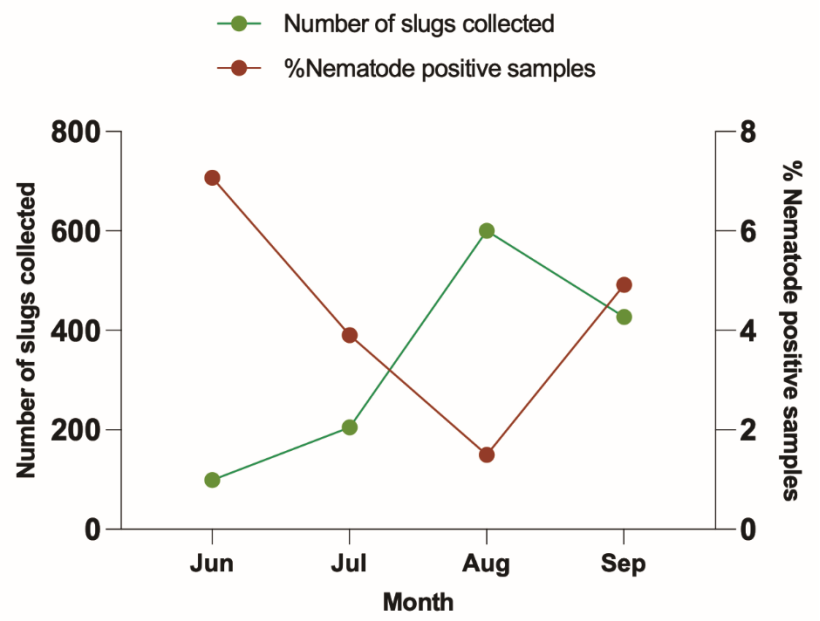

Fig. 2.2: Temporal pattern of the total number of slugs collected from agriculture sites, nurseries/ greenhouses (green line), and nematodes isolated (%) from slug cadavers (brown line).

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Chapter 3

Chemotaxis response of *Phasmarhabditis californica* (Family: Rhabditidae) and *Pristionchus entomophagus* (Family: Neodiplogasteridae) to pest slugs

3.1.Introduction

Soil nematodes are among the most diverse groups of soil-dwelling organisms (Rasmann *et al*., 2012). This group contains but is not limited to free-living bacteriophagous nematodes (e.g., the genera *Rhabditis*, Oscheius*, Plectus*, *Cephalobus*, *Caenorhabditis*), plant-parasitic nematodes (e.g., *Meloidogyne* and *Globodera* spp.), and animal-parasitic nematodes such as entomopathogenic and malacopathogenic nematodes (Wilson & Grewal, 2005; Rasmann *et al*., 2012). Both free-living (scavengers, microbivores, and predators) and parasitic nematodes use specific recognition mechanisms to orientate to their food source, host, or prey (Zuckerman & Jansson, 1984). Both experimental evidence and morphological configurations in the cephalic region provide consensus that these nematodes, especially species that are parasitic, use chemical or physical stimuli originating from their potential hosts to locate them (Zuckerman & Jansson, 1984; Laznik *et al*., 2022). These chemotaxis behaviors can be species-specific and influence the nematode's motility, direction, and ability to find hosts (Wang *et al*., 2019). Sensory modalities involved in the host-seeking behaviour of parasitic nematodes include olfaction, gustation, thermo-sensation, vibration, and humidity sensation (Dillman *et al*., 2012, Santhi *et al*., 2021). The chemotaxis responses by soil nematodes have been extensively studied in certain groups such as bacteriophagous nematodes (e.g., *Caenorhabditis elegans*), some plant-parasitic nematodes (e.g., *Meloidogyne* and *Globodera* spp.) (Rasmann *et al.,* 2012), entomopathogenic nematodes (e.g., *Heterorhabditis bacteriophora*) (O'halloran & Burnell, 2003) and a few malacopathogenic nematodes, including *Phasmarhabditis* spp. (Rae *et al.,* 2009; Cutler & Rae, 2021) to better understand ecological interactions between hosts and parasites.

P. californica is a facultative parasite of gastropods with biocontrol potential (Nermut *et al.,* 2022). *P. californica* is one of eighteen nominal species of the genus *Phasmarhabditis* (Wilson & Grewal, 2005; De Ley *et al.,* 2016; Howe *et al*., 2020; Ivanova *et al*., 2022; Rae, 2023). Host-seeking behaviors are typically exhibited by the infective juvenile (IJ) stage, a developmentally arrested third larval stage analogous to the dauer larvae of some free-living nematodes (Wilson & Grewal, 2005; Dillman *et al*., 2012). The chemotaxis behaviour of

infective juveniles of a few *Phasmarhabditis* spp. has been tested with different cues (live slug samples, foot mucus, mantle mucus, faeces, and volatile cues) associated with the slug, *D. reticulatum* (Rae *et al*., 2006). The nematodes showed a strong attraction towards live slugs and mucus from the foot and mantle of *D*. *reticulatum*. Andrus and Rae (2019) tested variation in chemoattraction of two strains of *P. californica* (DMG0018 and DMG0019) to the mucus of seven slug species: *Deroceras invadens, D. reticulatum, A. valentianus, Milax sowerbyi, Arion hortensis, Arion ater,* and *L. flavus*. Different chemoattraction profiles were observed: DMG0018 was more attracted to the mucus of *D. reticulatum* and *M. sowerbyi*, while DMG0019 showed no significant attraction toward *D. reticulatum* compared to the control, distilled water (Andrus & Rae, 2019). These differences in chemoattraction between different strains of the same species call for further investigation into the host preference of other reported strains of *P. californica*.

P. californica has been reported in California, Oregon, New Zealand, and Europe (De Ley *et al*., 2016; Howe *et al*., 2020; Wilson *et al*., 2016; Carnaghi *et al*., 2017). It was developed into a biocontrol product (Stenberg *et al.,* 2021) in 2022 and is currently commercially available (Nemaslug 2.0) in the UK. However, its use is currently restricted to England, Scotland, and Wales. *P. californica* has only recently been documented in Canada; it was first reported in a seasonal nursery in Edmonton, Alberta (Brophy *et al*., 2020a; Brophy *et al*., 2020b). Given that certain strains of *P. californica* display different chemoattraction profiles on different slug species, it is important to investigate the host preferences of the novel Canadian strain. Thus, the first objective of this study was to test the chemoattraction of the Canadian strain of *P. californica* (accession number MT135094, Brophy *et al*. 2020b) to slug mucus of the following four common pest slug species in Alberta, Canada: *D. reticulatum, A. valentianus, A. rufus*, and *A. fasciatus*. This study provides information on the preferred hosts of *P. californica* under laboratory conditions, which could have implications for the efficacy of *P. californica* as a biocontrol agent.

In a more recent survey, another nematode, *Pristionchus entomophagus* (Family: Diplogasteroidea) was also isolated in association with the slug *D. reticulatum* (Patuwatha Withanage *et al.,* 2023). Nematodes in this genus often associate with beetles (Herrmann *et al.*, 2006; Brown *et al.,* 2011; Cinkornpumin *et al.*, 2014). They have a cosmopolitan distribution and are characterized as necromenic, hermaphroditic microbivores (Michaud, 2013; Félix *et al.,*

2018). *Pristionchus* use a mixture of insect and plant-derived odors released during beetle feeding to recognize their hosts (Cinkornpumin *et al.*, 2014). They have also been reported hitchhiking (or in association with) other insects, e.g., fig-pollinating wasps (Susoy *et al.,* 2016), termites (two species), and corn borer moths (*Ostrinia*)(Félix *et al.,* 2018). In the soil, *P. entomophagus* occupy similar microhabitats (e.g., decaying plants, humus, compost, moss, rotten potatoes, rotten wood, and decomposing fungi) as slugs (Félix *et al.,* 2018; Getz, 1959); consequently, nematodes may be accidentally ingested and, in some instances, pass-through the gut intact (Sudhaus, 2018). The microflora found in *P. entomophagus* can exhibit significant variability and potentially play a role in causing mortality in insect hosts. As a result, *P. entomophagus* has attracted research attention due to its potential biocontrol properties against its hosts (Michaud, 2013).

Given that there is potential for niche overlap between *P. entomophagus* and *P. californica,* antagonistic interaction between these two species is possible. It would further be interesting to investigate the preference of *P. entomophagus* for different slug species to determine if they have an overlapping host preference with *P. californica*. Therefore, my second objective was to investigate the chemoattraction profiles of *P. entomophagus* on the slug species: *D. reticulatum, A. valentianus, A. rufus,* and *A. fasciatus*. Here, I used the same slug species that were used to investigate the host preference of *P. californica.* The results are expected to establish baseline information on the chemoattraction profiles of *P. entomophagus* on non-target gastropod hosts.

3.2. Materials and method

3.2.1. Slug collection and maintenance

A. valentianus, A. fasciatus, and *A. rufus* were collected from agricultural sites, nurseries, and greenhouses in the Edmonton area (Alberta, Canada) from June to October 2021. *D. reticulatum* were collected and donated for research by residential gardeners. Groups of <40 slugs were maintained in breathable plastic containers (3.5 L, 23.49 cm x 23.49 cm x 11.43 cm) lined with a damp absorbent paper towel. All the slugs (except *A. fasciatus*) were fed carrot slices *ad libitum* and approximately 5ml of Fluker's High-Calcium Cricket Diet® (Louisiana, USA); *A. fasciatus* were fed iceberg lettuce as they did not prefer carrots. All the slug containers were incubated at 18°C, 80% RH, 12 h light; 12°C, 60% RH, 12 h dark cycle and cleaned weekly.

3.2.2. Nematode isolation, identification, and maintenance

3.2.2.1. P. californica

The infective juveniles of *P. californica* (accession number MT135094, Brophy *et al.* 2020b) were collected from ongoing laboratory cultures. The culture was originally established from nematodes collected from a single *A. rufus* slug specimen found during a survey conducted in Edmonton, Alberta in 2019 (Brophy *et al.* 2020a; Brophy *et al.* 2020b). Subsequent generations were cultured on modified White traps with previously frozen, nematode-free *D. reticulatum* slug cadavers. Nematodes were maintained under the same laboratory conditions as slugs (18°C, 80% RH, 12 h light; 12°C, 60% RH, 12 h dark cycle) for 10-14 days until infective juveniles (IJs) were released. The aqueous media with IJs of *P. californica* were then collected into 50 ml plastic conical tubes and allowed to settle for approximately 30 minutes. The nematodes were then pipetted into a 10 ml beaker for quantification (individually counted 50 infective juveniles for each setup).

3.2.2.2. P. entomophagus

A single adult nematode suspected to be *P. entomophagus* (based on the morphology: stoma contains teeth-like structures, lack of a conoid tail with a longer hyaline tip as in *P. californica*) was isolated from a *D. reticulatum* slug cadaver. It was then inoculated onto a frozen, nematode-free adult slug cadaver (*D. reticulatum*) in a White trap. The slugs were considered to be nematode-free if they exhibited no indications of swollen body regions, discoloration, excess mucus secretion, or the presence of nematodes on their body surfaces. Subsequently, twelve isofemale lines were generated using the same methods. The nematodes were then incubated at 18°C, 80% RH, 12 h light; 12°C, 60% RH, 12 h dark cycle for 4-5 days until they released juveniles. From each isofemale line, an individual nematode was fixed in 95% ethanol and later transferred to a proteinase-K-based lysis buffer for DNA extraction. Primer sets 18A (5′-AAAGATTAAGCCATGCATG-3′) and 26R (5′CATTCTTGGCAAATGCTTTCG-3′) were used in PCR amplification and direct-end sequencing of the 18S ribosomal DNA (Blaxter *et al*., 1998; Denver *et al.*, 2003). The resulting 18S rRNA sequences were compared against GenBank's non-redundant (nr) database using blastn (NCBI Resource Coordinators). The specimen was confirmed to be *P. entomophagus* (99.28% match to KT188843). For the below experiments, infective juveniles were collected from White traps as described in 3.2.2.1.

3.2.3. Chemotaxis assay

3.2.3.1. Preparing mucus samples

Mucus swabs were taken from the foot and mantle of healthy, adult *D. reticulatum* specimens collected from colony containers. The selected slugs were of similar size (>200mg), exhibited active feeding behavior, and showed no visible signs of pathology. To collect mucus, two drops of distilled water were applied to a 1 cm^2 piece of Grade 1 Whatman™ Qualitative Filter Paper and it was gently rubbed (30 seconds) against the foot and mantle region of the slug. Mucus samples were collected separately from 20 individuals. The treated papers were immediately placed on the relevant test quadrant of the agar plate (see 3.2.3.2.). The same procedure was followed to collect mucus from *A. valentianus, A. fasciatus*, and *A. rufus.*

3.2.3.2. Chemotaxis assays

The experimental setup was adapted from Rae *et al*. (2006), Hapca *et al*. (2007), Rae *et al*. (2009), and Andrus and Rae (2019). All experimental arenas comprised of 9 cm diameter Petri dishes with agar-A (a nutrient agar medium was not used to minimize propagation of the nematodes during the incubation period). The underside of each Petri dish was marked equally into quadrants (before adding agar) from the outside of the dish with a permanent marker. A 2 cm diameter circle was also drawn in the middle of the dish (Fig. 3.1).

A piece of Whatman paper previously treated with mucus of the test slug species was placed in one of the quadrants approximately 0.5 cm away from the Petri dish wall. A second piece of Whatman paper was treated with two drops of distilled water (control) and placed at the opposite end at a similar distance away from the edge of the Petri dish.

The Petri dishes were then incubated at 18°C for approximately 2 hours before the nematodes were inoculated (to allow better diffusion of the chemicals of the mucus sample) (Hapca *et al*. 2007). Two hours after the mucus application, 50 active IJs of *P. californica* were introduced to the middle circle of the petri dish, sealed with Parafilm™ and incubated at 18°C, 80% RH, 12 h light; 12°C, 60% RH, 12 h dark cycle for 24 hours to allow sufficient time for the nematodes to migrate to the test quadrants from the initial point of introduction. Twenty assays using mucus of *D. reticulatum* were tested against a control (distilled water). After 24 hours of incubation, both Whatman paper pieces were removed, placed in separate Petri dishes, and washed with distilled water to collect any nematodes. The water in each dish was thoroughly inspected under a dissecting microscope and the nematodes were counted. Each region in the petri dish (X, X', Y, Y'; fig.3.1), including the center, was inspected for nematodes.

The same procedure was followed to test the chemoattraction of *P. californica* to other slug species: *A. valentianus, A. fasciatus*, and *A. rufus. P. californica* was further tested against all possible pairwise host combinations (twenty assays for each pair) to check for host preference. However, due to unforeseen circumstances, the *P. californica* culture collapsed, so I was unable to test *A. valentianus* against *A. fasciatus* and *A. fasciatus* against *A. rufus.*

The chemoattraction of *P. entomophagus* to *D. reticulatum, A. valentianus, A. fasciatus*, and *A. rufus* was tested following the procedure above (n=20 per species).

