Trematodes in the limelight: Division of labour, life cycles and interactions with other symbionts

by

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Abstract

Parasites that require multiple hosts to complete their life cycles can significantly affect the ecology of their hosts and ecosystems. Trematodes are obligatory parasites that use snails as first intermediate hosts and vertebrates as definitive hosts. There are four genera of freshwater snails in Alberta (*Lymnaea*, *Physa*, *Planorbella* and *Stagnicola*) whose trematode communities remain largely undocumented. Effects of these parasites on their snail hosts are also unknown. I surveyed multiple sites that varied in trematode prevalence and species richness. Through DNA barcoding, I reported for the first time in Alberta the presence of *Ribeiroia ondatrae* and *Drepanocephalus spathans*, two trematodes of importance for wildlife conservation. Using *in-vitro* techniques and behavioural analysis, I provided for the first time in Canada evidence for the existence of division of labour in freshwater trematodes, a strategy only documented in some marine species so far. I tested for effects of trematode infection on snail host survival and showed that survival differed among snail genera, influenced by location and intensity of infection. I studied symbiotic relationships among snails, trematodes, oligochaete worms (genus *Chaetogaster*) and leeches (genus *Helobdella*). Oligochaete worms are likely attracted to snails shedding cercariae, whereas leech presence did not seem to be related to trematode infection of their snail host. I also provided, for the first time in Alberta, plausible complete life cycles for 20 trematode species that use snails as first intermediate hosts, snails, leeches, fish and amphibians as second intermediate hosts, and birds and mammals as definitive hosts.

Preface

This thesis is an original work by Mónica Ayala-Díaz. No part of this thesis has been previously published. This work was supported by The Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant, an Alberta Conservation Association Grant in Biodiversity, a Consejo Nacional de Ciencia y Tecnología (CONACYT) Programa de Becas en el Extranjero grant, and teaching assistantships provided by the Department of Biological Sciences, University of Alberta.

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Chapter 1: Introduction

Ecological interactions: definitions and ecological importance of parasitism

Interactions among species in an ecosystem vary depending on the evolutionary history and environmental conditions in which these interactions arise. For this reason, ecological interactions among species are usually hard to define and measure, because these interactions also depend on scale and context (Lang and Benbow, 2013). Ecological interactions can be intra-specific, when the interacting individuals belong to the same species, or inter-specific, when each individual belongs to a different species (Lang and Benbow, 2013). Depending on their nature, ecological interactions can be divided in broad terms into competition, predation, herbivory and symbiosis (Lang and Benbow, 2013). In this thesis, I will focus on competition and symbiosis. In competitive interactions, individuals share resources and thus, all individuals involved incur costs. However, costs are not necessarily equally shared. One individual can suffer lower costs when it outcompetes another individual that, for a variety of reasons, is at a disadvantage in the competitive interaction (Lang and Benbow, 2013).

Symbiotic relationships are very common in nature (Paracer and Ahmadjian, 2000). Symbioses establish among two or more different species that live in close proximity, interact with each other, and share resources. Symbioses are categorized depending on the effects of one species on the other, and reciprocally. In this sense, the most widely accepted types of symbioses are mutualism, commensalism and parasitism. In mutualistic symbioses, both species benefit from the association. In commensalism, only one of the symbiont species benefits from the association, while the other species neither benefits nor is harmed. In parasitism, one of the symbionts benefits from the association at the detriment of the other, which suffers costs. Symbiotic relationships can be obligate if one or both of the organisms involved depend on the association for survival. When both symbionts can survive without each other, the symbiosis is facultative. Another dichotomy in symbioses relates to the location of one symbiont relative to the body of the other. In ectosymbiosis, the smaller-bodied symbiont lives on the outside surface of the larger-bodied host, and in endosymbiosis the symbiont lives inside the body of its host.

Parasitism has evolved hundreds of times in both prokaryotic and eukaryotic clades. It is likely the most common life strategy on the planet. Most multicellular organisms have at least one species of parasite living in or on them at one point in their lifetime (Bush et al., 2001). Parasites are thus likely the most abundant organisms on earth and their biomass can exceed that of top predators in some ecosystems (Kuris et al., 2008). Parasitic organisms can alter host population dynamics by reducing individual survival and fecundity, altering host behaviour and changing processes of sexual selection, acting as a selection agent on their host's phenotype and decreasing the reproductive potential of their host (Claereboudt and Bouland, 1994; Takahashi et al., 1997; Abbot and Dill, 2001; Marino et al. 2017; Friesen et al. 2020). Ecologically, parasites affect host populations similarly to predators' sublethal effects on prey behaviour. The "ecology of fear" also applies to parasites as hosts invest resources in immune responses to infection and avoidance behaviours (McLaughlin et al., 2020; Koprivnikar and Penalva, 2015; Koprivnikar et al., 2021). When incorporated into studies of energy flow and food web dynamics, parasites increase complexity and connectivity of food webs. They can dominate food web structures, being part of up to 75% of trophic interactions in an ecosystem (Lafferty et al., 2006; McLaughlin et al., 2020; Bennett et al., 2024). In some cases, parasites can effectively alter energy flow through a whole ecosystem and across ecosystems, by creating an alternative nutrient-rich food source that would not exist if parasites did not facilitate the process. For example, parasitic nematomorphs alter the behaviour of their insect hosts and drive these to jump into streams and lakes, where the parasite completes its life cycle (Thomas et al., 2002). The biomass of insects driven to the water by the parasite can be so large that in some ecosystems, fish can switch to feeding primarily on terrestrial insects (higher nutritional value) instead of benthic crustaceans (lower nutritional value) (Sato et al., 2011). In spite of the key influence of parasites on hosts and ecosystems, these organisms are often overlooked in ecological studies. It is thus necessary to increase our knowledge of parasite abundance, diversity and ecology to build a more holistic understanding of ecological and evolutionary processes.

Parasite ecology focuses on the study of parasite communities and the processes that influence parasite assemblages associated with particular hosts (Poulin, 1995). Most studies are based on descriptions of the composition of communities of multicellular organisms (i.e., macroparasites) and the roles of different parasite species in the infracommunity (i.e., within a single host), interactions among individuals of different parasite species, and interactions between parasites and their host (e.g.,

Esch et al., 2001; Poulin, 2023; Presswell et al., 2023; Dutra and Poulin, 2024). How other symbiotic relationships such as mutualism, competition and predation are affected by parasitism is generally less studied. Effects of parasitism on predation of infected hosts are influenced by the transmission strategy of the parasite. For example, trophically transmitted parasites benefit from increasing the predation risk of their host (Hasik et al., 2023). Conversely, parasite effects on host competition appear to be reliant on the nature of the host's habitat. Because parasites affect relationships between predator and prey differently depending on the host habitat, parasitism is generally more detrimental to host competitive abilities in freshwater and marine environments than in terrestrial ecosystems (Hasik et al., 2023).

Biology of trematodes

Trematodes (Platyhelminthes) are a diverse group of obligate parasitic flatworms that infect a range of vertebrate species as definitive hosts. Depending on species, trematodes require between one and four hosts of different species to complete their life cycles (i.e., a single generation). It is currently accepted that the ancestral trematode life cycle comprises three different hosts. From this ancestral life cycle, shorter or longer life cycles have evolved (Poulin, 1998; Bush et al., 2001; Esch et al., 2001). The three-host life cycle is typical for the majority of trematode species. Adult trematodes live and sexually reproduce in a vertebrate definitive host, releasing their eggs into the environment with the host feces. Depending on trematode species, eggs hatch into the external environment, most often water, as a free-living larval stage called miracidium, which actively seeks and penetrates the skin of the trematode's first intermediate host, usually a snail. In other trematode species, first intermediate snail hosts acquire trematode infections by accidentally ingesting trematode eggs while grazing. In the latter case, the ingested egg hatches into a miracidium only when inside the snail host. In both transmission strategies, miracidia that successfully infect the snail develop into clonal colonies of either sporocysts or rediae, depending on trematode species. Rediae have mouthparts and a rudimentary gut used to ingest snail tissue. Sporocysts lack these structures and feed by absorption of nutrients through the tegument (Esch and Fernandez, 1994). Sporocysts and rediae both reproduce asexually and produce hundreds of cercariae, the next free-living larval stage for trematodes. Cercariae then usually leave the snail to actively infect other invertebrates, fish or amphibians that are used as second intermediate hosts. Some trematode species use snails as both first and second intermediate

hosts (Evans and Gordon, 1983; Fried et al., 1997; Esch et al., 2002; Zimmerman et al., 2015). Cercariae can either relocate to different and more suitable organs within the snail individual where they were produced, or they can leave the snail of origin and actively infect other snail individuals by swimming into their pneumostomes (Huffman and Fried, 2012). Once inside the second intermediate host, cercariae shed their tail and mature into metacercariae, which can either develop protective layers, form a cyst, or can remain inside the second intermediate host without encysting. In most cases, metacercariae reach the vertebrate definitive host, where they fully mature, when second intermediate hosts are preyed upon by the definitive host (Esch et al., 2002).

Some trematode genera are of concern for veterinary and human health. Among those are the species responsible for schistosomiasis, a chronic and acute disease affecting livestock, wildlife and millions of people in tropical regions. Schistosomiasis is difficult to treat and eradicate. The disease affects primarily urogenital, lung and liver functions, but can also cause life-threatening complications during pregnancy in humans (Aribodor et al., 2024; Carrim et al., 2024; Tallima et al., 2024). Schistosome trematodes have a reduced two-host life cycle, snails being their only intermediate host. Cercariae are produced asexually then leave the snail and actively seek and infect their definitive host by penetrating through the skin. Some schistosomes do not use humans as definitive hosts, but cercariae can still penetrate skin and cause skin irritation, known as swimmer's itch. Swimmer's itch is generally mild, but allergic reactions to cercarial penetration can be very severe. With increasing temperatures due to climate change, swimmer's itch ranges are shifting (Sangiorgio et al., 2024). It is thus important to document the current species composition and distribution of trematode fauna in temperate regions and monitor range shifts to evaluate potential risks to human health.