3.2.4. Statistical analysis

An attraction index (AI) was calculated for each individual slug assayed following the equation below; (modified from Hapca *et al*., 2007).

$$\text{Attraction Index (AI) for Host sp. } \mathbf{X} = \frac{\alpha[N(\mathbf{X}) - N(\mathbf{Y})] + \beta[N(\mathbf{X'}) - N(\mathbf{Y'})]}{\alpha[N(\mathbf{X}) + N(\mathbf{Y})] + \beta[N(\mathbf{X'}) + N(\mathbf{Y'})]}$$

The coefficients α and β equal 2 and 1, respectively, capture the weighted proportion of the nematodes in X and Y (quadrants containing the cues) relative to X' and Y' (quadrants adjacent to the cue). In this study, the nematode populations in each zone of the Petri dish were assessed, including zones X, X', Y, and Y' (Fig. 3.1). The quantification of nematodes in each zone allowed for the calculation of their relative abundance. To determine the Attraction Index (AI) for a specific host slug species, the converted values of the nematode populations (i.e., relative abundance) in the zones of interest were utilized in the above equation. The notation N(X) refers to the relative abundance of nematodes on and underneath the Whatman paper treated with mucus of the host species X. The notation N(Y) refers to the relative abundance of nematodes on the paper treated with distilled water or as in the second set of experiments, the mucus of a second host species Y; N(X') and N(Y') refer to the relative abundance of nematodes in the adjacent area to X and Y (i.e., X' and Y'). The AI values range from -1 to +1, such that -1 indicates absolute repulsion to cue X (or attraction to cue Y) while +1 indicates an absolute attraction to the cue. A Wilcoxon signed-rank test with continuity correction was performed to determine the statistical significance of the AI values (R v4.2.2; R Core Team, 2022).

3.3. Results

The objective of this study was to examine the chemoattraction of both *P. californica* and *P. entomophagus* to the mucus of four different slug species (i.e., *D. reticulatum, A.*

valentianus, A. fasciatus, and *A. rufus*). The results show these two nematodes share similar slug hosts. Relative to blank controls, the Canadian strain of *P. californica* preferred slug species, *A. valentianus* (AI= 0.43± 0.12, p=0.005)*, A. fasciatus* (AI= 0.41±0.15, p=0.014), and *A. rufus* (AI= 0.42±0.14, p=0.014); however, surprisingly, the attraction of *P. californica* to the mucus of *D. reticulatum* compared to the control was non-significant (AI= 0.28±0.13, p= 0.073) (Fig. 3.2). We further examined the preference of *P. californica* for the mucus of two host species in pair-wise comparisons. For a given pair (e.g., X vs. Y), a positive attraction index (AI) value would indicate an attraction towards host X, whereas a negative value would suggest an attraction towards host Y. The attraction index (table 3.1) was significantly different from 0 when nematodes were given a choice between the mucus of *D. reticulatum* and *A. fasciatus*, with a strong preference for the former (AI= 0.30±0.11, p=0.026). The nematodes did not show a clear preference when presented with *D. reticulatum* and *A. rufus* (AI= 0.2±0.12, p=0.097). There was no preference for either *A. valentianus* or *A. rufus* (AI= -0.13±0.15, p=0.420) in the pairwise tests. There was also no clear preference for either *D. reticulatum* or *A. valentianus* (AI= 0.06±0.12, p=0.668) (fig.3.3).

The AI values for *P. entomophagus* (relative to control) indicated a significant attraction towards *D. reticulatum* (AI= 0.42±0.07, p< 0.05) and *A. fasciatus* (AI= 0.32±0.11, p=0.01) compared to a control. The attraction towards *A. valentianus* was not significantly different from the control (AI= 0.24±0.17, p=0.15) (Table 3.1). In contrast, the AI for *A. rufus* was negative (AI= -0.30±0.14, p=0.049), which indicates repulsion to the mucus of this species. Interestingly, both *P. californica* and *P. entomophagus* had an attraction towards *D. reticulatum* (AI= 0.28±0.13, and AI= 0.42±0.07, respectively), however, these attractions were not significantly different from each other (p= 0.86, Fig. 3.4). Further, non-significant results were observed for the preference of *P. californica* and *P. entomophagus* toward *A. valentianus* (AI= 0.43± 0.12, AI= 0.24±0.17, p=0.65 respectively) and *A. fasciatus* (AI= 0.41±0.15, AI= 0.32±0.11, p=0.25 respectively). In contrast, there was a significant difference in the preference for *A. rufus* by the two nematodes (Fig. 3.4) in which *P. californica* had a strong attraction to the cue while *P. entomophagus* was repulsed (p= 0.001).

3.4. Discussion

In this study, I assessed the host preference of *P. californica* and *P. entomophagus* using the mucus of four different slug species. The results show that *P. californica* was strongly attracted to all slug species compared to a control, except for *D. reticulatum,* for which I observed a mild attraction (AI= 0.28±0.13, p=0.073). The Canadian strain of *P. californica* used in this study was originally isolated from an *A. rufus* slug specimen (Brophy *et al*. 2020a; Brophy *et al*. 2020b); however, it was subsequently cultured on freeze-killed *D. reticulatum* slug cadavers. It is worth noting that the attraction of this strain toward *A. rufus* compared to control was significant (AI= 0.42±0.14, p=0.014). In a pairwise test between *D. reticulatum* and *A. rufus, P. californica* did not show a clear preference for either species (AI= 0.2±0.12*,* p= 0.097), which suggests that the Canadian strain of *P. californica* equally prefers both *D. reticulatum,* and *A. rufus*. Andrus & Rae (2019) tested the chemoattraction of two strains of *P. californica* (DMG0018 & DMG0019) to the mucus of different slug species including *L. flavus, D. reticulatum, L. valentiana* (*A. valentianus*) and *A. ater.* Both strains DMG0018 and DMG0019 had no significant difference in their attraction towards *L. flavus* compared to a control (Andrus & Rae*,* 2019). Thus, both strains did not prefer the mucus of *L. flavus.* These two nematode strains were initially isolated from the snail, *Oxychilus draparnaudi*, but have been subsequently grown on *L. flavus* cadavers. However, these results show that these nematodes are not necessarily attracted to *L. flavus* despite being grown on *L. flavus* for generations. Therefore, the weak attraction I observed in the Canadian strain of *P. californica* on *D. reticulatum* is unlikely due to their continuous growth on freeze-killed *D. reticulatum* cadavers, but to their inherent attraction to the cues of that host species. Further, *P. californica* strain DMG0018 preferred *D. reticulatum* over *L. valentiana* and *A. ater*, while the strain DMG0019 preferred *A. ater,* but showed no attraction toward *D. reticulatum* or *L. valentiana* compared to a control. Thus, there are differential chemoattraction profiles for different strains of *P. californica*. A careful examination of the host preference profiles of each *P. californica* strain is thus highly recommended before any formulation of these nematodes as a biocontrol product. In comparison, our study showed that *A. valentianus* ranked higher (AI= 0.43± 0.12) in terms of the chemoattraction profiles of *P. californica* (accession number: MT135094), followed by *A. rufus* (AI= 0.42±0.14)*, A. fasciatus* (AI= 0.41±0.15), and *D. reticulatum* (AI= 0.28±0.13). Therefore, these results also support the fact that there are different chemoattraction profiles for related strains of *P. californica.* I further noticed that this nematode species was significantly attracted to

D. reticulatum when presented in a pairwise with *A. fasciatus* (AI= 0.30±0.11, p=0.03), despite *D. reticulatum* being a weak attractant compared to the control. The mucus of undisturbed or non-stressed *D. reticulatum* slugs typically contains a low concentration of divalent ions, resulting in clear and low-viscosity mucus (Luchtel & Deyrup-Olsen, 2001). However, when the slugs are disturbed or stressed, the mucus undergoes changes. Rubbing individual slugs on Whatman paper caused the mucus to become milky white and significantly thicker. It has also been discovered that the mucus of disturbed slugs has a higher content of calcium salts (Luchtel & Deyrup-Olsen, 2001). Consequently, it is speculated that this white mucus, rich in ions, may not be attractive to nematodes (Luchtel & Deyrup-Olsen, 2001), which could explain the nonsignificant results in the attraction of *P. californica* to *D. reticulatum* mucus compared to a control with no ions (distilled water). However, when paired with *A. fasciatus*, it is possible that the mucus from both hosts has different chemical compositions due to the rubbing of the slugs to collect mucus. Hence, it is possible that *P. californica* demonstrates a preference for mucus containing a lower concentration of defensive substances in the pair. Additionally, the different diets provided to the two slug species (carrot slices for *D. reticulatum* and iceberg lettuce for *A. fasciatus*) could also influence the chemical composition of their mucus. Hence, it is recommended for future research that both slug species are fed with the same food type, specifically iceberg lettuce (avoiding carrot due to the extremely low survival rate of *A. fasciatus* when fed with carrot). Further, collecting non-stressed mucus by allowing slugs to crawl on Petri dishes lined with damp Whatman paper is suggested for more reliable results.

In this study, I further used chemotaxis assays to test the host preference of *P. entomophagus* on four different slug species. *P. entomophagus* had a significant attraction to the mucus of *D. reticulatum* (AI= 0.42±0.07, p< 0.05) but was strongly repulsed by *A. rufus* (AI= -0.30±0.14, p=0.049). These results contrast with what I observed in the assays for *P. californica.* During the study, *P. entomophagus* was originally isolated from *D. reticulatum* cadavers and was subsequently cultured on freeze-killed *D. reticulatum* until they were used in the experiments. *Pristionchus* species often associate with beetles in their dauer diapause stage and wait until their host dies to resume development and feed on microbes in the decomposing cadaver (Félix *et al*., 2018). Apart from this necromenic lifestyle, they have also been reported in phoretic associations with other non-beetle insects, e.g., fig-pollinating wasps (Susoy *et al*., 2016), termites (two species), corn borer moths (Lepidoptera) (Félix *et al*., 2018) and non-insect species such as slugs

including *D. reticulatum* (Brophy *et al*., 2020b, Patuwatha Withanage *et al*., 2023), *Arion circumscriptus, Lehmannia* (*Limax*) *marginata*, *Malacolimax* (*Limax*) *tenellus*, and snails such as *Succinella* (*Succinea*) *oblonga* (Sudhaus, 2018). A recent study (Patuwatha Withanage *et al.,* 2023), isolated both *P. californica* and *P. entomophagus* from *D. reticulatum* slug samples collected from a residential garden. Given that *P. entomophagus* has strong preferences for both *D. reticulatum* and *A. fasciatus*, these two nematode species may compete for similar resources. *P. entomophagus,* like *P. pacificus,* a related species of the same genus has a eurystomatous mouth form which likely is used to prey on other nematodes in crowded conditions (Werner *et al.,* 2017; Lightfoot *et al.,* 2021). *P. pacificus* has been reportedly involved in intraguild predation of *C. elegans* to achieve immediate access to resources while reducing competition (Quach & Chalasani, 2020). Thus, I hypothesize that *P. entomophagus* has an antagonistic relationship with *P. californica* either through direct predation or indirect competition for resources. While other studies have focused on the infectivity and pathogenicity of *P. californica* and other related species of the genus *Phasmarhabditis* on different slug species, there are no studies to date that test the ability of *P. californica* to cause mortality in slugs in the presence of another potentially antagonistic nematode. Future studies should examine the type and mechanism of interaction between *P. californica* and, *P. entomophagus*. The Canadian strain of *P. californica* has the potential to be used as a biocontrol agent against pest slugs, therefore, I suggest further research on the infectivity and pathogenicity of *P. californica* on host slug species in the presence of other nematode species carrying potentially antagonistic properties.

Overall, this study investigated the chemoattraction of the Canadian strain of *P. californica* and *P. entomophagus* to the mucus of four different slug species that are common in plant nurseries, greenhouses, agricultural sites, and residential gardens in Alberta. However, attraction to the mucus of a particular pest slug host may not be sufficient to conclude that this nematode would be suitable as a biocontrol agent. It is equally important that they inhibit the feeding activities of the pest hosts upon infection and cause mortality to their hosts within a short period of time. In this study, I also demonstrated that *P. californica* and *P. entomophagus* can share host species and have potentially overlapping host ranges; thus, they may directly or indirectly compete for the same resources. Further research is, therefore, needed to test the efficacy (in both infectivity and pathogenicity) of *P. californica* as a biological agent against slugs in the presence of *P. entomophagus* and other antagonistic nematode species.

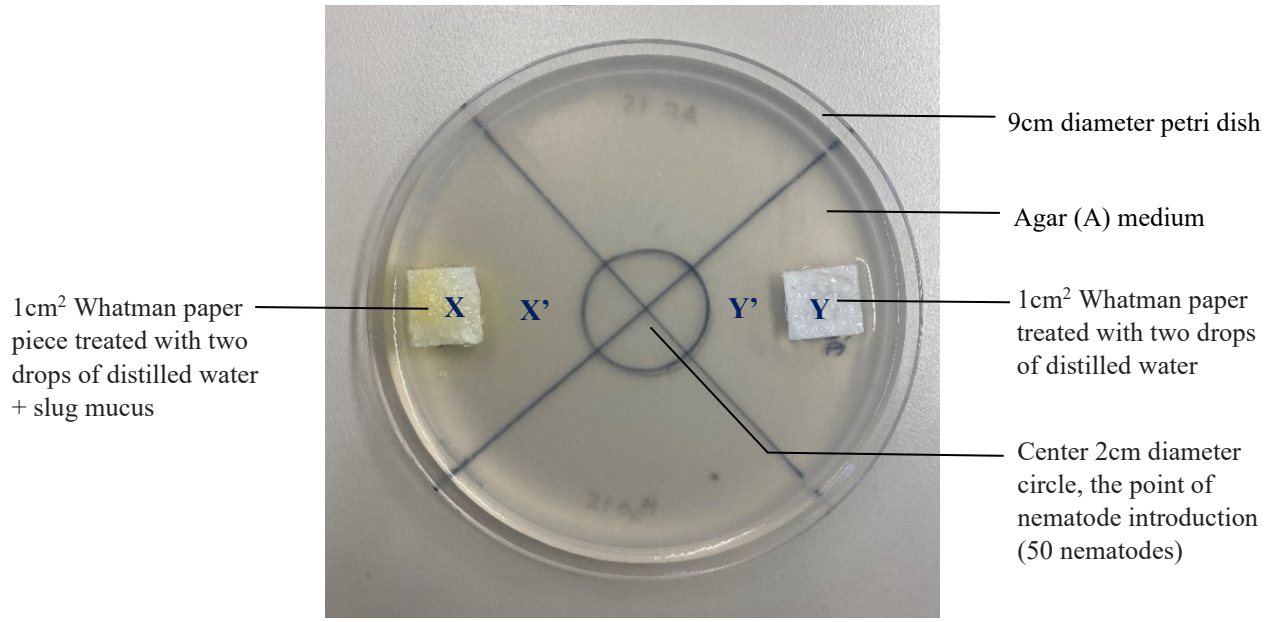

Fig 3.1. Chemotaxis assays were performed on a 9 cm Petri dish with agar divided into four quadrants: X: 1cm^2 Whatman filter paper with mucus of slug species X (treatment 1), X': adjacent area to treatment 1, Y: 1cm^2 Whatman filter paper with distilled water (control) or mucus of slug species Y (treatment 2), Y': adjacent area to control or treatment 2

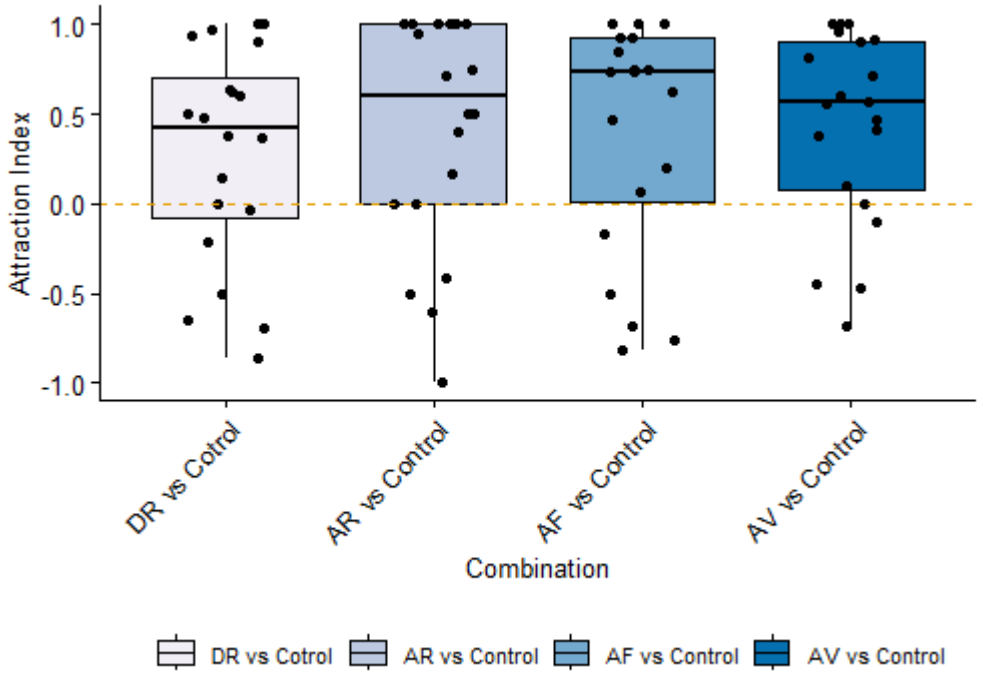

Fig 3.2. Attraction indices of *Phasmarhabditis californica* to the mucus of slugs: *D. reticulatum, A. valentianus, A. fasciatus*, and *A. rufus.* The values range from +1 to -1. Positive values indicate attraction toward mucus while negative values indicate repulsion. (*D. reticulatum,* DR; *A. valentianus,* AV; *A. rufus,* AR; and *A. fasciatus,* AF).

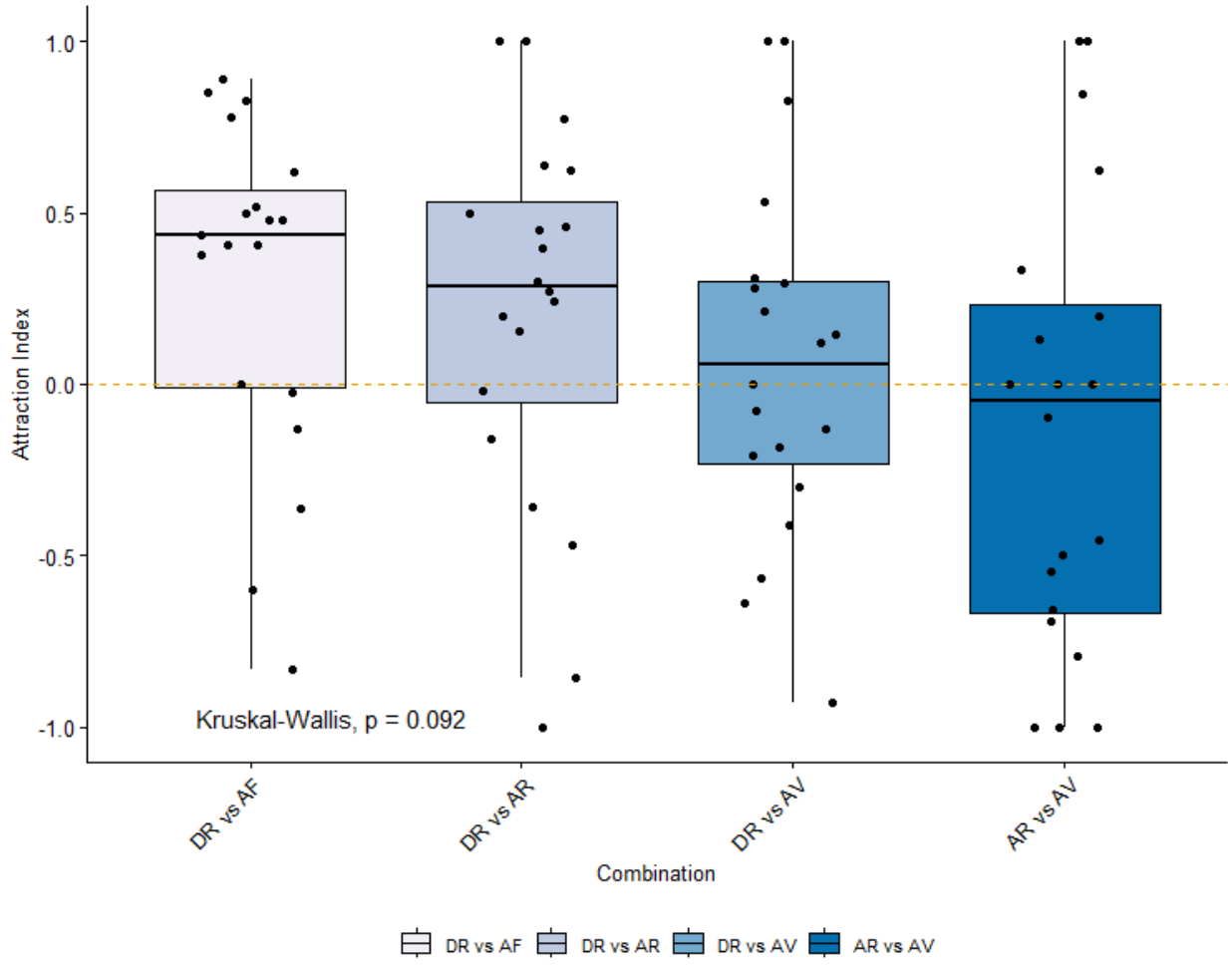

Fig 3.3. Attraction indices of *Phasmarhabditis californica* to the mucus of *D. reticulatum, A. valentianus, A. fasciatus*, and *A. rufus* in pairwise comparisons. The values range from +1 to -1. Positive values indicate attraction toward *D. reticulatum* (DR) while negative values indicate attraction toward the other host species in the comparison (*D. reticulatum,* DR; *A. valentianus,* AV; *A. rufus,* AR; and *A. fasciatus,* AF).

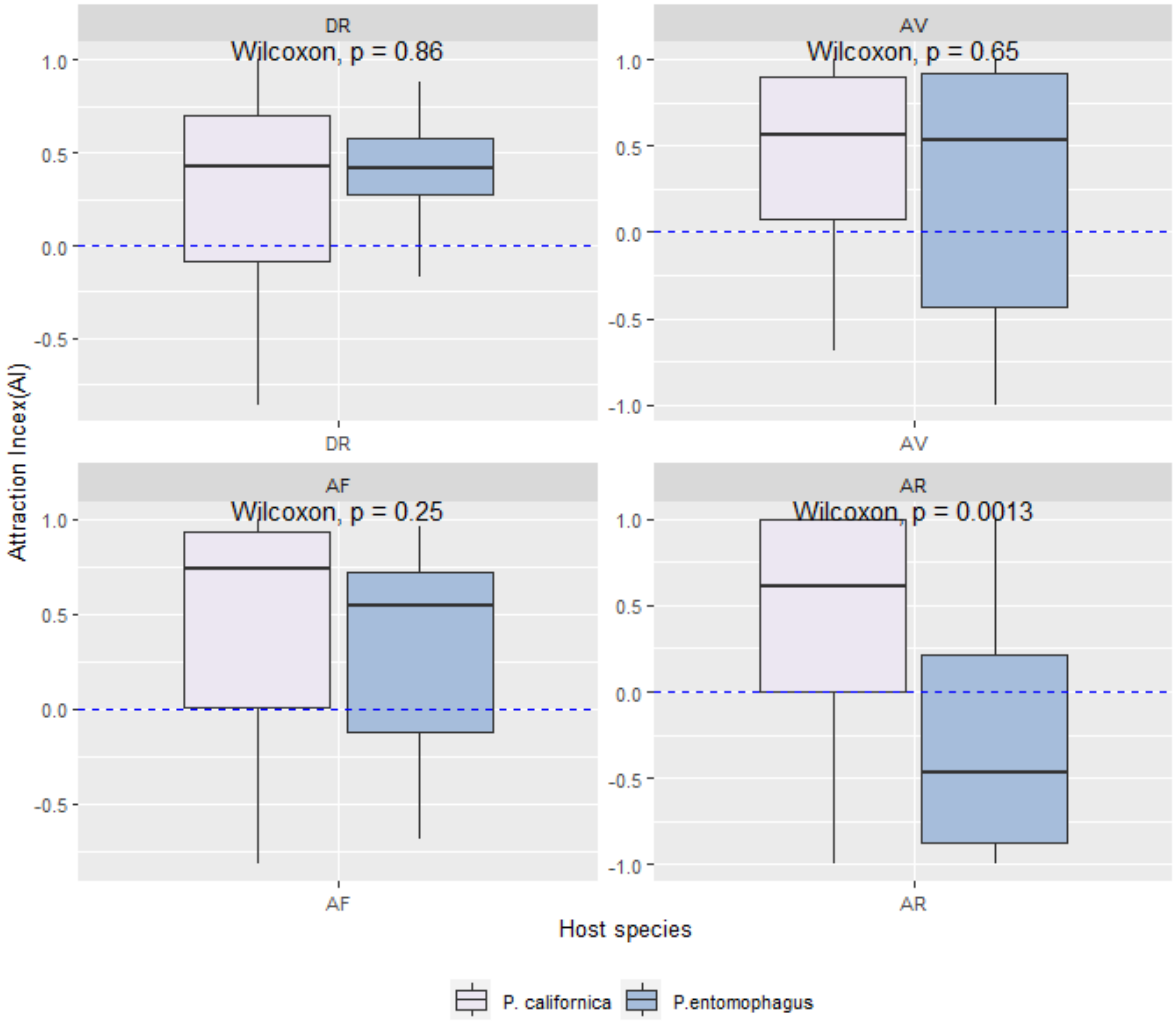

Fig 3.4. Comparison of the attraction indices of *Phasmarhabditis californica* and *Pristionchus entomophagus* to the mucus of *D. reticulatum, A. valentianus, A. fasciatus*, and *A. rufus*. The values range from +1 to -1. Positive values indicate attraction toward the host cue while negative values indicate repulsion to the cue. (*D. reticulatum,* DR; *A. valentianus,* AV; *A. rufus,* AR; and *A. fasciatus,* AF).

Table 3.1. Mean attraction indices of *Phasmarhabditis californica* and *Pristionchus entomophagus* on four different slug species: *D. reticulatum,* DR; *A. valentianus,* AV; *A. rufus,* AR; and *A. fasciatus,* AF. ***** *Indicates statistical significance*

Nematode species	Host species	**DR**	**AF**	**AV**	**AR**
P. californica	**Control**	**+0.28** p=0.073	**+0.41*** p=0.014	**+0.43*** p=0.005	**+0.42*** p=0.015
	AF	**+0.30*** p=0.026		**Did not perform AV vs AF**	**Did not perform AR vs AF**
	AV	**+0.06** p=0.668	**Did not perform AV vs AF**		**-0.13** p=0.420
	AR	**+0.21** p=0.097	**Did not perform AR vs AF**	**-0.13** p=0.420	
P. entomophagus	**Control**	**+0.42*** P<0.05		**+0.24** p=0.150	**-0.30*** p=0.0496

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Chapter 4

Efficacy of *Phasmarhabditis californica* (Family: Rhabditidae) on causing mortality to *Deroceras reticulatum*, in the presence of *Pristionchus entomophagus* (Family: Neodiplogasteridae)

4.1. Introduction

Slugs, especially *Deroceras reticulatum,* are economically important pests in agriculture and horticulture worldwide (Barker, 2002; Koslowski, 2012). They can cause serious damage to a wide range of crops, especially at the seedling stage (Kunkel *et al.,* 2023). Management of these pests is mostly dependent on chemical applications such as iron phosphate, carbamate compounds (methiocarb and thiodicarb), and metaldehyde (Pieterse *et al.,* 2017). Even though these pesticides can be efficient at killing slugs, some can cause serious harm to other organisms if accidentally ingested (Kunkel *et al.,* 2023). They may negatively impact the environment if leached into ground water (O'Brien *et al*., 2008; Kunkel *et al.,* 2023). The usage of parasitic nematodes has been tested as an alternative to these chemicals in Europe (Kunkel *et al.,*2023). There are eight nematode families that are associated with mollusks hosts. Those families include Agfidae, Alaninematidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogastridae, Mermithidae, and Rhabditidae (Grewal *et al*., 2003; Ross *et al.,* 2016; Kunkel *et al.,* 2023). Among them, *Phasmarhabditis* (family Rhabditidae) is a genus of bacterial-feeding soil-dwelling nematodes that are also facultative parasites of gastropods (Wilson and Grewal, 2005). *P. hermaphrodita*, *P. papillosa*, *P. neopapillosa,* and *P. californica* are known for their host-parasitic associations with terrestrial gastropods (Wilson & Grewal, 2005; De Ley *et al*., 2016). Their parasitic cycle is initiated by the third larval or dauer stage, a developmentally arrested phase of the nematode. They enter a slug host via its dorsal integumental pouch (Wilson *et al.*, 1993; Tan & Grewal, 2001). Once inside, the dauers resume growth, start feeding within the host, and develop into self-fertilizing hermaphrodites, eventually killing the slug within 4-21 days depending on the intensity of the infection (Wilson et al., 1993; Wilson & Grewal 2005; Rae *et al.* 2007). When the food source, the cadaver, is depleted, the nematodes then form new dauer larvae and leave the cadaver in search of new slug hosts, and the cycle continues. *P. hermaphrodita,* the most studied species in this genus is known to cause mortality in a wide range of snails and slugs belonging to 15 gastropod families (Kunkel *et al.,* 2023). In

comparison, *P. papillosa* has been shown to be more lethal than other *Phasmarhabditis* species (De Ley *et al*., 2016). But, only *P. hermaphrodita* has been developed as a biological molluscicide and was commercially released in 1994 by MicroBio Ltd, Littlehampton, UK (formally Becker Underwood, now BASF) under the tradename Nemaslug® (Rae *et al*., 2007). *P. californica* was first discovered in California (De Ley *et al*., 2016) but has been subsequently found in New Zealand, Europe, and recently in Canada (Brophy *et al.,* 2020a; Brophy *et al.,* 2020b; Patuwatha Withanage *et al.,* 2023). This nematode has been developed as a biocontrol product against slugs (Nemaslug® 2.0) (Stenberg *et al.,* 2021; Mc Donnell *et al*., 2023). However, unlike the product Nemaslug® which is currently available in 15 European countries (Rae *et al.,* 2007; Laznik *et al*., 2020; Mc Donnell *et al.,* 2020), Nemaslug® 2.0 is only commercially released in England, Scotland, and Wales (Mc Donnell *et al*., 2023).