Trematodes in community ecology

Since the completion of trematode complex life cycles requires that all necessary species are present in an ecosystem, trematodes have been proposed as bioindicators of ecosystem health (Campião et al., 2012; Shea et al., 2012; Shah et al., 2013). Trematode cercariae are released to the environment in high numbers, and can thus be an important nutrient rich food source for other non-host organisms (Morley, 2012; Babaran et al., 2021; Koprivnikar et al., 2023). When trematode cercariae are preferentially preyed upon by non-host species, cercariae can have a considerable impact on food web dynamics in their ecosystem (Mironova et al., 2020; Schultz and Koprivnikar, 2021;

McLaughlin et al., 2020). Further, trematode metacercariae can alter the behaviour and morphology of their intermediate host, affecting host population dynamics. A widely known example of host manipulation is *Dicrocoelium dendriticum* (Rudolphi, 1819) Looss, 1899 (Dicrocoeliidae), which encysts in the suboesophageal ganglion of ants. An ant infected with *D. dendriticum* climbs to the top of a grass blade, where it remains overnight, jaws tightly clasped to the grass blade until temperature rises in the morning (Manga-González et al., 2001). This behaviour is strikingly different from the behaviour of uninfected ants, and is believed to increase the probability that sheep or cattle, the trematode definitive hosts, ingest the infected ant while grazing (Badie et al., 1973). Likely the best-known trematode species that alters its intermediate host morphology is *Ribeiroia ondatrae* (Price, 1931) Price, 1942 (Psilostomidae), which causes severe malformations in frogs. Cercariae of *R. ondatrae* penetrate the skin of tadpoles and encyst as metacercariae in their growing limb buds, mechanically and/or chemically disrupting cell development in the limb buds and affecting hind limb development, resulting in multiple hind limbs in infected frogs (Stopper et al., 2002; Szuroczki et al., 2012). Deformed frogs have mobility issues and are thus more susceptible to predation by birds and reptiles, which are definitive hosts for *R. ondatrae* (Johnson et al., 2001; 2002; Goodman and Johnson, 2011). Excluding examples of host behavioural manipulation and morphological alterations caused by trematodes, metacercarial effects on second intermediate hosts are believed to be either benign or very virulent, depending on trematode species and host age at the time of cercarial penetration (de Montaudoin et al., 2012; Marino et al., 2017; Pathirana et al., 2019a; 2019b; Calhoun et al., 2020). In cases where metacercarial infection affects survival of second intermediate hosts, these alterations can have a great impact at the population, community and ecosystem level.

Trematodes and snails

Except for a small number of marine trematodes, all known species (approximately 25 000 described) use snails as first intermediate hosts (Esch et al., 2001). Trematodes inhabit the digestive and reproductive glands, hepatopancreas and mantle of their snail host, rediae feed directly on snail tissue, whereas sporocysts absorb nutrients from the surrounding tissue (Esch and Fernandez, 1994). Trematode infection in the gonadic region of the snail eventually causes total castration of the host, which in turn will affect host population dynamics and the evolutionary history of snails (Esch and Fernandez, 1994). Through diverting energy from gamete production, trematode infection can also

increase snail growth and survival, changing the snail life history (Minchella, 1985). In freshwater ecosystems, the majority of snails are pulmonates, obtaining oxygen directly from air entering the highly vascularized mantle cavity through a small opening called pneumostome (Clifford, 1991). In Alberta, Canada, there are four families of aquatic pulmonate snails: Acroloxidae, Lymnaeidae, Physidae and Planorbidae. All are hermaphroditic and herbivorous, feeding primarily on periphyton (Clifford, 1991). Lymnaeid and planorbid snails can live up to five years in the wild and reproduce yearly laying egg masses on vegetation or other solid objects (Clifford, 1991). Lymnaeid, physid and planorbid snails are hosts to a variety of trematode species in Alberta (Gordy et al., 2016; 2017; Gordy, 2018; Gordy and Hanington, 2019). These gastropod groups also host ectosymbiotic leeches and oligochaete worms that live between the shell and mantle of the snail. The relationship between these ectosymbionts and their snail host is currently unclear. It has been proposed that leeches are either commensals, obligate or opportunistic parasites, facultative predators, predators or scavengers of snails (Klemm, 1976; Brooks and Welch, 1977; Damborenea and Gullo, 1996; McCaffrey and Johnson, 2017; Gullo and Lopretto, 2018). Oligochaete worms are either commensals or mutualistic ectosymbionts of snails, potentially defending their snail hosts against trematode infection (Sankurathri and Holmes, 1976; Fashuyi and Williams, 1977; Muñiz-Pareja and Iturbe-Espinoza, 2018).

Competition and division of labour in trematodes

Interactions among trematode species within snail hosts have been studied since the 1930s (Wesenberg-Lund, 1934; Cort et al., 1937), and hierarchies have been observed among trematode species based on competition advantages some have over others. Trematode species that develop rediae in the snail host have a competitive advantage over trematodes that develop as sporocysts. Rediae are capable of attacking and consuming competitor species (Esch and Fernandez, 1994). More recently, two morphologically distinct types of rediae were described in marine trematodes (Hechinger et al., 2011; Miura, 2012; Lloyd and Poulin, 2014a; Nielsen et al., 2014). These two morphotypes seemingly have different roles in their colony. Large rediae are in charge of reproduction, whereas small morphotypes defend the trematode colony against competition from other trematode species (Hechinger et al., 2011; Miura, 2012; Lloyd and Poulin, 2014a; Mouritsen and Halvorsen, 2015). This phenomenon is now recognized as an example of division of labour, and resembles the colony

structure present in social insects such as ants, bees and termites. Division of labour allows different members of a colony to become specialized in different duties, increasing colony success and fitness. In this sense, fitness is increased by allowing reproductive members of the colony to focus specifically on producing new individuals, thus increasing reproductive output of the colony. In trematodes, members of the small caste do not reproduce and actively attack other co-occurring trematodes that compete for resources within the snail host (Hechinger et al., 2011; Miura, 2012; Lloyd and Poulin, 2014a; Mouritsen and Halvorsen, 2015). The most accepted drivers of the existence of division of labour in trematode colonies are interspecific competition combined with long-lived snail hosts (Hechinger et al., 2011; Poulin et al., 2019).

With only one exception (Metz, 2022), strong evidence for division of labour in trematodes has been observed only in marine environments, in the trematode families Philophthalmidae, Himasthlidae and Heterophyidae (Hechinger et al., 2011; Leung and Poulin, 2011; Miura, 2012; Kamiya et al., 2013; Nielsen et al., 2014; Garcia-Vedrenne et al., 2016, 2017; Lagrue et al., 2018). Only two studies have assessed division of labour in freshwater trematodes, and both failed to provide support for this phenomenon (Garcia-Vedrenne et al., 2016; Neal et al., 2024). However, in their study of *Echinostoma liei* (Echinostomatidae) Garcia-Vedrenne et al. (2016) indicate that the trematode colonies used in their study had been isolated in lab conditions for multiple generations. With a complete lack of competition in laboratory conditions, colonies might have lost the smaller caste over time. Neal et al. (2024) could not rule out the existence of two different castes in trematode colonies from 20 different species and 10 families. Most small rediae possessed either germinal balls or embryos, their appendages were not more developed than appendages of large rediae, they were as active as large rediae, and they did not display attack behaviours. However, small rediae in their system possess large pharynges, similar to the enlarged pharynges of marine trematodes with division of labour. It is thus necessary to increase the number of studies focusing on division of labour in freshwater trematodes to elucidate if division of labour is exclusive to marine environments due to the comparatively long lifespans of marine snails, or if division of labour might exist in high competition freshwater environments as well.

Thesis goals

Freshwater ecosystems (both natural and artificial) in Alberta support highly diverse invertebrate communities, used as food source by fish, amphibians, mammals and migratory birds. Invertebrate communities include several species of snails, which can serve as hosts for 39 known trematode species, some occurring at prevalence as high as 74% (Gordy et al., 2016). High trematode species richness and prevalence might lead to highly competitive trematode infracommunities inside snail hosts. As high interspecific competition is likely one of the main selective pressures for the evolution of division of labour (Hechinger et al., 2011), Albertan snail populations are thus excellent options to look for division of labour in trematodes in freshwater environments. As trematode communities in Alberta have been poorly studied, complete life cycles of trematode species in the province remain unknown. In this thesis, I aim to expand the knowledge on trematode communities from freshwater snails in Alberta. In Chapter 2, I describe trematode community composition at seven sites around Edmonton: Lafarge, Morinville (i.e. Heritage Lake), Pigeon Lake, St. Albert, Lac Ste. Anne, Strathcona County and Wabamun Lake (Fig. 1). In Chapter 3, using snails sampled from three of the same sites, I documented and tested the potential occurrence of division of labour in two freshwater trematode morphospecies. In Chapter 4, I used snails collected from five of those sites plus two others (Narrow Lake and water bodies north of Ardrossan) (Fig. 1) to observe the impacts of trematode metacercariae on survival of their snail hosts. In Chapter 5, I researched the effects of ectosymbiotic oligochaete worms and leeches on trematode infection in snail hosts, used as both first and second intermediate host by trematodes. Lastly, in Chapter 6, I use molecular techniques (i.e. barcoding) and literature searches to provide, for the first time, a comprehensive, verified and plausible set of second intermediate and definitive hosts for trematodes present in Alberta.