Biotic interactions between nematodes in the soil should be taken into consideration when assessing the efficacy of a malacopathogenic nematode (Barbercheck & Kaya, 1991; Schurkman & Dillman, 2021). Nematodes used as biocontrol agents may come in contact with other nematode species near, on, or within the target host. The nematodes in the genus *Pristionchus* (family Diplogastridae) often associate with scarab beetles (Herrmann *et al.*, 2006; Brown *et al.,* 2011) as well as other host species such as fig-pollinating wasps (*Ceratosolen*) (Susoy *et al.,* 2016), termites (*Odontotermes*) (Von Lieven & Sudhaus, 2008) and moths in the family Crambidae (*Ostrinia*) (Félix *et al.,* 2018). But more importantly, they have been isolated from slugs including *Arion circumscriptus*, *L. marginata, Malacolimax* (*Limax*) *tenellus,* and snails such as *Succinella* (*Succinea*) *oblonga* (Sudhaus, 2018). *Pristionchus* spp. often co-occur in large numbers with *Caenorhabditis* spp. and are found in different feeding stages on rotting vegetable matter, also a known food source for slugs (Félix *et al.,* 2018). Recently, *P. entomophagus* was isolated from *D. reticulatum* (Patuwatha Withanage *et al.,* 2023), one of the major host species of *P. californica.* The coexistence of these two nematode species, i.e., *P. californica* and *P. entomophagus,* both of which are microbivores could be problematic from a biocontrol perspective if they compete for the same food resource. Competition is usually the strongest between parasites that are most alike with respect to their physiological demands on the host (Norton, 1989; Eisenback, 1993). Given that both *Phasmarhabditis* and *Pristionchus* share a common host, *D. reticulatum,* and share food resources on the host cadaver, it is expected that these two species may compete directly or indirectly for resources.

If *P. entomophagus* outcompetes *P. californica,* the efficacy of *P. californica* as a molluscicide may be compromised. A few experiments have been conducted on the efficacy and progeny production of certain entomopathogenic nematodes in the presence of potential competitors (Koppenhöfer *et al.,* 1995; Campos-Herrera *et al.,* 2015; Blanco-Pérez *et al.,* 2017; Quach & Chalasani, 2020). However, no study has yet examined the efficacy of malacopathogenic nematodes in the presence of other nematodes. Therefore, the objective of this study was to investigate the ability of *P. californica* to cause mortality in *D. reticulatum,* the single model host, in the presence of *P. entomophagus.* Further, I compared the establishment success of the nematodes and progeny production in single and mixed infections for both nematode species. A better understanding of the ecological relationships of soil nematodes, especially antagonistic interactions, is important to achieving extended nematode persistence in the field and effective use of these malacopathogenic nematodes in agroecosystems. Thus, information gained from this study would help understand how competition can influence the efficacy of *P. californica* as a biocontrol agent.

4.2. Materials and method

4.2.1. Slug rearing and maintenance

We maintained 70 juvenile laboratory-reared *D. reticulatum* (approximately 2cm in length when stretched) in perforated plastic containers (3.5L, 23.49cm x 23.49cm x 11.43cm) lined with a damp absorbent paper towel. Groups of <40 slugs were maintained in a single container. All the slugs were regularly monitored for general health including signs of infection (e.g., swollen mantle, discoloration, reduced activity, and feeding) and fed carrot slices *ad libitum* with a supplement of approximately 5ml of Fluker's High-Calcium Cricket Diet® (Louisiana, USA). All the slug containers were incubated at 18°C, 80% relative humidiy (RH), 12 h light; 12°C, 60% RH, 12 h dark cycle and cleaned weekly.

4.2.2. Nematodes for experiments

P. californica (Strain DL 310; Accession number MT472242) samples were supplied by Denver lab, Department of Integrative Biology, Oregon State University, Corvallis, OR, USA, and subsequently cultured on modified White traps with freeze-killed, nematode-free *D. reticulatum* slug cadavers. Nematodes were maintained under laboratory conditions: 18°C, 80% RH, 12 h light; 12°C, 60% RH, 12 h dark cycle for 10-14 days until IJs were produced. The IJs

were then collected into Falcon™ 50 mL Conical Centrifuge Tubes and allowed to settle for approximately 30 minutes. The nematodes at the bottom of the tubes were then pipetted out into a 10 ml beaker in which they were well mixed before quantification. The beaker containing nematodes was then filled with distilled water up to a volume of 10 ml. To estimate the concentration of nematode larvae in the beaker, I counted the number of nematodes in three 10µl drops of the homogenized nematode sample. The average number of nematodes in the three drops was used to determine the final concentration of the nematodes in the beaker. *P. entomophagus* (isolated in Edmonton, Canada 99.28% match to KT188843) were similarly cultured, collected, and quantified.

4.2.3. Infection vials

Infection arenas were prepared using a plastic vial (3.5cm in diameter, 5 cm in height) lined with a damp piece of Whatman paper (Grade 1 Whatman™ Qualitative Filter Paper). I prepared 15 infection vials for each of the following treatments: (i) *P. californica* only, (ii) *P. entomophagus* only, and (iii) mixed infections with both *P. californica* and *P. entomophagus*. Control vials had no nematodes, only distilled water in similar volume. For single species infections, I used 1800 infective juveniles (IJ)/vial but halved the number of each nematode species in mixed infections, i.e., 900IJ of *P. californica* and 900IJ of *P. entomophagus* (to control for nematode density). The concentration of nematodes was quantified as described above (see 4.2.2). Each nematode species was pipetted and inoculated into each vial. Healthy, equal-sized *D. reticulatum* juveniles were selected and individually placed in each vial in direct contact with the nematodes. Soon after placing the slugs in tubes, they were confined to a limited space with a breathable foam plug placed approximately 1cm from the bottom of the tube. This was to ensure that the slugs were constantly in direct contact with the nematodes while allowing some movement by the slug (Fig.4.1). The tube was then capped with a perforated lid. All infection vials were incubated under laboratory conditions (18°C, 80% RH, 12 h light; 12°C, 60% RH, 12 h dark cycle) for five days with regular monitoring. Slugs were inspected daily for any mortality (no signs of movement upon poking with a blunt needle). Dead slug cadavers were placed in individual Petri dishes (6 cm diameter) lined with damp Whatman paper, covered, and sealed with Parafilm™.

After the five-day infection period, the remaining live slugs were transferred into new nematode-free, breathable plastic vials for 14 days and fed equal size carrot slices (Fig.4.2) twice during the 14 days period (i.e., on 1st day and the 7th day). They were monitored regularly; slugs that died during this period were individually placed in 6 cm diameter Petri dishes lined with a damp Whatman paper and incubated for a maximum of five days to isolate any emerging nematodes. After the incubation period, the nematodes (by then adults or parental population- P) in single infection (see below for mixed infections) treatments were rinsed with distilled water from each slug cadaver, transferred to separate Falcon™ 15 mL Conical Centrifuge Tubes, and allowed to settle before they were pipetted out for quantification. If there were <200 individuals, they were counted, and for values greater than 200, the number of nematodes was estimated. To make estimations, I prepared 1ml homogenized nematode solution using the nematode pellet from the Falcon™ 15 ml Conical Centrifuge Tubes. Then I pipetted out three 10µl drops of homogenized nematode sample and counted the number of nematodes in each droplet to get an estimate of the total number of nematodes in 1ml. As each 1ml sample contained all the nematodes present in the cadaver (the cadavers were rinsed to collect the nematodes), it was considered representative of the total nematode count per slug cadaver. On each occasion, only live nematodes (mobile) were counted. The progeny of *P. californica* (F1) from each cadaver was transferred into new white traps and incubated for another five days until they matured enough to ensure both accurate identification of the nematodes and quantification. Adult stages of *P. californica* (Fig.4.3a) and *P. entomophagus*(Fig.4.4a) can be readily distinguished based on morphology: *P. entomophagus* are smaller in size, the stoma contains teeth-like structures (Fig.4.4b) (eurystomatous mouth form) (Lightfoot *et al.,* 2021), and lack the conoid tail (Fig.4.4c) with a longer hyaline tip as in *P. californica* (Fig.4.3c). I also collected the progeny from single infection treatments of *P. entomophagus* and quantified the reproductive success.

In mixed treatments, the adult nematodes that established in the host were individually identified based on morphological features and counted (if <200) or estimated (if >200). Then, all the F1 individuals produced on each slug cadaver were transferred into separate white traps and incubated for five days until both nematode species reached maturity with identifiable external features (Fig.4.3 -4.4). Any juveniles observed in the subsequent generation (F2) after five days were disregarded. After 14 days post-infection, any remaining live slugs were censored

(decapitated) and incubated in white traps as described above. Nematodes (P and F1) of each species were counted (or estimated) following the aforementioned procedure.

4.2.4. Statistical analysis

The slug mortality rates were visualized using Kaplan-Meier survival plots and analyzed with a log-rank test. The mortality of slugs in single *P. californica* treatments was compared with that of the mixed treatments and the control, separately. The proportion of nematodes established inside each slug was calculated as follows: in single infections, the number of nematodes in each species established inside a slug was divided by 1800; while in mixed infections the number of nematodes in each species was divided by 900, corresponding to their initial inoculum concentration. I further counted (or estimated >200) the progeny production (per slug sample) in each treatment group. Both the proportion of nematodes established inside the slugs and their corresponding progeny numbers were analyzed in the R statistical program with generalized linear models using the MASS package and a negative binomial family (R.v4.2.2; R Core Team, 2022).

4.3. Results

4.3.1. Mortality of Slugs

Overall, all the slugs exposed to *P. californica* in the single treatment were infected (all the live slugs were euthanized at the end of the experiment to check for nematodes: proved 100% infections), of which six of the 15 slugs died, five within the first five days. Only a single death was reported post-exposure. Out of the 15 slugs exposed to *P. entomophagus,* 12 slugs (80%) were infected, and three remained uninfected. However, no deaths were observed during the exposure and post-exposure period. Significantly higher mortality was observed in slugs exposed to *P. californica* compared to the controls (Log-rank, *df*=28, p= 0.007) (Fig. 4.5). In mixed nematode cultures in which the slugs were exposed to both *P. californica* and *P. entomophagus,* all (15/15) got infected with *P. californica*, 13 were co-infected with *P. entomophagus* infections (86.67%). Only four deaths were observed during the 5-day exposure period; no other mortality was observed thereafter. Slug mortality in the mixed infections was significantly higher compared to the controls (Log-rank, *df*=28, p= 0.035) (Fig. 4.6), but was not any different from single *P. californica* infections (Log-rank, *df*=28, p= 0.38) (Fig. 4.7). Since *P. entomophagus*

alone caused no mortality to the slugs, the deaths in mixed infections were assumed to be caused by *P. californica.*

4.3.2. Nematode establishment and progeny production

I counted (or estimated if >200 nematodes) the number of adult nematodes in the slug host (parental nematode group, P) to evaluate the establishment success of each species of nematode in the single and mixed infections. The results revealed that the proportion of *P. californica* established in slug cadavers collected from single treatments was not significantly different from those collected from mixed infection treatments (z=1.635, p= 0.102, Fig. 4.8). However, I observed a significant reduction in the number of offspring (F1) produced by the established nematodes (P) in mixed compared to single infections; the mean number of F1 of *P. californica* (matured into adults) in single treatments was 2140.0 ± 921.0 S.E., while in mixed treatments it decreased to a mean of 12.10 ± 4.36 S.E (z= 6.98, *df* = 28, p<0.001) (Fig. 4.9). Note that the analysis of progeny was offset by the number of larvae in the initial inoculum. The proportion of *P. entomophagus* adults that established (P) in the cadavers was not significantly different between single, and mixed treatments (z= -0.964, *df* = 28, p=0.335) (Fig. 4.10). Further, the mean number of nematode progeny (F1) of the established *P. entomophagus* was also not significantly different (z= 0.892, *df* = 28, p=0.373) in the single (16.9 ± 8.23 S.E.) and mixed (8.20 ± 6.61 S.E.) infection groups (Fig. 4.11).

4.4. Discussion

In this study, I investigated the infection outcomes of slugs either single or doubly exposed to *P. californica* and *P. entomophagus* (mixed treatments). The results revealed that the ability of *P. californica* to cause mortality in slugs remained comparable even in the presence of *P. entomophagus.* I observed a 40% mortality in slugs exposed to *P. californica* compared to controls, while in mixed nematode treatments, *P. californica* caused 27% mortality. However, this difference in mortality between the two treatments (single vs. mixed) was not statistically significant. Further, there was no significant difference in the proportion of *P. californica* established inside the host slugs in both mixed and single infections. However, the reproductive success of *P. californica* was severely reduced in mixed treatments, which resulted in significantly lower progeny production. Hence, the co-occurrence of these two nematodes

resulted in asymmetrical outcomes, with *P. californica* reproductive success being adversely affected by the presence of *P. entomophagus*, but not the other way around.

Campos-Herrera *et al.* (2015) studied two entomopathogenic nematode (EPN) species (*Steinernema kraussei* and *Heterorhabditis megidis)* separately in the presence of scavenger, free-living nematode (FLN) isolates of the genus *Oscheius* to assess the interspecific competition of these species sharing a common host (larvae of the greater wax moth, *Galleria mellonella*). Their results revealed that the host larval mortality was significantly lower in EPN-FLN mix treatments compared to the corresponding EPN-single application treatments. Thus, the pathogenicity of the two EPNs was negatively influenced by the FLNs. However, in my study, the mortality rate of the slugs due to *P. californica* remained unchanged even in the presence of *P. entomophagus.* I also observed no difference in the establishment success (after successful penetrations) of *P. californica* in single and mixed treatments, hence would hypothesize no interference between adults when it comes to establishment. The number of *P. entomophagus* established in slug cadavers was also comparable in both single and mixed treatment groups. The nematodes that establish in a host are important in determining the rate of progeny production inside the host (Koppenhöfer *et al.,* 1995). A higher intensity of infection inside the host should yield a higher total number of progenies. *P. californica* showed no difference in their success in invading the host in mixed and singles. Yet, I observed a lower reproductive success of *P. californica* in mixed treatments compared to single treatments, likely due to competition with *P. entomophagus*, even as *P. entomophagus* progeny production remained unchanged in the presence of *P. californica. P. entomophagus* as eurystomatous (wide-mouthed) morphs with omnivorous feeding habits have the potential of supplementing its bacterial diet by predating on the larvae of other nematode species (Lightfoot *et al.,* 2021). Certain species of the genus *Pristionchus* such as *P. pacificus* possess intraguild predatory properties on the larvae of other nematodes, especially *C. elegans* (Quach & Chalasani, 2020). As a close relative, *P. entomophagus* may be preying on the larvae of *P. californica* resulting in a low *P. californica* progeny yield. Since *P. californica* and *P. entomophagus* can occupy similar host species, *D. reticulatum* (Brophy *et al.,* 2020b; Patuwatha Withanage *et al.,* 2023), it is plausible that they could be involved in resource competition. Given that *P. entomophagus* has a short (faster) life cycle (4-5 days) compared to *P. californica* (4-21 days), it is plausible that both adult and F1 individuals of *P. entomophagus* indirectly compete with *P californica* for bacteria on the cadaver,

resulting in low juvenile production and/ or survival of *P. californica* to adulthood. I also noticed that *P. entomophagus* progeny production remained unchanged in the presence of *P. californica.*

An application of *P. californica* as a biocontrol agent against slugs might be less efficient if *P. californica* progeny production is decreased by *P. entomophagus.* This might significantly impact the recommended persistence of *P. californica* as advised by the manufacturer under field conditions (e.g., Nemaslug 2.0® persists approximately for six weeks- BASF product label). If a single application does not successfully control slugs long enough, any product based on *P. californica* would cost extra for more frequent applications under field conditions. However, I coinfected the slugs with these two nematodes in an extremely restricted space (3.5cm in diameter tubes), thus there is a possibility that limited space might have worsened the effects. Further, these effects might be influenced by other biotic factors such as nematode density. At higher densities, we may expect to see *P. entomophagus* engaging in intraguild predation on the dauer larvae of *P. californica.*

Campos-Herrera *et al.* (2015) and Blanco-Pérez *et al.* (2017) also revealed differential reproductive abilities of *S. kraussei* and *H. megidis in the presence of Oscheius*. They observed low progeny production for both *S. kraussei* and *H. megidis* in the FLN–EPN mix treatments compared to single species treatments. Koppenhöfer *et al.* (1995) coinfected the larvae of *G. mellonella* with *Steinernema carpocapsae* and *Steinernema glaseri*. Overall, both EPNs had lower reproductive success, but in comparison, *S. carpocapsae* had a lower progeny production than *S. glaseri.* Koppenhöfer *et al.* (1995) suggested that *S. glaseri* had an intrinsic advantage due to its faster development, outcompeting *S. carpocapsae* for resources. Related to these findings, it would be beneficial to investigate the intrinsic competitive abilities among different *Phasmarhabditis* species as this may determine which species is a better candidate (e.g., more efficient, persistent, and sustainable) for controlling pest slugs.