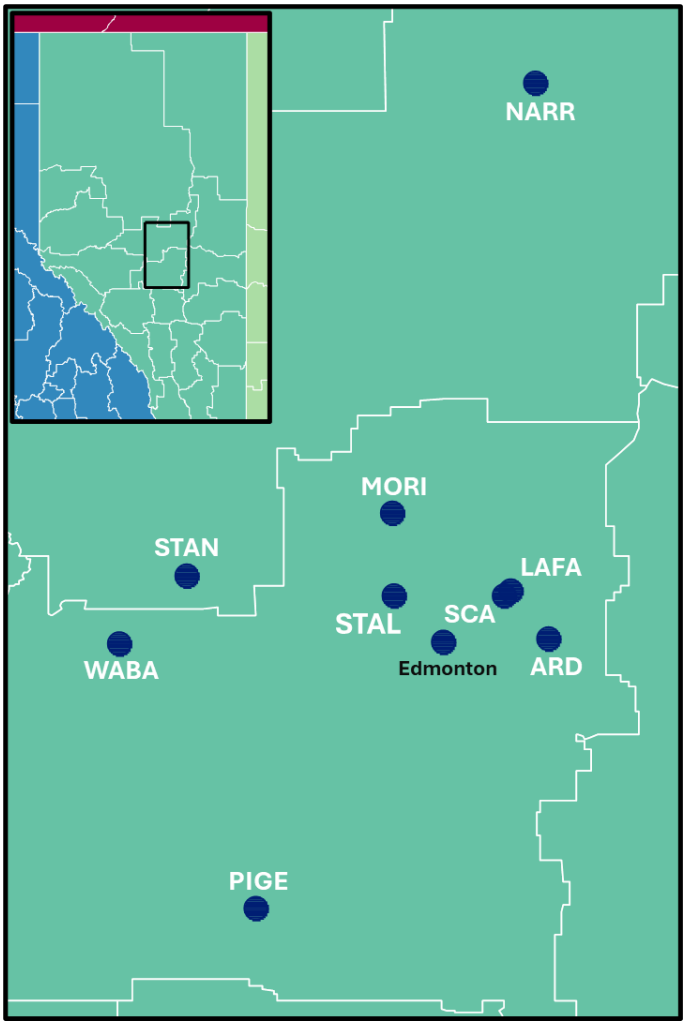


Figure 1. Map showing the nine collection sites located near the city of Edmonton, Alberta: Ardrossan (ARD; Lat. 53.56°, Long. -113.15°), Lafarge (LAFA; Lat. 53.65°, Long. -113.27°), Morinville (MORI; Lat. 53.80°, Long. -113.67°), Narrow Lake (NARR; Lat. 54.64°, Long. -113.19°), Pigeon Lake (PIGE; Lat. 53.03°, Long. -114.12°), St. Albert (STAL; Lat. 53.64°, Long. -113.67°), Lac Ste. Anne (STAN; Lat. 53.68°, Long. -114.35°), Wabamun Lake (WABA; Lat. 53.54°, Long. -114.58°), Strathcona (STRA; Lat. 53.64°, Long. -113.29°).

Chapter 2: Trematode community composition

Introduction

Parasites play important roles in the ecology of their hosts, because they can alter several aspects of the host's individual fitness and population dynamics by reducing host survival and fecundity, or by affecting host behaviour and processes of sexual selection (Hamilton and Zuk, 1982; Claereboudt and Bouland, 1994; Takahashi et al., 1997; Abbot and Dill, 2001; Marino et al., 2017; Friesen et al., 2020). In spite of the diverse impacts of parasites on their hosts, they are often ignored in ecological studies. Another important aspect of parasite ecology is composition and dynamics among parasite species in an ecosystem. Parasite assemblages and the processes that influence assemblage structure within a host can have cascading effects on parasites, hosts and ecosystems (Poulin, 1995). In parasite ecology, definitions of 'infracommunity' and 'component community' differ greatly depending on the number of parasite taxa studied and the scale at which parasite infection is described (e.g., Esch et al., 2002; Poulin, 2019; Lula Costa et al., 2021). In this chapter, I use the term 'infracommunity' as the parasite assemblage (i.e., all parasites) contained within an individual host, and 'component community' as all parasites in a host population, across different localities (Poulin, 2019).

One ecologically important group of multicellular parasitic organisms (i.e., macroparasites) are trematodes. Trematodes are obligatory parasitic flatworms with complex life cycles. A single parasite generation requires multiple hosts to be completed (see Chapter 1 for details). Trematodes are important in their ecosystem because they can impact several aspects of the population dynamics of their hosts. They affect energy flow in food webs and can alter the behaviour of their intermediate hosts to increase transmission rates to definitive hosts (Manga-González et al., 2001; Lafferty et al., 2006; Sato et al., 2011; McLaughlin et al., 2020; Bennett et al., 2024). Some trematode species cause severe illness in humans (see Chapter 1 for details). Trematodes are so deeply weaved into food webs and ecosystems that they can be used as bioindicators (Campião et al., 2012; Shea et al., 2012; Shah et al., 2013).

Generally, information about trematode parasites remains scarce in Canada. Trematode diversity has only been recently studied in Alberta (Gordy et al., 2016). Natural and artificial lakes and ponds support a high diversity of invertebrates in Alberta. These invertebrate communities feed resident fish,

amphibians and mammals, and provide important stops for several species of migrating birds. Freshwater snail species in Albertan lakes can host up to 39 species of trematodes, with some lakes reaching overall trematode prevalence of 74% in snails (Gordy et al., 2016). This previous work is based on surveys of six lakes in central Alberta. Given that trematode assemblages can vary greatly across different geographical regions, it is important to increase trematode diversity surveys across as many localities as possible to obtain a better understanding of the factors that might drive trematode component communities in Alberta. This in turn can be used to better inform conservation and human health policies. Thus, to increase our knowledge of trematode diversity in central Alberta, I documented and described trematode assemblages from freshwater snails in two lakes previously surveyed by Gordy et al. (2016) and five sites that have not been studied before in central Alberta.

Methods

Snail collection and husbandry

Between June 3rd and September 4th, 2019, I surveyed trematode prevalence at seven different localities (lakes and artificial ponds) in Alberta (Fig. 1). Lafarge pond is situated on the northeastern outskirts of Edmonton (simultaneously surveyed with Brooke McPhail, of Patrick Hanington's lab from the University of Alberta School of Public Health), Heritage Lake in Morinville, Pigeon Lake (previously surveyed by Gordy et al., 2016), an artificial pond in St. Albert, Lac Ste. Anne, a pond in Strathcona County (simultaneously surveyed with the Hanington lab), and Wabamun Lake (previously surveyed by Gordy et al., 2016) (Fig. 1).

At these sites, I collected snails using a dip net (2 mm mesh opening) to scoop up macrophytes growing close to the sediment. Snails > 3 mm in shell length were picked out of the macrophytes at the site and were transported to the laboratory and housed individually in plastic specimen cups filled with 60 mL of water from the lake or pond of origin of the snail. Snails were maintained in an incubator at 15°C and fed *ad libitum* with small pieces of commercial Hikari© algae wafers. I identified the snails to genus level based on morphology, using Prescott and Curtenau (2004) as reference. In total, I collected 1641 snails from four genera belonging to three families: Lymnaeidae (*Lymnaea* n = 538; *Stagnicola* n = 371), Physidae (*Physa* n = 272) and Planorbidae (*Planorbella* n = 460).

Snail and data processing

I dissected all snails by gently cracking their shell using a small hammer, carefully dislodging the columellar muscles from the shell axis, and removing the snail from its shell. Under a dissecting scope, I then separated the gonads and digestive gland of each snail from head/foot tissue using dissecting scissors. After dissection, the head/foot tissue of each snail was immediately submerged in 95% ethanol to euthanize the snail. Gonads and digestive glands were further inspected by teasing apart the tissue membrane along the ventral side of the gonad/digestive gland. Trematode cercariae found during dissection were observed through a dissecting scope and identified based on morphological comparison with the images in Gordy et al. (2016). I also recorded the number of snails that were infected with metacercariae, which were categorized as unknown trematode species. Metacercarial identification based on morphology is unreliable, unless scanning electron microscopy is available (e.g., Saville et al., 1997). I calculated overall trematode prevalence of infection in each snail genus as the percentage of individuals infected with at least one trematode taxon. At each of the seven study sites, I calculated site-specific infection prevalence as the percentage of snails of any genus infected with each of the trematode taxa. I then used the function nmds in the ecodist package (Goslee and Urban, 2007) in R to make an ordination based on prevalence per trematode morphotaxon by building a non-metric multidimensional scaling plot (NMDS) of the seven sites based on trematode assemblages. For the NMDS, I used a Bray Curtis distance matrix to observe potential patterns followed by trematode taxa that might depend on locality.