Egleton *et al.* (2021) tested the relative efficacy of Nemaslug® alone compared to a combination of Nemaslug® and malacopathogenic-baited refuge traps in cool climate vineyards in Southwestern England. The active organism in this product, *P. hermaphrodita* (DMG0001) had no significant effect in controlling bud damage in plot studies, however when in combination with malacopathogenic-baited refuge traps, the reliability of the Nemaslug® treatment was increased. Overall, it was shown that this product has its limitations under field conditions.

Further, the DMG0001 strain of *P. hermaphrodita* has been in the product for 25 years, long enough that this strain has become less virulent to slugs (Cutler & Rae, 2020). Therefore, it would be useful to investigate other potential malacopathogenic nematodes, especially those in the genus *Phasmarhabditis* as alternatives to DMG0001. The trade of Nemaslug® is also limited to certain European countries where *P. hermaphrodita* naturally occurs. To date, the use of this product is not yet available in Canada. *P. californica* might be a better alternative for a malacopathogenic product in Canada since it has been isolated in several invasive slug species including *D. reticulatum* and *A. rufus* (Brophy *et al.,* 2020a; Brophy *et al.,* 2020b; Patuwatha Withanage *et al.,* 2023) in Canada. In my study, I demonstrated that this strain of *P. californica* (DL 310) is capable of causing mortality in *D. reticulatum,* even in the presence of a potentially antagonistic nematode. However, given that the reproduction of *P. californica* can be hindered by the presence of other nematodes, it is important to understand the implications of co-occurrences/co-infections on the long-term persistence of *P. californica* in the soil and its efficacy as a biological control agent.

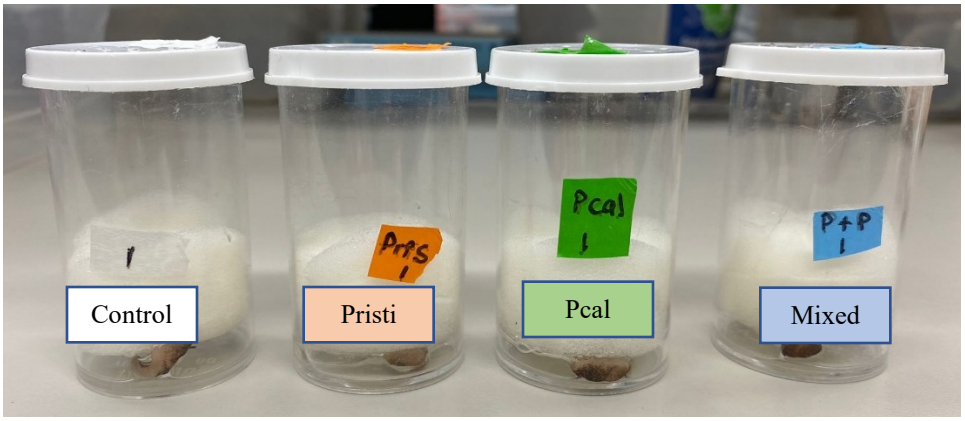

Fig 4.1. Infection vials for each treatment group: single *P. californica* (Pcal), single *P. entomophagus* (Pristi), mixed infections with both *P. californica* and *P. entomophagus* in combination (mixed) and control. Each vial contains a foam plug pushed approximately 1cm to the bottom to ensure direct contact between the slug and the nematodes (if present). The exposure period lasted five days.

Fig 4.2. Slugs are individually maintained in vials with food (carrots) post-exposure until censored.

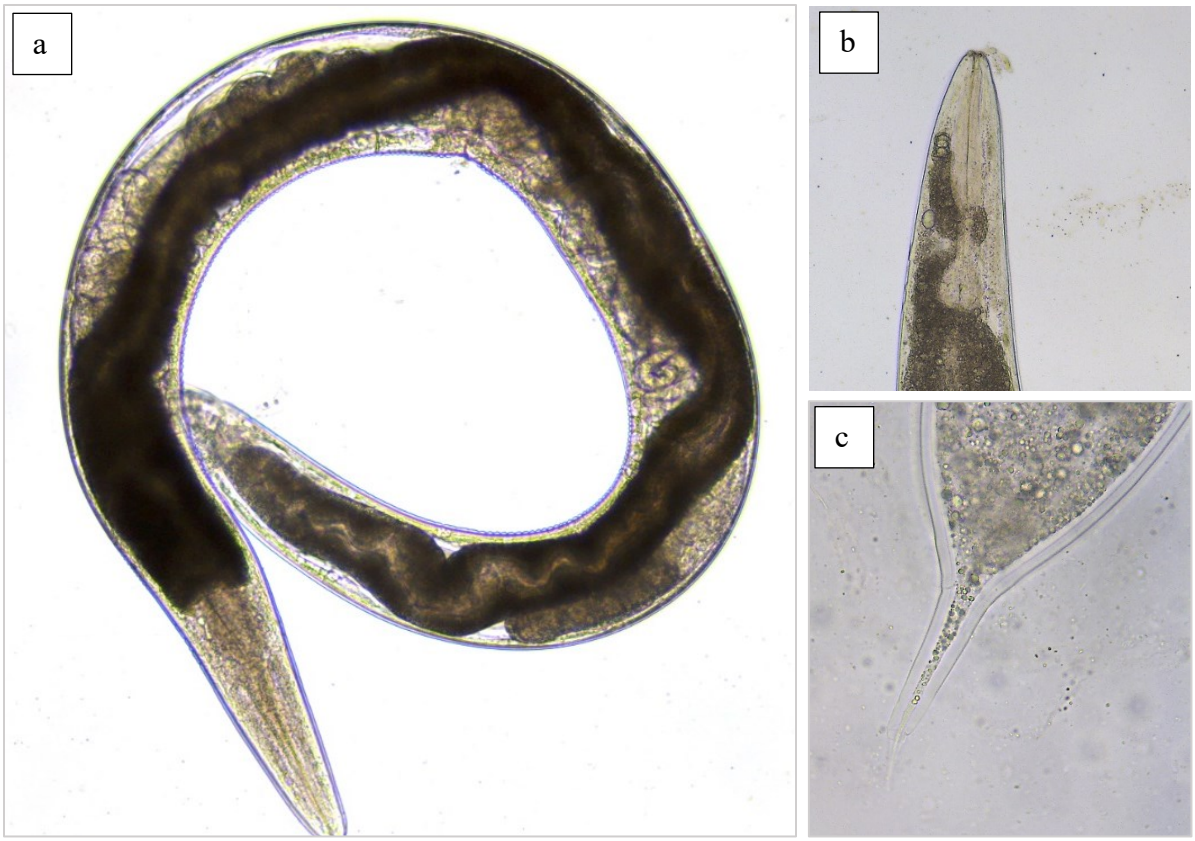

Fig 4.3. *Phasmarhabditis californica*: a) a gravid female, b) oral region without any teeth-like structures, and c) the conoid tail with a hyaline tip.

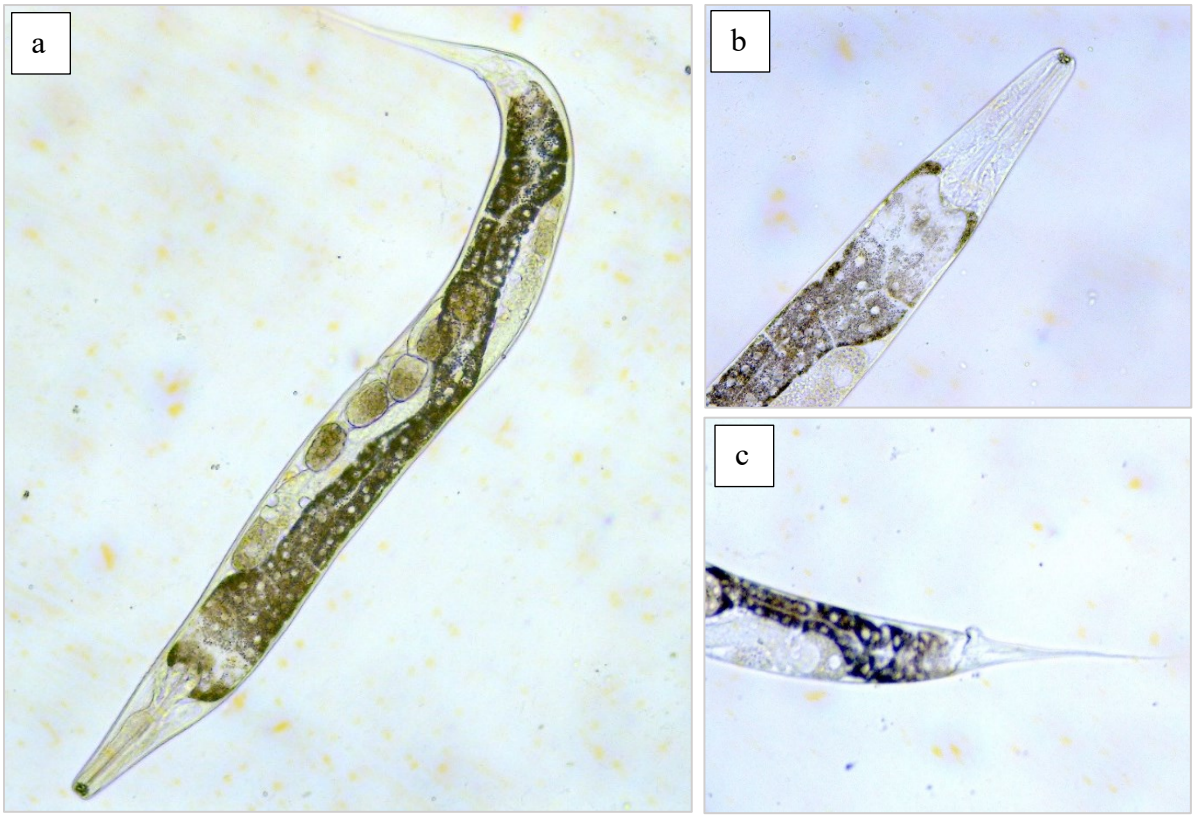

Fig 4.4. *Pristionchus entomophagus*: a) a gravid female, b) oral region with teeth-like structures, and c) the elongated tail.

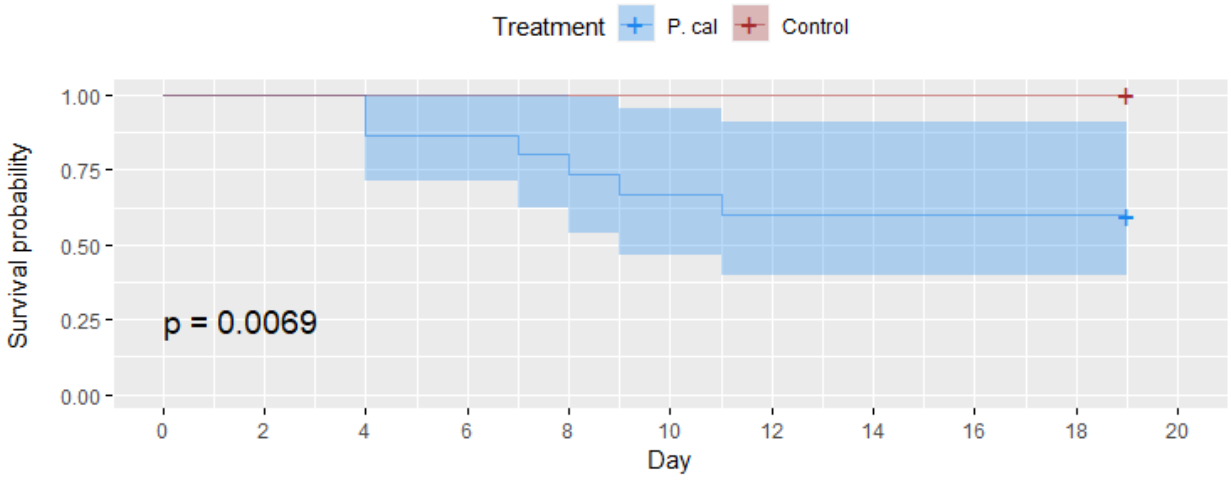

Fig 4.5. Survival probability estimates of single *Phasmarhabditis californica* infections versus control (the Kaplan-Meier method) including 95% confidence bands. The infections lasted five days, followed by the post-infection period of days. The experiment was censored on day 19.

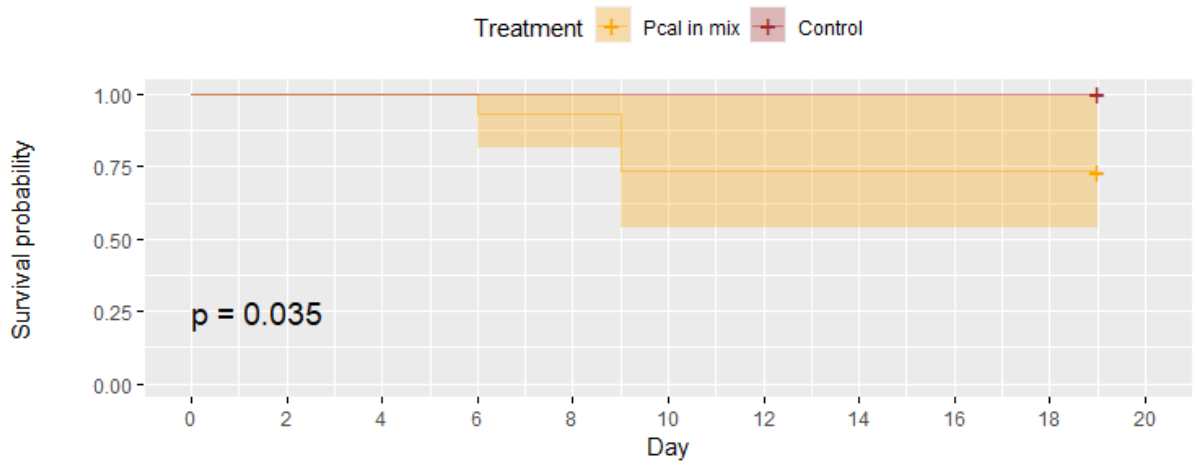

Fig 4.6. Survival probability estimates of Mixed *Phasmarhabditis californica* infections versus control (the Kaplan-Meier method) including 95% confidence bands. The infections lasted five days, followed by the post-infection period of 14 days. The experiment was censored on day 19.

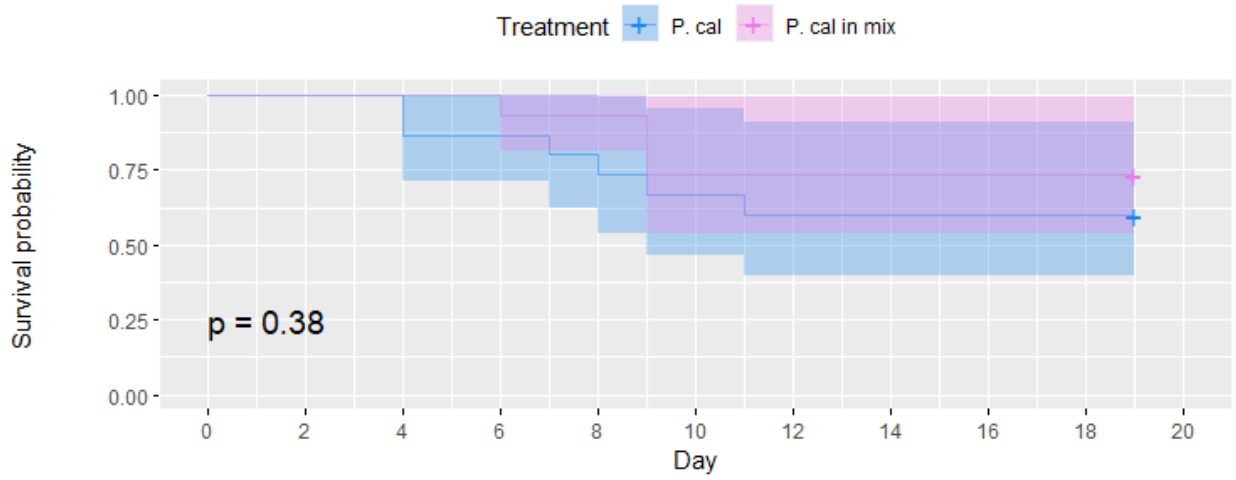

Fig 4.7. Survival probability estimates of Mixed *Phasmarhabditis californica* infections versus single *P. californica* (the Kaplan-Meier method) including 95% confidence bands. The infections lasted five days, followed by the post-infection period of 14 days. The experiment was censored on day 19.

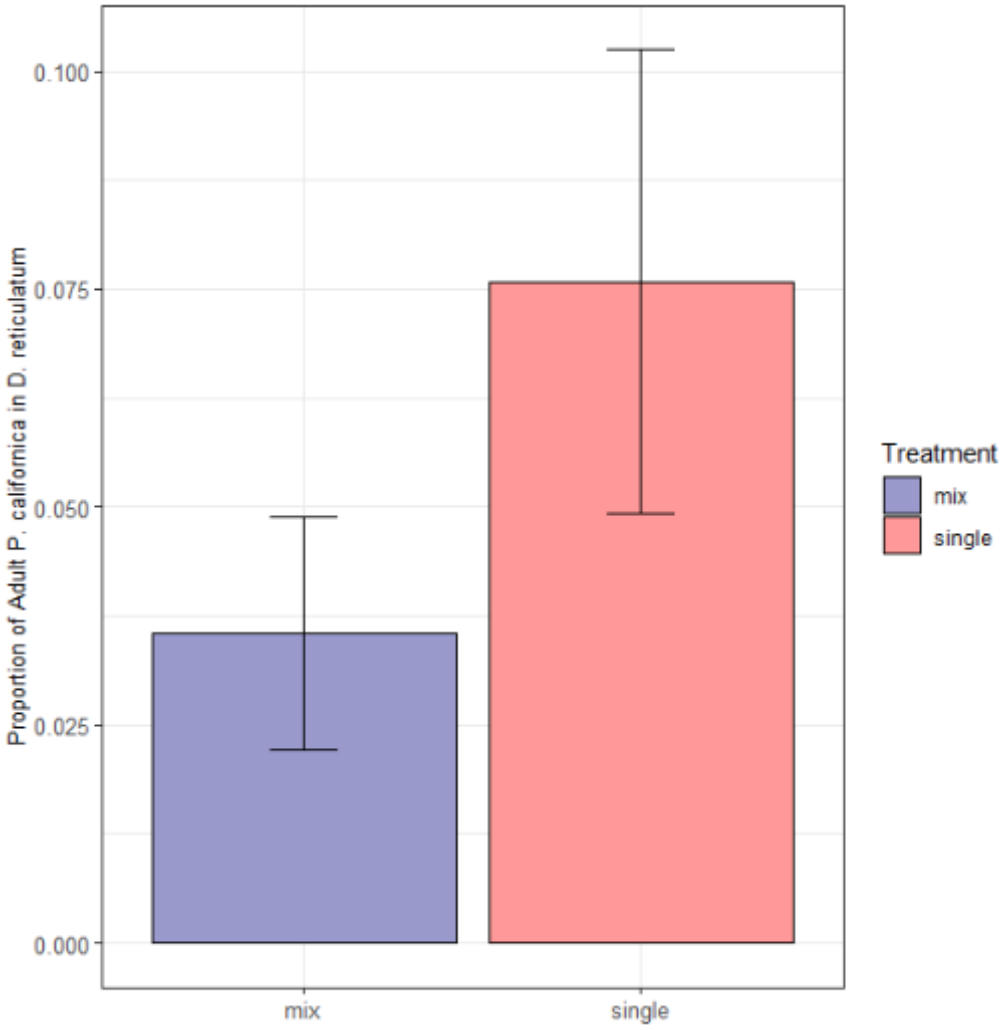

Fig 4.8. The proportion of adult *Phasmarhabditis californica* that established given the initial inoculum in single versus mixed infection groups (z=1.635, *df= 28,* p= 0.102). In single and mixed treatments 1800 and 900 IJs of *P. californica* were inoculated respectively. Error bars represent standard error.

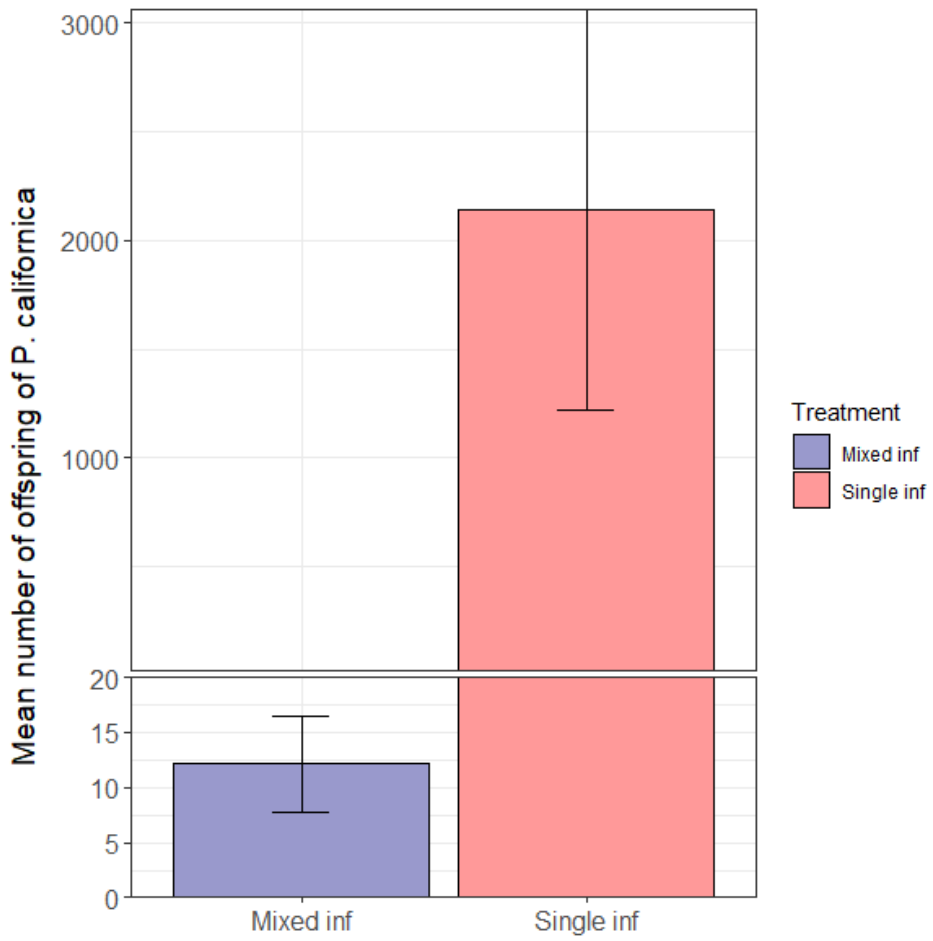

Fig 4.9. The mean number of offspring (F1) *Phasmarhabditis californica* produced on slug cadavers from single (2140±921 S.E.) and mixed infection groups (12.1±4.36 S.E.) ($z= 6.979$, *df= 28,* $p<0.001$). Y-axis- break at 20 individuals to better visualize the number of F1 individuals in mixed treatments.

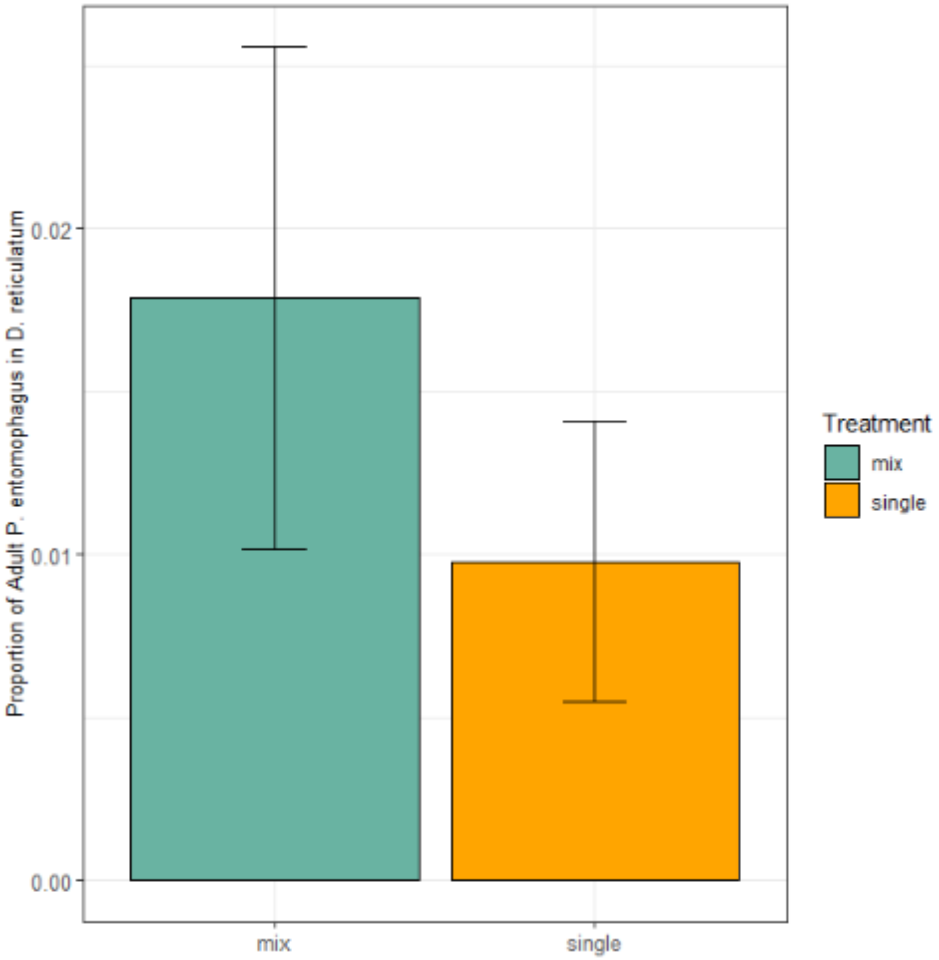

Fig 4.10. The proportion of adult *P. entomophagous* in slug cadavers given the initial inoculum from single and mixed infection groups (z= -0.964, *df* = 28, p=0.335). In single and mixed treatments 1800 and 900 IJs of *P. entomophagus* were inoculated respectively.

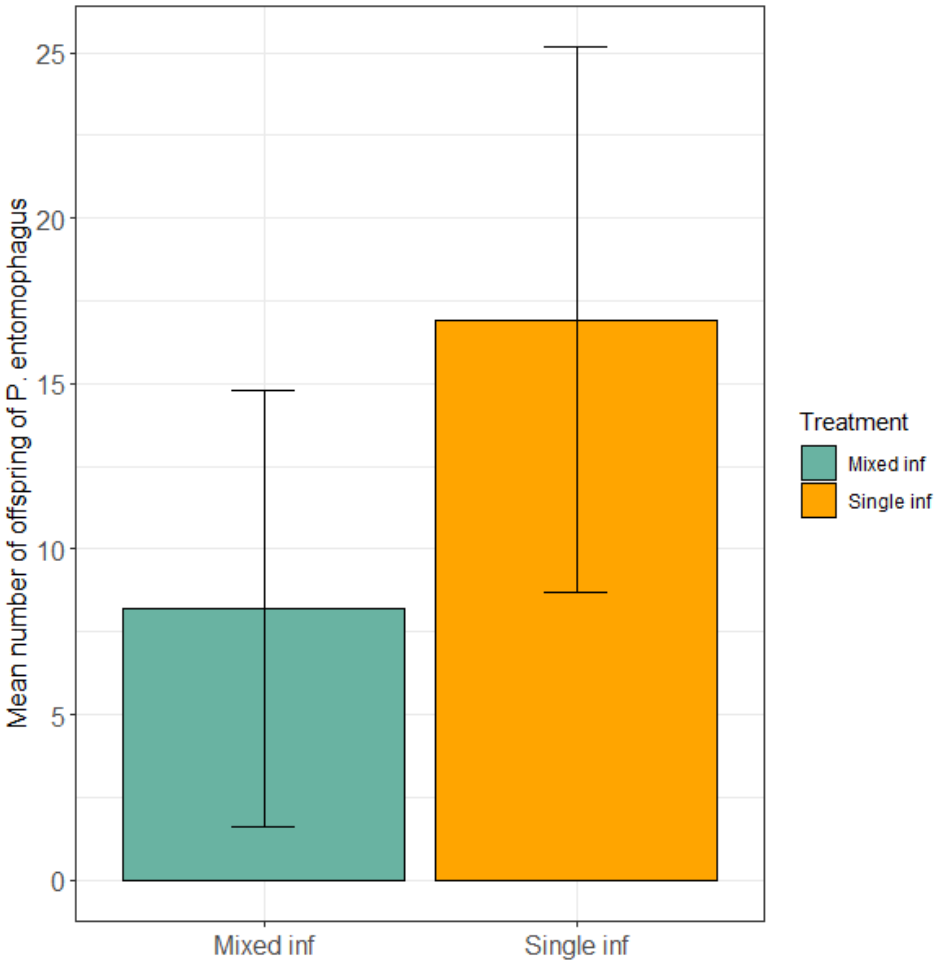

Fig 4.11. The mean number of juvenile *Pristionchus entomophagus* produced on slug cadavers in single (16.9±8.23 S.E.) and mixed (8.2±6.61 S.E.) infection groups (z= 0.892, *df* =28, p=0.373).