Molecular identification

To augment and potentially confirm trematode identifications based on morphology, I prepared a subset of trematode larvae for molecular identification. I isolated samples of sporocysts from two lymnaeid snails, rediae from one physid and 40 planorbids. I also isolated unencysted metacercariae from two *Lymnaea* and two *Stagnicola* individuals, and encysted metacercariae from four *Physa* and eight *Stagnicola* snails. Sporocysts, rediae and metacercariae were preserved in 95% ethanol and stored at -20°C prior to DNA extraction. For each sample of trematode tissue, I extracted genomic DNA (gDNA) following extraction protocols from DNeasy® Blood & Tissue kits (Quiagen, cat. no. 69506). I then amplified partial fragments of the mitochondrial gene *ND1* from rediae samples identified morphologically as *Petasiger* sp. using the primers NDJ11 and NDJ2a described in

Kostadinova et al. (2003). For each sample of sporocysts, rediae (except for rediae samples that were identified morphologically as *Petasiger* sp.) and metacercariae, the mitochondrial *cox1* gene fragment was amplified using the primers Dice1F and Dice11R as described in Van Steenkiste et al. (2015). PCR was done in 20 µl volumes using 10 µl of the AccuStart II GelTrack® PCR SuperMix (Quanta Bio, cat. no. 95136-500), 0.5 µl of each primer (concentration = 10 µM), and 9 µl of gDNA. I then electrophoresed PCR products (i.e. amplicons) in 1% agarose and purified them following the instructions from Truin Science PCR cleanup kits (Truin Science, model: KTS1115). Purified amplicons were then sent to Macrogen Inc. (Korea) for Sanger sequencing, using the same PCR primers to amplify both strands of each gene fragment. I then trimmed and analyzed resulting sequences for quality with SnapGene© Viewer (Dotmatics) software, and subsequently MUSCLE aligned and BLASTed using Geneious Prime® (version 2023.2.1) software. Library sequences with the highest pairwise comparison percent match rates were then selected and, when possible, a cut-off of 5% nucleotide divergence of both gene sequences was used to determine identity of trematode species. This threshold was used because it is a conservative standard of maximum intraspecific mitochondrial DNA (mtDNA) divergence (Gordy et al., 2016). However, in some cases, the highest pairwise comparison percent match rates exceeded the 5% ideal divergence threshold. Thus, for all samples, I used morphology traits of cercariae to validate barcoding IDs, as it has been suggested that identifications of sequences stored in GenBank are not always fully reliable (e.g., Cheng et al., 2023).

Results

Component communities

From sporocysts, rediae and metacercariae infecting the four snail genera, I identified 21 different trematode morphotaxa. Metacercariae were either unencysted or encased in cysts within the digestive gland of their snail hosts. Due to the difficulty of identifying metacercariae morphologically, I separated unencysted metacercariae into different morphotaxa based on body size and pattern of internal structures (i.e. named UK1 to UK5 in Table 1 and Fig. 2). Some sporocyst and redial infections could not be identified morphologically due to the lack of distinguishable cercariae (i.e. named UK in Table 1 and Fig. 2). Morphology data suggest that most taxa are present at most sites, but infection prevalence was higher at Lafarge, St. Albert, Lac Ste. Anne, Morinville and Wabamun Lake sites (Fig. 2). Morinville and Wabamun Lake had very similar trematode prevalence and

taxonomic richness (Table 1), and were relatively close in assemblage structure (Fig. 2). Assemblage structure at Lac Ste. Anne contrasted strongly with that of Morinville and Wabamun, the St. Albert pond was distinct based on high prevalence of several taxa (vectors in Fig. 2), whereas the Strathcona pond and Pigeon Lake were characterized by a smaller number of taxa. Trematode diversity was positively correlated to snail sample size when all trematode taxa and snail genera were pooled (Fig. 3). Trematode prevalence was highly variable among snail host species, with *Physa* having the lowest (18.38%) and *Stagnicola* the highest (70.89%) prevalence of infection among the four snail genera. *Lymnaea* (49.26%) and *Planorbella* (47.83%) had intermediate trematode prevalence.

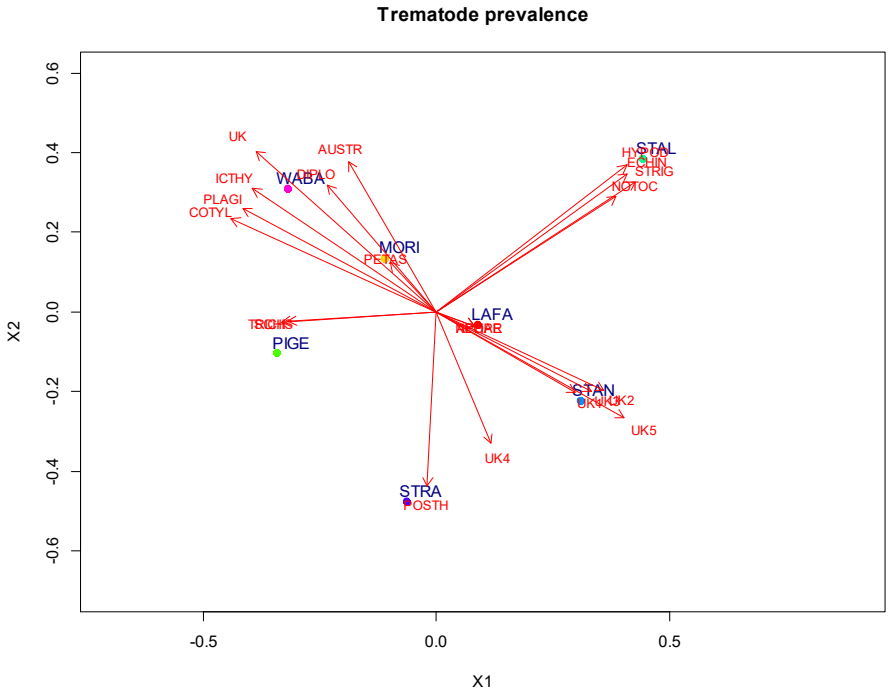


Figure 2. NMDS for trematode genera prevalence in host snails (%). Blue captions represent sampling sites: Pigeon Lake (PIGE), Wabamun Lake (WABA), Strathcona (STRA), Morinville (MORI), St. Albert (STAL), Lafarge (LAFA), Lac Ste. Anne (STAN). Red captions represent trematode genera: *Apharyngostrigea* (APHAR), *Australapatemon* (AUSTR), *Cotylurus* (COTYL), *Diplostomum* (DIPLO), Echinostomatidae (ECHIN), *Hypoderaeum* (HYPOD), *Icthyocotylurus* (ICTH), *Neopetasiger* (NEOPE), *Notocotylus* (NOTOC), *Petasiger* (PETAS), *Plagiorchis* (PLAGI), *Postodiplostomum* (POSTO), Schistosomatidae (SCHIS), Strigeidae (STRIG), *Trichobilharzia* (TRICH), unidentified sporocysts and rediae (UK) and metacercariae (UK1, UK2, UK3, UK4, UK5).

Table 1. Trematode prevalence at the seven study sites. Sample size of snails examined (n) is specified in parentheses below the name of each site and next to each prevalence value. Sampling occasions (so) are specified in parentheses below the name of each site. Prevalence is given in percentage. All trematode taxa were identified morphologically. The majority of taxa were identified to genus level. However, some trematodes could not be identified below family level. UK = unidentified sporocysts and rediae. UK1, UK2, UK3, UK4, UK5 = unidentified metacercariae. * = Total prevalence does not match the sum of prevalence for each species, as some snails were infected with more than one species of trematode.

Trematode taxa	**Site**						
	Pigeon Lake (n = 65; so = 1)	Wabamun Lake (n = 25; so = 1)	Strathcona (n = 208; so = 8)	Morinville (n = 125; so = 2)	St. Albert (n = 93; so = 2)	Lafarge (n = 867; so = 8)	Lac Ste. Anne (n = 258; so = 2)
Apharyngostrigea	--	--	--	--	--	0.23 (n = 2)	--
Australapatemon	--	4 (n = 1)	--	--	1.08 (n = 1)	0.35 (n = 3)	0.39 (n = 1)
Cotylurus	4.62 (n = 3)	8 (n = 2)	--	--	--	0.46 (n = 4)	0.78 (n = 2)
Diplostomum	--	20 (n = 5)	3.37 (n = 7)	3.2 (n = 4)	4.3 (n = 4)	2.54 (n = 22)	2.71 (n = 7)
Echinostomatidae	--	4 (n = 1)	--	--	86.02 (n = 80)	8.19 (n = 71)	1.55 (n = 4)
Hypoderaeum	--	--	--	5.6 (n = 7)	22.58 (n = 21)	3.92 (n = 34)	0.39 (n = 1)
Icthyocotylurus	1.54 (n = 1)	8 (n = 2)	--	0.8 (n = 1)	--	0.12 (n = 1)	--
Neopetasiger	--	--	--	--	--	0.12 (n = 1)	--
Notocotylus	--	--	0.48 (n = 1)	0.8 (n = 1)	2.15 (n = 2)	0.35 (n = 3)	--
Petasiger	--	--	--	3.2 (n = 4)	--	0.58 (n = 5)	--
Plagiorchis	3.08 (n = 2)	12 (n = 3)	2.4 (n = 5)	9.6 (n = 12)	--	4.38 (n = 38)	2.33 (n = 6)
Posthodiplostomum	--	--	0.48 (n = 1)	--	--	--	--
Schistosomatidae	--	4 (n = 1)	3.37 (n = 7)	0.8 (n = 1)	--	0.35 (n = 3)	--
Strigeidae	--	--	--	--	1.08 (n = 1)	0.23 (n = 2)	--
Trichobilharzia	--	4 (n = 1)	2.88 (n = 6)	1.6 (n = 2)	--	0.81 (n = 7)	0.39 (n = 1)
UK	26.15 (n = 17)	48.00 (n = 12)	9.13 (n = 19)	40 (n = 50)	17.20 (n = 16)	27.10 (n = 235)	17.05 (n = 44)
UK1	--	--	--	--	--	2.54 (n = 22)	2.71 (n = 7)
UK2	--	--	--	0.80 (n = 1)	1.08 (n = 1)	2.19 (n = 19)	10.47 (n = 27)
UK3	--	--	0.96 (n = 2)	4 (n = 5)	1.08 (n = 1)	9.46 (n = 82)	12.40 (n = 32)
UK4	--	--	1.44 (n = 3)	--	--	2.19 (n = 19)	0.39 (n =)
UK5	--	--	2.40 (n = 5)	1.60 (n = 2)	2.15 (n = 2)	3.34 (n = 29)	12.02 (n = 31)
Total prevalence*	**30.77**	**66**	**23.07**	**66**	**93.55**	**52.36**	**40.7**
Taxon richness	**4**	**9**	**10**	**12**	**10**	**20**	**13**