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Chapter 5

Conclusions and future directions

In my first study, I conducted a comprehensive slug-nematode survey as a follow-up to the survey conducted by Brophy *et al.* (2020a, 2020b) to confirm the presence of the novel Canadian strain of *P. californica* in Alberta. Even though I was unable to isolate it in my survey of agricultural and horticultural sites, I was able to isolate *P. californica* from *D. reticulatum* samples received from a residential garden in Lethbridge, Alberta. Therefore, I suspect a fragmented distribution of *P. californica* within the province. This might open up future research avenues on using malacopathogenic nematodes (MPN) in a sustainable control strategy of pest slugs in Canada. I further summarized the terrestrial slug species found in agricultural fields, nurseries, and greenhouses. However, this study does not address the ecological and economic impacts of the slug species found in agricultural and horticultural sites in Alberta, which is in dire need of attention. For instance, the following have been reported in the 51st Annual Meeting of Western Committee on Crop Pests (2011):

"Grey garden slug infestation in MB[Manitoba]destroyed a late-seeded canola crop."

"Once again, several reports of slug damage in crops. One report was very severe on a late seeded canola crop in central Alberta. This is most likely the common grey garden slug (Deroceras reticulatum)." – Alberta crop insect update 2011

"Wet conditions were favourable for slugs. There have been reports in previous years in various areas in the province. However, infestations were largely restricted to low lying, wetter areas of fields. In 2011 reports of infestations included large portions to full fields. Some reports were from the southwest, a normally semi-arid region of the province. There are control options but not economically viable on a large field scale situation."- 2011 Saskatchewan Insect Report

S. Hartley, a provincial specialist on insect and vertebrate pests from the Saskatchewan Ministry of Agriculture, highlighted an increase of the slugs in one of the high-value crops, strawberries. According to Hartley's report, *"Slug populations were above average in a few patches and seem to be more prevalent in the central to northwest regions".* However, the report does not provide any information regarding the extent of crop damage caused by slugs, or the specific species of slugs involved.

However, identifying economically important pest slug species in Alberta, or on a large scale throughout Canada, is a huge research gap that needs to be filled. Over the past two decades, there were about 89,000 google scholar hits for the keywords "Agricultural pests, Canada", however only 7,480 hits were reported for "Agricultural pests, **slugs**, Canada" during this time frame. In contrast, the search words "Agricultural pests, **insects**, Canada" revealed about 24,800 articles. These numbers indicate a greater focus on entomological pests than gastropod pests in Canada. In this study, I tried to fill the gap a little by confirming the identity of nine different slug species in the survey sites. *D. reticulatum* was the most common in agricultural sites while *D. laeve* (likely both native and introduced populations) was the most common in nurseries and greenhouses, both of which are considered major pests in North America (Chíchester & Getz 1969; Prystupa, 1983). Further, less attention has been paid to slug-associated nematodes. Given that *P. californica* is malacopathogenic and has the potential to be an important biocontrol agent, it is necessary to study this organism more thoroughly. However, a Google Scholar search for *P. californica* results in only 109 hits likely due to the fact that this nematode species was only discovered in 2016. In comparison, *P. hermaphrodita,* which was first discovered almost 150 years ago (Rae *et al.,*2007), yielding about 564 articles.

To further our knowledge on *P. californica,* I demonstrated the chemoattraction profile of the novel strain of *P. californica* to the mucus of four pest slug species in Alberta. I also investigated the chemoattraction profile of a necromenic nematode species, *P. entomophagus*. The results revealed chemoattraction to a single host, *D. reticulatum* by both nematodes. This could raise the possibility of interspecific interaction between the two species occupying the same host. The attraction and co-occurrence of both species to a shared host may lead to a decrease in the long-term efficacy of *P. californica* in causing mortality to slugs. The fourth chapter of my thesis examines the mortality of the host slug, *D. reticulatum* by *P. californica*, establishment success, and progeny production of *P. californica* in the presence of *P. entomophagus.* The chapter reveals that the mortality caused by *P. californica* in slugs remained comparable even in the presence of *P. entomophagus*. Although the reproductive success of *P. californica* was significantly reduced in mixed treatments, there was no significant difference in the establishment of *P. californica* within the host slugs between the single and mixed infections. The study further suggests that *P. californica* could be a promising biocontrol agent for slugs, as its infectivity and establishment within the host were not affected by *P. entomophagus*, yet the

reduced reproductive success in the presence of *P. entomophagus* may impact the long-term persistence and efficacy of *P. californica* as a recommended biological control agent. Hence, future studies on the cost-effective dose of MPNs used, length of infection, host biology, nematode biology, and development, are important in deciding whether *P. californica* is a good candidate for biocontrol of pest slugs in Canada. The nematodes do not exist alone but interact with other nematodes in the soil community. Therefore, it is equally important to consider the local nematode fauna associated with slugs to better understand the consequences of this niche overlap. In situations with multiple pest infestations (e.g., pest insects and pest slugs), simultaneous application of EPNs and MPNs could result in interactions of different species in the same environment. Therefore, studying the interaction between EPNs and MPNs would be another direction for future research. I highly recommend a risk assessment be made to determine the impact of *P. californica* on non-target host species before any decision is undertaken. Other than biotic factors, abiotic factors such as soil pH, soil temperature, and soil humidity affecting the viability and persistence of this nematode. Given that there is only a limited set of pesticide options against slugs, managing these agricultural pests with sustainable and effective biocontrol solutions should be pushed forward. There are only two malacopathogenic nematode products currently available (Nemaslug®, Nemaslug® 2.0), both based on nematodes in the genus *Phasmarhabditis,* thus more research should be conducted to investigate other nematode families with biocontrol properties (e.g., EPNs with killing properties) that can be formulated into commercial biocontrol products against pestiferous slugs.

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Appendices

Information adapted from:
Slugs: a guide to the invasive and native fauna of California (Mc Donnell, 2009)
https://idtools.org/id/mollusc/index.php

Photos by: Dayani Buddhika Maheshini Patuwatha Withanage

Figure 1: 1A-1D: *Deroceras reticulatum*: 1A, Extended and contracted *D. reticulatum,* the arrowhead points towards the keel; 1C(a) concentric striations on the mantle and (b) the milky color mucus upon poking; 1D, mating of *D. reticulatum*

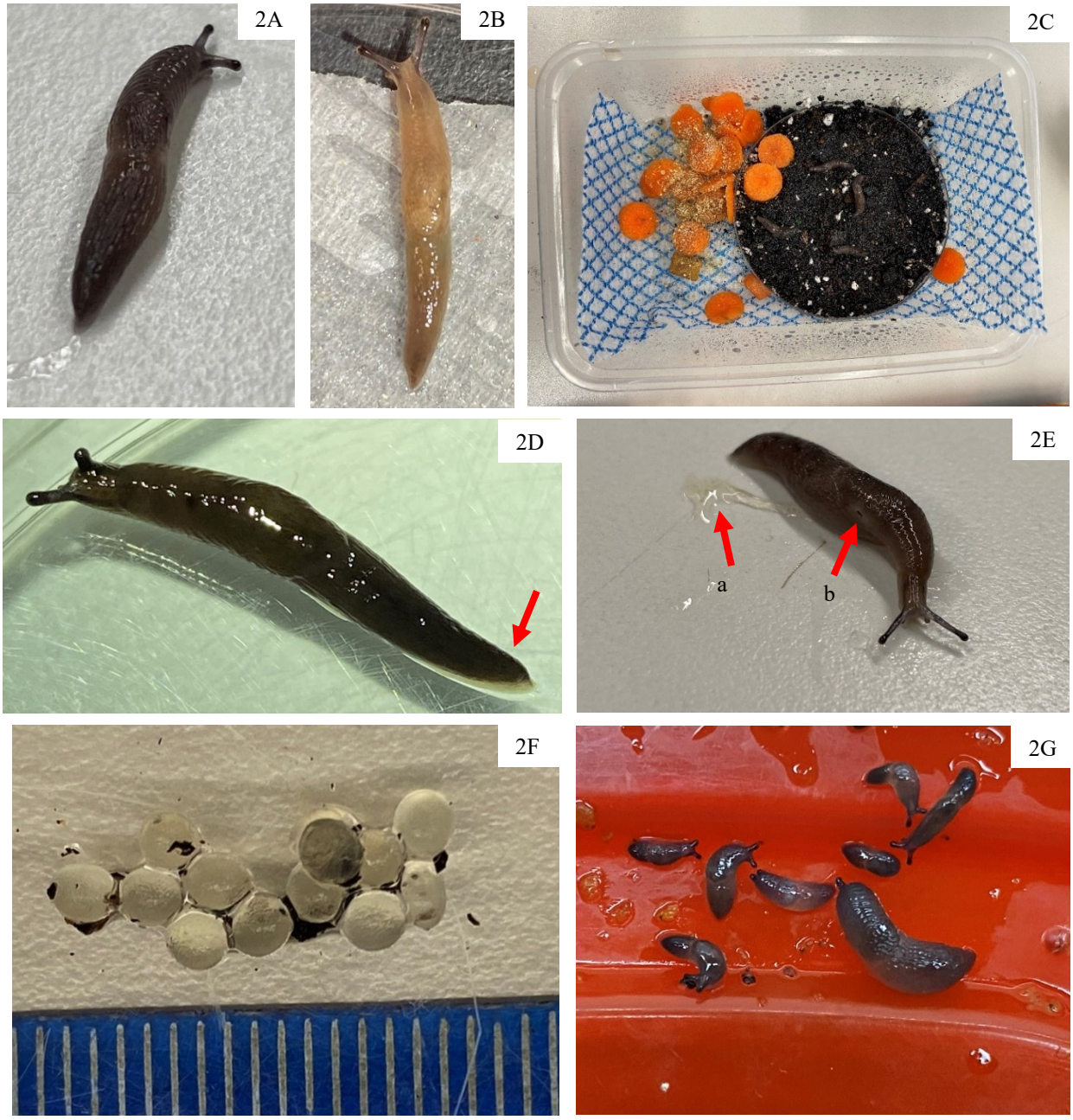

Figure 2: 2A-2G: *Deroceras laeve*: 2A-2B, different color morphs of *D. laeve*; 2C, a habitat of the breeding colony of *D. laeve*; 2D, showing the keel; 2E (a)colorless mucus; 2E (b)the position of pneumostome; 2F, eggs of *D. laeve*; 2G, neonates of *D. laeve*

Figure 3: 3A-3F: *Ambigolimax valentianus*: 3A, a pair of *A. valentianus*; 3B-3C, different morpho types of *A. valentianus* encountered during the survey; 3D, the sole of the slug (Ventral side); 3E, the breeding colony; 3F, the eggs of *A. valentianus.*

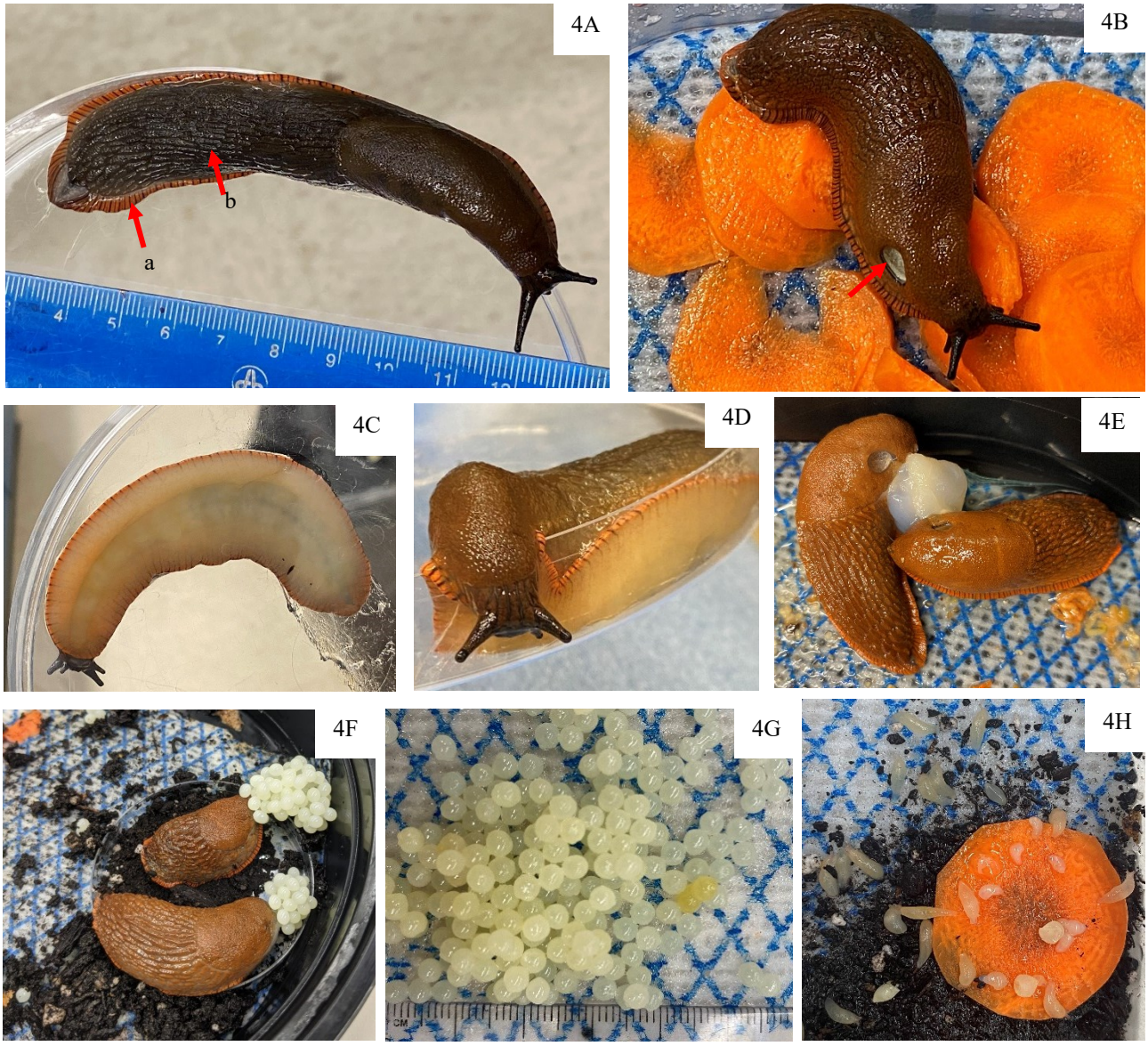

Figure 4: 4A-4H: *Arion rufus*: 4A, A fully extended (~90mm length) *A. rufus* showing (a)the foot fringe and (b)the course tubercles on the back; 4B, *A. rufus* showing the pneumostome; 4C, the tripartite foot (pale color foot with three clearly distinguishable sections); 4D, the head region showing the two pairs of tentacles; 4E, mating of *A. rufus;* 4F, *A. rufus* with egg clutches; 4G, An egg clutch(each egg ~2mm in diameter); 4H, the neonates of *A. rufus.*