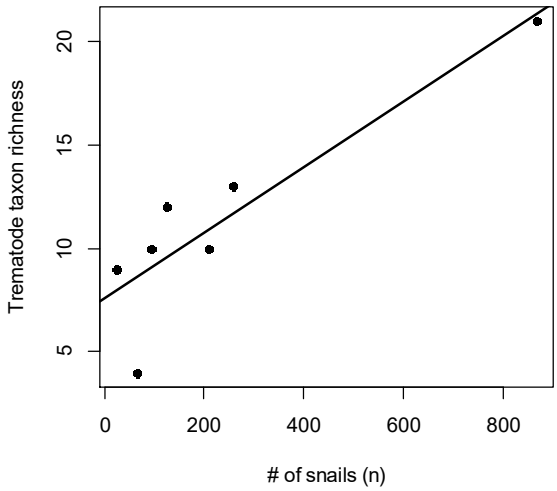


Figure 3. Correlation between sample size of snails per site (n) and trematode taxon richness. Data from seven sites sampled in 2019.

Molecular identification

Both sporocyst samples from *Lymnaea* were successfully barcoded and identified as *Diplostomum* sp. and *Trichobilharzia szidati* Neuhaus, 1952 (Schistosomatidae) (Table 2). Rediae from *Physa* were identified as *Notocotylus* sp., while the majority of rediae sampled from *Planorbella* were identified as *Ribeiroia ondatrae* (Price, 1931) Price, 1942 (Psilostomidae) or *Echinostoma trivolvis* (Cort, 1914) Kanev, Vassilev, Lie & Fried, 1988 (Echinostomatidae) (Table 2). Both samples of unencysted metacercariae isolated from *Stagnicola* were identified as *Cotylurus* sp. E and *Cotylurus cornutus* (Rudolphi, 1809) Szidat, 1928 (Strigeidae), respectively (Table 2). Unencysted metacercariae found in *Lymnaea* and all metacercarial cysts from *Physa* and *Stagnicola* either failed to amplify or produced unusable sequences, and could not be matched to sequences from GenBank. Thus, identities of unencysted metacercariae infecting *Lymnaea* and metacercariae that encyst in *Stagnicola* and physid snails remain unknown.

Table 2. Species identification results based on sequencing of fragments from mitochondrial gene *cox1* from trematode sporocysts, rediae and metacercariae found in freshwater snails used as first intermediate hosts. * = closest match to GenBank, but below 5% divergence ideal threshold. ** = molecular ID does not match cercaria morphotype typical for family, thus not reliable. Information of original morphological identification in parentheses after molecular IDs.

Site	Host ID	Barcoding ID	% identity	GenBank accession match
	Lymnaeidae			
		Sporocysts		
St. Albert	*Lymnaea*	*Diplostomum* sp. VVT4	98.9	MZ323295
Lafarge	*Lymnaea*	*Trichobilharzia szidati*	97.1	MK433246
		Metacercariae		
Lafarge	*Stagnicola*	*Cotylurus sp. E*	100	OM102969
Lafarge	*Stagnicola*	*Cotylurus cornutus*	98.6	MH369544
	Physidae			
		Rediae		
Strathcona	*Physa*	*Notocotylus* sp. A	100	MH369415
	Planorbidae			
		Rediae		
St. Albert	*Planorbella*	***Cotylurus cornutus* (unedentified rediae)	*88.3	MH369539
St. Albert	*Planorbella*	***Diplostomum spathaceum* (*Hypoderaeum*)	95.5	KR271424
St. Albert	*Planorbella*	***Diplostomum spathaceum* (unidentified rediae)	95.5	KR271454
Lafarge	*Planorbella*	*Drepanocephalus spathans*	100	JX468067
Morinville	*Planorbella*	***Echinoparyphium* sp. A (*Petasiger*)	95.7	MH369039
Lafarge	*Planorbella*	*Echinoparyphium* sp. lineage 3	96.7	MH369270
Lafarge	*Planorbella*	*Echinoparyphium* sp. lineage 3	*89.3	MH369270
St. Albert	*Planorbella*	*Echinostoma trivolvis* (*Hypoderaeum* sp.)	98.3	MH369271
St. Albert	*Planorbella*	*Echinostoma trivolvis* (*Hypoderaeum* sp.)	99.2	MH369271
St. Albert	*Planorbella*	*Echinostoma trivolvis* (*Hypoderaeum* sp.)	99.7	MH369271
St. Albert	*Planorbella*	*Echinostoma trivolvis* (*Hypoderaeum* sp.)	100	MH369271

Site	Host ID	Barcoding ID	% identity	GenBank accession match
St. Albert	*Planorbella*	*Echinostoma trivolvis* (*Hypoderaeum* sp.)	99.4	MH369271
St. Albert	*Planorbella*	*Echinostoma trivolvis* (*Hypoderaeum* sp.)	98.4	MH369271
St. Albert	*Planorbella*	*Echinostoma trivolvis* (*Hypoderaeum* sp.)	96.4	MH369271
St. Albert	*Planorbella*	*Echinostoma trivolvis* (*Hypoderaeum* sp.)	97.4	MH369271
St. Albert	*Planorbella*	*Echinostoma trivolvis* (*Hypoderaeum* sp.)	96.5	MH369271
St. Albert	*Planorbella*	*Echinostoma trivolvis* (*Hypoderaeum* sp.)	100	MH369271
St. Albert	*Planorbella*	*Echinostoma trivolvis* (*Hypoderaeum* sp.)	*94.6	MH369271
St. Albert	*Planorbella*	*Echinostoma trivolvis* (*Hypoderaeum* sp.)	*94	MH369271
Lafarge	*Planorbella*	*Petasiger* sp. 4	*77.2	MH369312
Lafarge	*Planorbella*	*Petasiger* sp. 4	100	MH369312
Morinville	*Planorbella*	*Petasiger* sp. 4	*91.3	MH369313
Morinville	*Planorbella*	*Petasiger* sp. 4	100	MH369312
St. Albert	*Planorbella*	*Psilostomidae* gen. sp. A (*Hypoderaeum* sp.)	*92.3	MH369475
Lafarge	*Planorbella*	*Psilostomidae* gen. sp. A (*Hypoderaeum* sp.)	99.3	MH369475
St. Albert	*Planorbella*	*Ribeiroia ondatrae* (*Hypoderaeum* sp.)	99	OK210492
St. Albert	*Planorbella*	*Ribeiroia ondatrae* (*Hypoderaeum* sp.)	95.7	OK210492
St. Albert	*Planorbella*	*Ribeiroia ondatrae* (*Hypoderaeum* sp.)	97.4	MH369473
St. Albert	*Planorbella*	*Ribeiroia ondatrae* (*Hypoderaeum* sp.)	100	OK210381
St. Albert	*Planorbella*	*Ribeiroia ondatrae* (*Hypoderaeum* sp.)	95.6	OK210381
St. Albert	*Planorbella*	*Ribeiroia ondatrae* (*Hypoderaeum* sp.)	97.3	OK210381
St. Albert	*Planorbella*	*Ribeiroia ondatrae* (*Hypoderaeum* sp.)	97	OK210492
St. Albert	*Planorbella*	*Ribeiroia ondatrae* (*Hypoderaeum* sp.)	98.3	OK210492
St. Albert	*Planorbella*	*Ribeiroia ondatrae* (*Hypoderaeum* sp.)	100	OK210492
Lafarge	*Planorbella*	*Ribeiroia ondatrae* (*Hypoderaeum* sp.)	99.7	OK210492

Site	Host ID	Barcoding ID	% identity	GenBank accession match
Lafarge	*Planorbella*	*Ribeiroia ondatrae* (*Hypoderaeum* sp.)	100	OK210492
Lafarge	*Planorbella*	*Ribeiroia ondatrae* (*Hypoderaeum* sp.)	100	OK210492
Lafarge	*Planorbella*	*Ribeiroia ondatrae* (*Hypoderaeum* sp.)	100	OK210492
Lafarge	*Planorbella*	*Ribeiroia ondatrae* (*Hypoderaeum* sp.)	100	OK210381
Lafarge	*Planorbella*	*Ribeiroia ondatrae* (*Hypoderaeum* sp.)	95	OK210492

Discussion

This chapter adds valuable information to our limited knowledge of trematode component communities in Canada. Here, I describe trematode taxa composition and prevalence from two lakes previously studied by Gordy et al. (2016), and from five bodies of water that had not been sampled before. These sites differed strongly in trematode prevalence, taxon richness (Table 1) and assemblage structure (Fig. 2), while trematode prevalence also varied among snail genera. Using molecular methods, I was able to identify two trematode species that are new records for Alberta: *Ribeiroia ondatrae* and *Drepanocephalus spathans* Dietz, 1909 (Echinostomatidae) (Table 2).