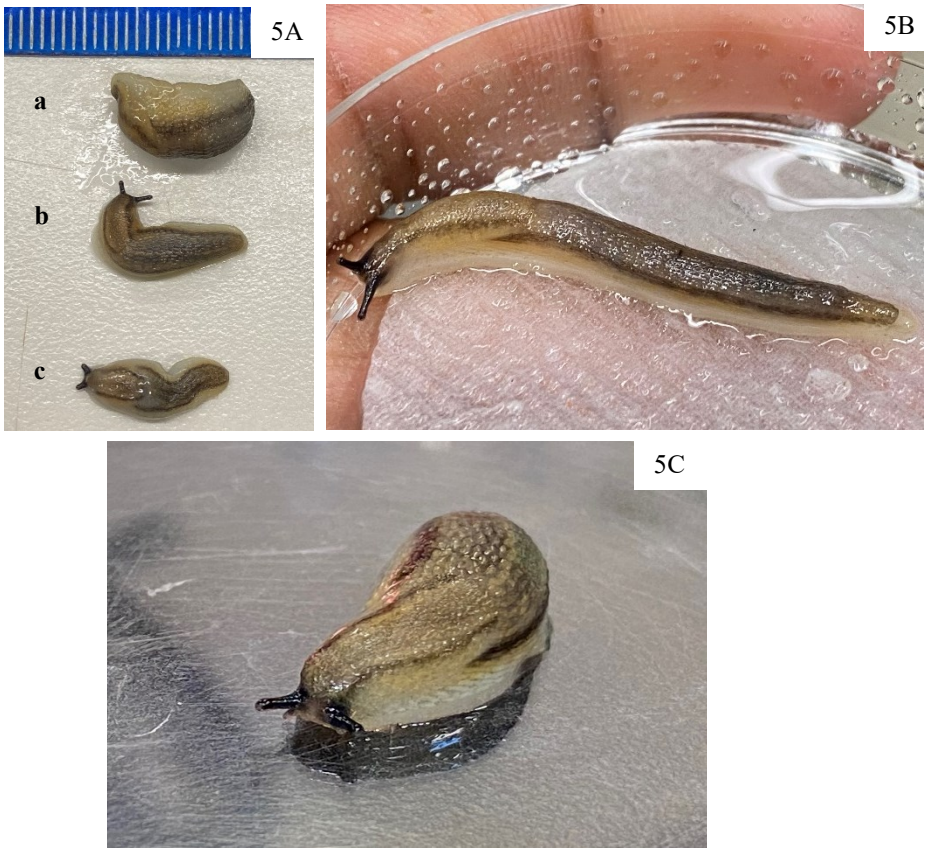

Figure 5: 5A-5C: *Arion fasciatus*:5A, *A. fasciatus* (a)with the contracted bell-shaped body (b-c) the dorsal side of the slug clearly displaying the discontinuous lateral lines at the end of the mantle region; 5B, fully stretched *A. fasciatus* with the yellow flush below the lateral line; 5C, *A. fasciatus* with black color tentacles and the rear mantle region with the discontinuous lateral line

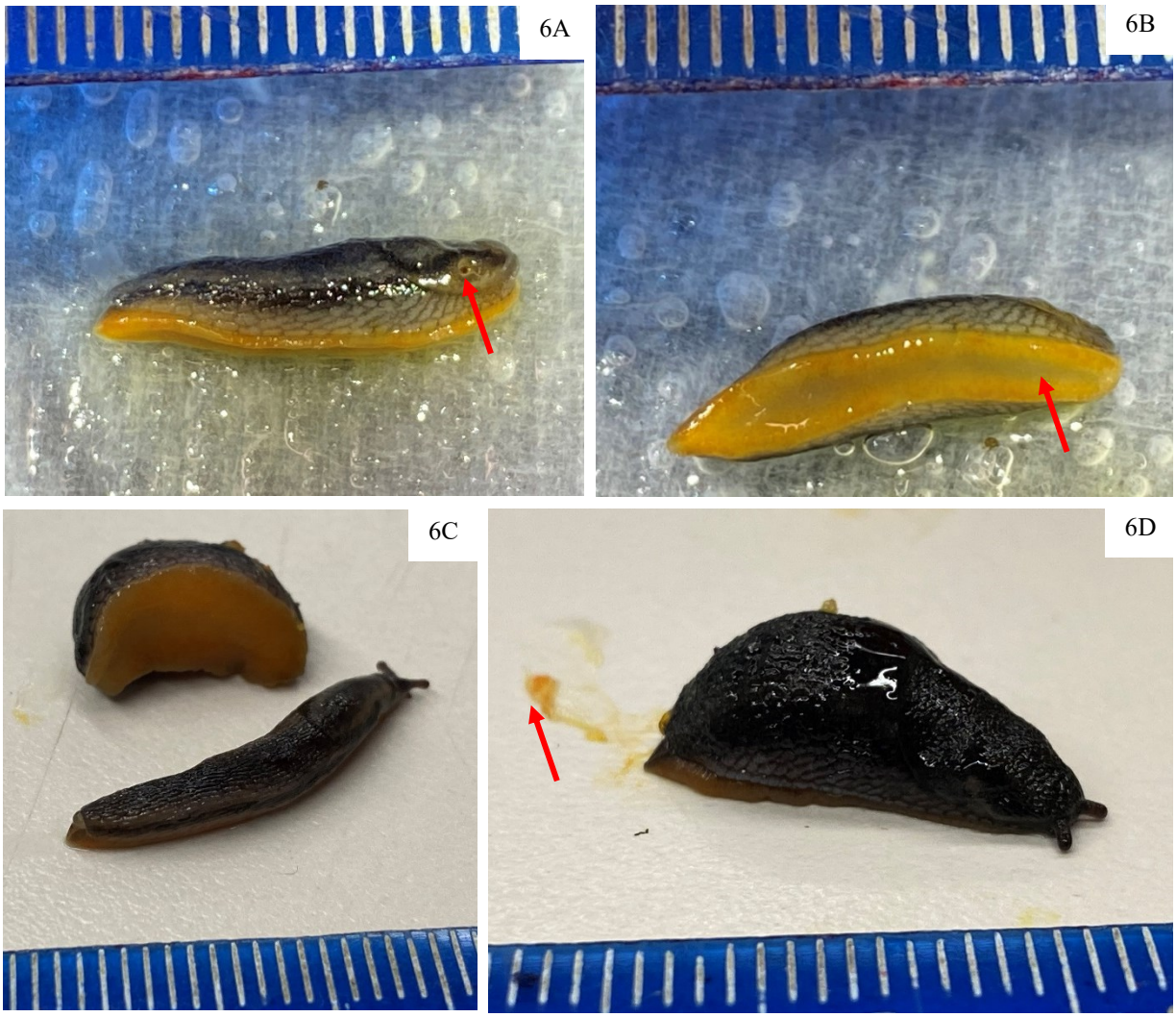

Figure 6: 6A-6B: *Arion hortensis*: 6A, *A. hortensis* displaying the lateral line encompassing the pneumostome(arrowhead); 6B, the yellow- orange color sole; 6C, *A. hortensis* (a) the contracted body and(b) the dorsal side of the slug; 6D, a contracted *A. hortensis* slug with yellow-orange color slime.

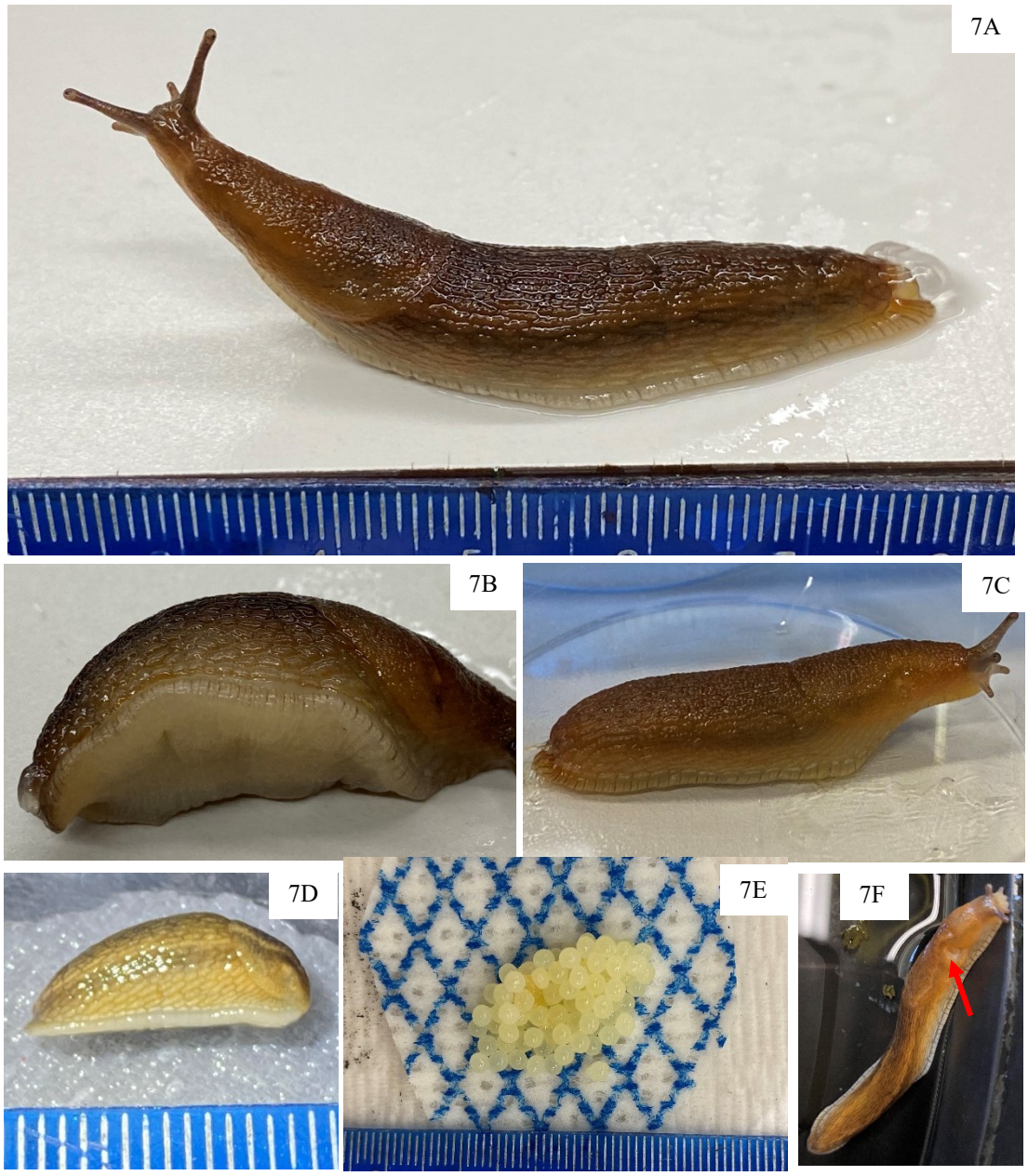

Figure 7: 7A-7F: *Arion subfuscus* :7A, a fully stretched *A. subfuscus* displaying the lateral line; 7B, the dirty white color sole; 7D, the bell-shaped body upon disturbance; 7E, the eggs of *A. subfuscus*; 7F, the pneumostome position of *A. subfuscus*

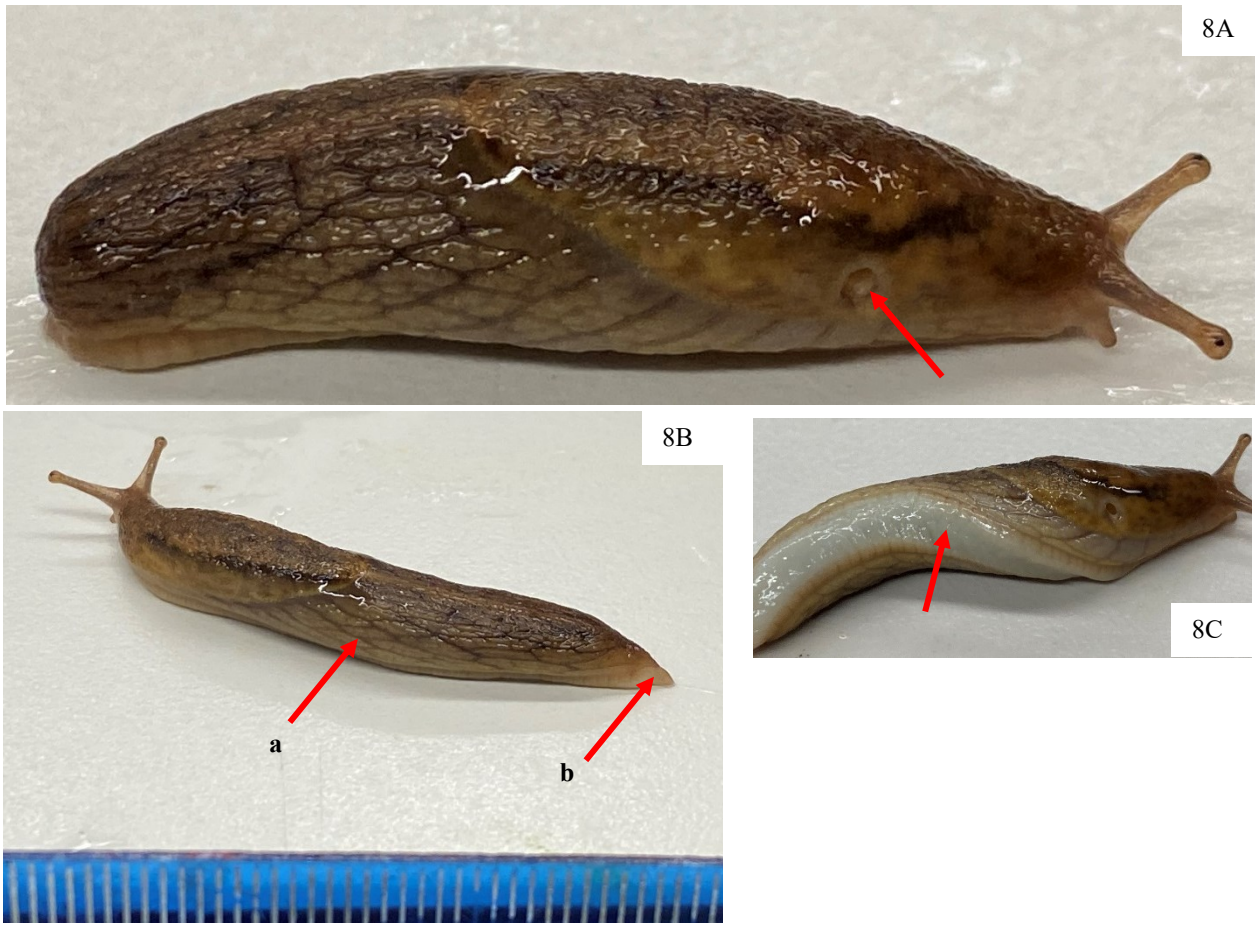

Figure 8: 8A-8C: *Prophysaon andersonii* :8A, an adult *Prophysaon* with its dropped tail, and only the mantle has the lateral line, the arrowhead points to the pneumostome; 8B, (a) the diamond mesh pattern on the back of body, (b) the droppable tail; 8C, the dirty white color sole