New records of trematodes

Both *R. ondatrae* and *D. spathans* are reported for the first time infecting snails from the genus *Planorbella* in central Alberta. Cercariae of these trematode species were initially identified morphologically as *Hypoderaeum* sp., as neither *R. ondatrae* nor *D. spathans* were observed by Gordy et al. (2016), which was used as reference to identify cercariae morphologically as the only existing report of trematodes from Alberta at time of writing. Morphological misidentification of *R. ondatrae* and *D. spathans* as *Hypoderaeum* sp. is also likely due to high similarity in cercarial morphology amongst these three echinostomes (Fig. S1). It is unlikely that redia samples in this study were misidentified genetically, given the large sample size for *R. ondatrae* (Table 2). Additionally, sequences of both *R. ondatrae* and *D. spathans* matched closely with sequences available in GenBank (Table 2). Further, *D. spathans* has been previously reported from Double-crested Cormorants in Ontario and Saskatchewan (Robinson et al., 2010; Wagner at al., 2012), and this species of fish-eating

bird is common in Alberta (see map at https://ebird.org/species/doccor/CA-AB-SI). *Drepanocephalus spathans* is an echinostome trematode that uses planorbid snails as first intermediate hosts and fish as second intermediate hosts. It is of importance in aquaculture as it can cause high mortality rates in farmed juvenile fish (Griffin et al., 2012). This first record of *D. spathans* in Alberta is also valuable information for wildlife conservation, as trematodes that use fish as second intermediate hosts, such as *D. spathans*, have the potential to infect and reduce survival of wild fish.

Ribeiroia ondatrae has also been previously reported from Canada, in this case in frogs and Double-crested Cormorants from British Columbia and Saskatchewan (Roberts and Dickinson, 2012; Wagner et al., 2012). *R. ondatrae* is of great interest for amphibian conservation, as it can infect several species of amphibians and fish, and causes deformities in frogs, making their amphibian host more susceptible to predation by birds (Szuroczki and Richardson, 2009; Roberts and Dickinson, 2012). *R. ondatrae* was highly prevalent, particularly at the St. Albert pond (Table1, as *Hypoderaeum* sp.), suggesting that frog populations might be at risk of increased mortality caused by this trematode (Szuroczki and Richardson, 2009). The information provided in this chapter is thus of great importance for wildlife conservation, as the presence of *R. ondatrae* and *D. spathans* may impact populations of amphibians and fish that can act as second intermediate hosts for the parasite. It is necessary to further survey trematodes in central Alberta to elucidate if distribution of these two trematode species varies among localities or with time, to better inform conservation policies and to evaluate risks for aquaculture in Canada.

Trematode component community composition

Among trematode taxa that could be identified morphologically, members of the Echinostomatidae had the highest prevalence and were found at four of the surveyed sites (Table 1). Most trematode taxa were found at two or more sites, except for *Apharyngostrigea* and *Neopetasiger* that were sampled only from Lafarge. These two genera also had the lowest prevalence of the 21 morphotaxa (Table 1). *Plagiorchis* and *Diplostomum* were present at six of the seven study sites, while *Australapatemon*, *Cotylurus*, Echinostomatidae and *Notocotylus* were sampled from four of the seven sites (Table 1), suggesting these six trematode taxa are common in central Alberta. These results are similar to the component community surveyed by Gordy et al. (2016), who found *Australapatemon burti* (Miller, 1923) Dubois, 1968 (Strigeidae), *Cotylurus gallinulae* (Lutz, 1928) Dubois, 1937 (Strigeidae),

Diplostomum, *Echinostoma trivolvis*, *Notocotylus* and *Plagiorchis* at four of six lakes. However, unlike Gordy et al. (2016), who reported highest prevalence of *Plagiorchis* sp. when sites were pooled, the most prevalent infection when my seven sites were pooled was by metacercariae (Table 1), some of which were genetically identified as *Cotylurus* sp. and *Cotylurus cornutus* (Table 2). This shows the importance of snail dissections while assessing trematode communities, as data from immature rediae/sporocyst (i.e. from snails that do not shed cercariae) and metacercarial cyst infections are missed when only focusing on snails shedding cercariae, and thus, prevalence estimates can be inaccurate and possibly highly underestimated.

When grouping all trematode taxa together, overall trematode prevalence was highest in *Stagnicola* and lowest in *Physa*. These results partially agree with Gordy et al. (2016), who also found that lymnaeid snails had the highest trematode prevalence. It is noteworthy that unlike in Gordy et al. (2016), the types of trematode larvae that contribute the largest amount of data to prevalence calculations in this chapter differ between snail genera, as *Physa* were mostly infected with rediae and sporocysts, while *Stagnicola* was mostly infected with metacercariae. This information is novel for studies of trematode component communities in Alberta, as Gordy et al. (2016) provided prevalence information only from cercariae being shed from snails, instead of including metacercarial infections. In addition, Gordy et al. (2016) provide prevalence pooled by snail families, whereas here, I present trematode prevalence estimates for each snail genus. This distinction is important because *Lymnaea* and *Stagnicola* belong to the same family (Lymnaeidae), but in my study system, trematode prevalence between these snail genera differs by almost 22%. Additionally, in Gordy et al. (2016), *S. elodes* contributed most to the pooled prevalence of trematode infection in lymnaeids, which is similar to my results, but in my study system high prevalence in *Stagnicola* is mostly driven by metacercarial infection. It is thus likely that prevalence estimates from Gordy et al. (2016) are underestimated. More studies that use snail dissections in multiple localities in Alberta are necessary to provide accurate estimates and descriptions of trematode component communities in the region.

Trematode taxon richness was highest at Lafarge, and lowest at Pigeon Lake (Table 1). Gordy et al. (2016) also observed the lowest trematode species richness at Pigeon Lake, but unlike my results, Wabamun Lake had the highest trematode species richness of the six sites they surveyed. This difference could be attributed to differences in sampling effort and timing between studies. Gordy et al. (2016) visited Wabamun Lake 14 times across two consecutive years, while I sampled this lake

only once in June, which is before trematode infection reaches its peak, and thus likely many trematode taxa are missing from my survey. Trematode taxon richness was positively correlated with snail sample size (Fig. 3). It is noteworthy, however, that due to logistic constraints, sampling efforts varied among sites. This likely explains lower snail sample sizes from sites that were visited less frequently than from sites that were visited on many occasions (Table 1). However, sampling effort alone does not fully explain differences in snail sample sizes and trematode taxon richness among sites. Strathcona County was visited as frequently as Lafarge, but snails were less abundant in the former on all our visits, leading to a much lower sample size at Strathcona. Thus, the variation in trematode taxon richness among sites might be a product of differences in snail abundance, but it could also be related to differences in taxon richness of the final hosts for trematodes (i.e., birds and mammals). To answer this question, it will be necessary to complete trematode community composition studies with equal snail sampling efforts among different localities and to carry out surveys of birds and mammals at the same sites (see Chapter 6).

Identification techniques: pros and cons

Identification of trematode taxa based only on cercarial morphology is not fully reliable, as trematodes tend to have higher cryptic diversity (i.e. morphologically identical species that are genetically different (Galipaud et al., 2019)) than any other group of parasitic helminths (Pérez-Ponce de León and Poulin, 2018). Cercariae from different trematode families can look very similar without the appropriate staining and microscopy identification techniques. For this reason, many recent studies rely fully on molecular techniques to reduce potential for misidentification of trematode cercariae (e.g., Neal et al., 2024). DNA barcoding matches sequences from unknown samples with sequences stored in databases built and accumulated from a variety of sources, ideally, making misidentifications less likely to occur. However, barcoding identification assumes that the correct taxon identification for the original sequences is stored in databases, the accuracy of which has frequently come into question (e.g., Sangster and Luksenberg, 2021; Cheng et al., 2023). It is thus important to combine information from both morphological and molecular identification of trematodes to have a more reliable taxon identification in ecological studies (Gordy et al., 2016).

Here, I used information from morphological characteristics of cercariae combined with information from snail dissections to assess whether molecular identification matched known traits of

trematodes in Alberta. Most samples identified genetically in this chapter matched morphological characteristics of cercariae used initially to identify trematode taxa and thus, prevalence calculations are mostly reliable. It is noteworthy, however, that most preserved samples identified initially as *Hypoderaeum* were identified genetically as either *Echinostoma trivolvis*, *Drepanocephalus spathans* or *Ribeiroia ondatrae*. The latter two misidentifications have already been addressed, and thus, I will only focus on *E. trivolvis* in this section. Cercariae of *E. trivolvis* are very similar to those of *Hypoderaeum*. Both cercariae have spine collars, a relatively large oral sucker, distinctive main excretory collecting tubes with excretory granules, and tail fin folds (Fig. S1). The main difference between taxa is cercarial size. Given these similarities among cercariae, the high percent identity match among sequences, and large sample size, it is highly likely that initial morphological identifications of those samples were incorrect. I am thus confident molecular identifications are correct in this case. However, from rediae isolated from four different planorbid snails, I obtained molecular identifications of *Cotylurus cornutus*, *Diplostomum spathaceum* and *Echinoparyphium* sp. that I view as suspect. Both *C. cornutus* and *D. spathaceum* develop in sporocysts only and their cercariae have a bifurcated tail, characteristics that do not match the morphology (Fig. S2) or life history of *Hypoderaeum* sp., which was my initial and more likely correct identification of those samples. The trematode sample matched with sequences from *Echinoparyphium* sp. through barcoding were initially identified as *Petasiger*, whose cercaria morphology is very characteristic (Fig. S2), and thus morphological misidentification is less likely.

Lastly, through barcoding, I was able to identify metacercariae of some trematodes that use snails as second intermediate hosts in Alberta as *Cotylurus* sp. and *Cotylurus cornutus*, information that at the time if writing, has not been published from anywhere in Canada. I was also able to corroborate the presence of *Trichobilharzia* at five sites and *Diplostomum* at six sites in central Alberta (Table 1). These trematode genera cause swimmer's itch in humans, and due to their presence at most of my sampling sites, my results suggest they are very common in central Alberta. This information is of high importance for the public, human health authorities and the provincial government, as it can be used to produce better-informed health and recreation policies (Gordy et al., 2018).

Chapter 3: Division of labour in trematode colonies

Introduction

Division of labour takes place when different members of a social group (i.e., colony) become specialized in different duties that are beneficial to their colony and ultimately increase colony success and fitness (Page and Mitchell, 1998). Increased fitness in this context is achieved by allowing some individuals to focus specifically on reproductive roles and thus the colony reproductive output is increased. Colonies of social insects such as ants and bees are composed of at minimum a reproductive caste and a worker caste. The reproductive caste produces new members of the colony while the worker caste is in charge of providing food, housekeeping, nursing the young, and defending the colony against threats. This caste system is considered a key trait giving social insects a competitive advantage over non-social insects (Page and Mitchell, 1998). Reproductive division of labour was first thought to be exclusive to social insects, but was later described in sea anemones (Francis, 1976), snapping shrimp (Duffy et al., 2002) and mole rats (Jarvis, 1981), whose colonies include reproductive and non-reproductive castes.

More recently, two morphologically different types of larvae were described in clonal colonies of trematode parasites (Hechinger et al., 2011; Miura, 2012; Lloyd and Poulin, 2014; Nielsen et al., 2014). Trematodes are flatworms of the phylum Platyhelminthes that typically require two intermediate hosts in which asexual reproduction and partial development may occur, and one definitive host in which they sexually reproduce and complete their life cycle (Esch et al., 2002). The first intermediate host of trematodes is usually a snail, in which these parasites develop into clonal colonies of larvae from a single fertilized egg. These larvae are called sporocysts or rediae depending on their morphology. Rediae possess mouthparts and a rudimentary digestive tract, while sporocysts do not. Rediae and sporocysts produce free-living larvae (cercariae) that leave the snail to infect a second intermediate host. In some trematode species, colonies of clonal rediae consist of two morphotypes that show division of labour (Miura, 2012; Lloyd and Poulin, 2014). Reproductive rediae are much larger than non-reproductives, produce cercariae, and have relatively small mouthparts (Hechinger et al., 2011; Garcia-Vedrenne et al., 2016). Non-reproductive rediae (termed ‘soldiers’) are smaller, more active, have larger mouthparts and pharynxes, and attack other, co-occurring trematodes

competing for host resources (Hechinger et al., 2011; Miura, 2012; Lloyd and Poulin, 2014; Mouritsen and Halvorsen, 2015).

In trematode species with division of labour, the proportions of reproductive and soldier rediae in the colony (= caste ratio) is influenced by environmental conditions within the individual snail host (Leung and Poulin, 2011; MacLeod et al., 2018). When coinfections of more than one species of trematode occur inside a snail, colonies with division of labour have higher proportions of non-reproductive soldier morphs than they do in snails not infected by other trematode species. Attacks from soldier rediae result in one of the competitor species being eliminated from the snail over time (Leung and Poulin, 2011; Kamiya and Poulin, 2013; Lagrue et al., 2018; Resetarits et al., 2020). Division of labour also appears to be locality specific, as snail hosts from sites with higher diversities of trematode species contain colonies with higher proportions of soldiers than snails from localities with lower trematode diversity (Lloyd and Poulin, 2014b; Resetarits et al., 2020). Trematodes with division of labour also produce more soldiers when the shell of their snail host is damaged. As a breach in the host shell potentially exposes the colony to bacterial or fungal contamination, the colony responds by increasing the proportion of soldiers (MacLeod et al., 2018). Caste ratio in trematode colonies also responds to conditions outside their snail host. In low pH environments, more soldiers are produced than in normal pH environments (Guilloteau et al., 2016), possibly a response to more acidic conditions that could affect colony survival by stressing the snail host and lowering available resources for trematodes.

Because resources are limited inside a snail host, it is unclear whether division of labour is a universally advantageous life strategy in trematode parasites. When competition from other trematode colonies is high, production of a large number of soldiers will reduce the number of competitors and increase the reproductive output of the colony, indicating that trematodes with division of labour have a selective advantage in highly competitive environments (Kamiya and Poulin, 2013b; Mouritsen and Andersen, 2017). In contrast, division of labour may be costly to trematode colonies when competition inside the snail host is low, as production of soldiers uses resources that could be allocated to producing more reproductive individuals, thus decreasing production of the next larval stage (cercariae) (Kamiya and Poulin, 2013b). Interestingly, when trematode co-infections occur, both reproductives and soldiers grow larger than in host individuals without competition (Kamiya et al., 2013). The rationale to explain this is that soldiers grow larger by consuming members of other

(nutrient- rich) trematode colonies, and reduce competition, allowing the reproductives to grow and produce more offspring, thus increasing the biomass of the overall colony (Kamiya et al., 2013).

With the possible exception of the freshwater trematode *Haplorchis pumilio* (Looss) (Heterophyidae) referred to in the abstract of an unpublished thesis (Metz, 2022), studies on division of labour in trematodes have focused mainly on the marine families Philophthalmidae, Himasthlidae and Heterophyidae (Hechinger et al., 2011; Leung and Poulin, 2011; Miura, 2012; Kamiya et al., 2013; Nielsen et al., 2014; Garcia-Vedrenne et al., 2016, 2017; Lagrue et al., 2018), and freshwater examples remain largely understudied. Alberta has numerous freshwater lakes and ponds (both natural and artificial) that harbour dense populations of many different snail species, which in turn, can host to up to 39 trematode species (Gordy et al., 2016). Trematode prevalence in snails in Alberta can be as high as 74% (Gordy et al., 2016). This suggests that interspecific competition for and within snail hosts may be very high among trematode species. High interspecific competition is one of the main factors believed to drive the evolution of division of labour in trematodes (Hechinger et al., 2011). Snails in Albertan lakes and ponds are thus excellent species in which to look for division of labour of trematodes in freshwater environments.

Here, I describe redia morphology and colony size-frequency distributions of trematodes from freshwater snails in central Alberta. As it has been suggested that size distribution on its own is not a reliable method to establish if small rediae can confidently be classified as non-reproductive soldiers (Galaktionov et al., 2015), I performed a series of behavioural *in-vitro* experiments to observe interactions among large reproductive rediae, small rediae and members of other trematode colonies. Reliability of *in-vitro* experiments to assess trematode larval behaviour has been demonstrated elsewhere (Kamiya and Poulin, 2013b). Due to the probability of high interspecific competition inside freshwater snails in Alberta, I hypothesized that there would be trematode species with division of labour in my study system. I thus predicted that (i) there would be a small redia morph that would be substantially smaller than reproductives and lack germinal masses, (ii) size-frequency distributions from trematode colonies would be bimodal, indicative of colonies with division of labour, and (iii) small redia morph would be more active than reproductives and attack members of other trematode species, but not rediae of their own colony.

Methods

Snail collection and husbandry

In preliminary trematode surveys at Morinville, Lafarge, St. Albert and Strathcona County in 2019 (Fig. 1), I found two redia morphotypes in freshwater snails; one small, slender redia type with large mouthparts that resembled soldiers previously described from trematodes in marine snails (e.g. Miura, 2012), and a large redia type producing cercariae (i.e., reproductive). Colonies with small rediae were found in planorbid snails and appeared to belong to two trematode genera, *Petasiger* and *Hypoderaeum* (both Echinostomatidae), identified morphologically by comparison with the images in Gordy et al. (2016). In total, twenty-five snails (mean shell width = 22.88 mm) were collected from the four study sites between June 8th and August 28th in 2020, and between June 22nd and August 31st in 2021. I collected snails using a dip net (2 mm mesh opening) by scooping and sorting vegetation growing close to the sediment. Snails were then transported to the laboratory where they were isolated in plastic specimen cups with 60 mL of pond water collected at the same sites as the snails. I identified snails as *Planorbella* sp. based on morphology, using pictures within Prescott and Curtenau (2004).

After isolating the snails, I kept them overnight at room temperature (between 25°C and 26°C) to promote shedding of cercariae. Snails shedding cercariae that morphologically resembled either *Petasiger* or *Hypoderaeum* were kept in an incubator at 15°C, and small pieces of store-bought organic lettuce were provided as food *ad libitum*. Once a week, I replaced the lettuce piece and exchanged half the water in the container with fresh pond water to maintain the water relatively clean without shocking the snails with potential changes in salinity or pH.

Redia collection

Redia colonies were collected from one snail per week for 25 weeks, and used for both behavioural and morphological studies. Snails were dissected by gently cracking their shell using a small hammer, carefully dislodging the columellar muscles from the shell axis, and removing the snail from its shell. Under a dissecting scope, I then separated the snail's gonad/digestive gland from the head/foot tissue using dissecting scissors. The snail's head/foot tissue was then immediately submerged in 95% ethanol to euthanize the snail. Gonad and digestive glands of each snail were further inspected under a dissecting scope by teasing apart the tissue membrane along the ventral side of the gonad/digestive

gland. Rediae were then carefully separated from snail tissue using fine forceps. Once separated from snail tissue, rediae were processed for both morphology and behavioural experiments.

Morphology

Rediae were placed in a 5 mm-gridded 50 mm plastic Petri dish with a glass pipette and were spread out to avoid overlapping individual rediae. I then imaged each trematode colony with a Zeiss Axiocam color microscope camera attached to a dissecting scope. Using the open-source software ImageJ and the gridlines for calibration, I then measured the body area of each redia within randomized subsamples taken from each trematode colony (20 colonies, total n of rediae = 26,327). In total, I measured redia areas for 10 colonies of trematodes identified morphologically as *Petasiger* and 10 colonies of trematodes identified morphologically as *Hypoderaeum*, each colony coming from a different *Planorbella* individual. I tested redia area distributions of each colony for unimodality using the dip.test function in the package diptest in R (Maechler, 2024).

Behavioural trials

Colonies of trematodes identified morphologically as *Petasiger* and *Hypoderaeum* were used in behavioural trials. These colonies were placed in a 30 mm glass Petri dish with Sterile CBSS (Cell Balanced Salt Solution; from: Table I, Part A in Chernin, 1963) media (provided by the Hanington lab at UofA) and were left to acclimate overnight in an incubator at 15°C. After the acclimation period, I isolated groups of four individual rediae in different combinations (Table 3). These combinations were: (1) four conspecific reproductive rediae from the same colony, (2) four conspecific small rediae, (3) two conspecific reproductive and two conspecific small rediae, (4) two conspecific reproductive rediae from the same colony and two competitor sporocysts, (5) two conspecific small rediae from the same colony and two competitor sporocysts and (6) one reproductive and two conspecific small rediae from the same colony, and one competitor sporocyst. Species that reproduce as sporocysts were used as the competitor species in all experimental setups, given the difficulty to distinguish between rediae of different trematode species visually. I kept numbers of trematodes constant throughout experimental setups to reduce potential effects of density. With a few exceptions due to logistical constraints, I ran 10 trials per experimental setup for each trematode colony, so my results were statistically robust. With the exception of two experimental setups, I recorded behaviour of 10 colonies of each trematode

species in all experimental setups. Behavioural trials required three consecutive days to complete; thus colonies were kept in the 30 mm glass Petri dish with culture media in the incubator at 15°C until video trials were completed. The CBSS media allowed me to maintain trematode larvae alive and motile in the incubator for 7 days, after which fungal growth usually began.

Each trial was recorded for 20 minutes through a dissecting microscope with an OMAX microscope digital camera attached to the ocular lens of the microscope. I analyzed each video trial using EthoVision® XT 15 software from Noldus Technologies. EthoVision uses pixel areas to track movement of individuals and thus, behavioural measurements are not subjected to observer bias, increasing precision and accuracy of results. EthoVision uses percent of time spent moving per sampling-time unit to assign a mobility status. I selected one second as sample rate and thresholds as follows: highly mobile = moving more than 50% of each second, mobile = moving between 5% and 49% of each second, and immobile = moving less than 5% of each second. I focused data collection on redia mobility status, measured as percent of time spent being either 'highly mobile', 'mobile' or 'immobile'. I also measured interactions between individual larvae (defined below) manually and recorded as percent of time spent attacking. An interaction between a redia and another individual was classified as an 'attack' when mouthparts of the redia latched onto another individual in the trial. If the redia did not latch its mouthparts to the individual but simply brushed against it, the interaction was classified as a 'non-attack'.

Table 3. Redia and sporocyst combinations for in-vitro behavioural experiments with trematode larvae identified morphologically as Petasiger sp. and Hypoderaeum sp. Experimental setup includes combinations of conspecific reproductive (REP) and small rediae (SMA) from the same colony, and members of the other species (= competitor species) developing as sporocysts (SPO). Code name for each treatment is given in parentheses. N = number of individuals of each type of trematode larva.

Experimental setup (code name)	N_{REP}	N_{SMA}	N_{SPO}
1. REP (R4)	4	--	--
2. SMA (S4)	4	--	--
3. REP, SMA (R2S2)	2	2	--
4. REP + SPO (R2SP2)	2	--	2
5. SMA + SPO (S2SP2)	--	2	2
6. REP, SMA + SPO (R1S2SP1)	1	2	1

Molecular identification

To confirm trematode species identity, after measuring morphology and/or running behavioural trials, I preserved redia samples from most trematode colonies in 95% ethanol and stored them at -20°C for COI barcoding. For each sample of trematode tissue, I extracted genomic DNA (gDNA) following extraction protocols from DNeasy® Blood & Tissue kits (Quiagen, cat. no. 69506). I then amplified the mitochondrial *cox1* gene fragment from each putative *Hypoderaeum* sample according to Van Steenkiste et al. (2015), using the primers Dice1F and Dice11R as described in Van Steenkiste et al. (2015). For putative *Petasiger* samples, partial fragments of the mitochondrial gene *ND1* were amplified following Kostadinova et al. (2003), using the primers NDJ11 and NDJ2a described in Kostadinova et al. (2003). PCR was done in 20 µl volumes using 10 µl of the AccuStart II GelTrack® PCR SuperMix (Quanta Bio, cat. no. 95136-500), 0.5 µl of each primer (concentration = 10 µM), and 9 µl of gDNA. I then electrophoresed PCR products (i.e. amplicons) in 1% agarose and purified them following the instructions from Truin Science PCR cleanup kits (Truin Science, model: KTS1115). Purified amplicons were then sent to Macrogen Inc. (Korea) for Sanger sequencing, using the same PCR primers to amplify both strands of each gene fragment. I then trimmed and analyzed resulting sequences for quality with SnapGene© Viewer (Dotmatics) software, and subsequently MUSCLE aligned and BLASTed using Geneious Prime® (version 2023.2.1) software. Library sequences with the highest pairwise comparison percent match rates were then selected and, when possible, a cutoff of 5% nucleotide divergence of both gene sequences was used to determine identity of trematode species. This threshold was used because it is a conservative standard of maximum intraspecific mitochondrial DNA (mtDNA) divergence (Gordy et al., 2016). However, in some cases, the highest pairwise comparison percent match rates exceeded the 5% ideal divergence threshold. Thus, for all samples, I used morphological traits of cercariae to validate barcoding IDs, as it has been suggested that identifications of sequences stored in GenBank are not always fully reliable (Cheng et al., 2023)

Statistical analysis of behaviour

To test if behaviour of reproductive rediae differed from behaviour of small rediae in the absence of competition and with conspecifics, I used a non-parametric PERMANOVA with the function adonis2 in the vegan package in R (Oksanen et al., 2022). I used a non-parametric approach as mobility status data are correlated, sample sizes varied among colonies, response variables included a high number of

zeroes and are bound between zero and 100. I used mobility status (i.e. highly mobile, mobile, or immobile) as response variables and experimental setup (R4 or S4 in Table 3) as the explanatory variable. I treated colony ID numbers as random effects to control for repeated measurements coming from the same colony (i.e. number of trials, ranging from two to 10).

To test whether reproductive rediae change their activity patterns depending on the presence of small rediae, competitor trematode species, or both, I assumed *a priori* that redia mobility would be different among treatments and conducted pairwise comparisons of all treatments directly, given that restricting results to general differences in mobility among treatments would not be very informative nor biologically relevant. I thus performed a pairwise comparison PERMANOVA using the function pairwise.adonis2 in the pairwiseAdonis package in R (Martinez Arbizu, 2017). I again used mobility status as response variables and treatment (R4, R2S2, R2SP2 or R1S2SP1 in Table 3) as the explanatory variable. As before, I treated colony ID numbers as random effects to control for repeated measurements coming from the same colony (i.e. number of trials, ranging from two to 10).

To test whether small redia mobility would differ depending on the presence of conspecific reproductives, competitor trematode species, or both, I performed another pairwise comparison PERMANOVA in the same manner as with reproductives. I again used mobility status as response variables and treatment (S4, R2S2, S2SP2 or R1S2SP1 in Table 3) as the explanatory variable. As before, I treated colony ID numbers as random effects to control for repeated measurements coming from the same colony (i.e. number of trials, ranging from two to 10). Results from barcoding suggested that putative *Hypoderaeum* and putative *Petasiger* colonies are in fact a few different species (Table 4), but as sample sizes would be reduced dramatically and statistical results would be unreliable, I analyzed the data as if they were only two species of trematodes. I thus analyzed both putative *Hypoderaeum* and putative *Petasiger* species separately for each of the statistical models stated above.

Results

Molecular identification

Most samples from putative *Hypoderaeum* colonies were identified genetically as *Ribeiroia ondatrae* (Price, 1931) Price, 1942 (Psilostomidae), and a few others as either *Echinostoma trivolvis* (Cort, 1914) Kanev, Vassilev, Lie & Fried, 1988 (Echinostomatidae), *Echinopariphium* sp. C

(Echinostomatidae) or *Cotylurus* sp. F (Strigeidae) (Table 4). However, the image of *Cotylurus* sp. F from Gordy et al. (2016) does not match morphological traits of cercariae of *Hypoderaeum* (see Fig. S2 in the appendix for images excerpted from Gordy et al., 2016), so I disregarded this barcoding ID. Although *Ribeiroia ondatrae*, *Echinostoma trivolvis* and *Echinoparyphium* belong to different families, for simplicity I will keep referring to this group as 'putative *Hypoderaeum*' throughout this chapter. Most putative *Petasiger* colonies were matched with *Petasiger* sp. 4, and a few others with *Petasiger* sp. 5, *Echinoparyphium* sp. Lineage 3 and *Cotylurus marcogliesei* Locke, Van Dam, Caffara, Alves Pinto, López-Hernández & Blanar, 2018 (Strigeidae) (Table 4). However, *Petasiger* cercariae are very distinctive and dissimilar to cercarial morphology of both *Echinoparyphium* and *Cotylurus* (see Fig. S2 in appendix for images); thus, I disregarded barcoding IDs for these two genera. I therefore treat *Petasiger* as the identity of these colonies throughout this chapter.

Table 4. Trematode species identification results based on sequencing of fragments from genes *cox1* for putative *Hypoderaeum* and *ND1* for *Petasiger* colonies. * = Closest match to GenBank, but above 5% divergence ideal threshold. ** = ID does not match cercarial morphotypes typical to family, thus not reliable.

Experiment	Colony	Putative ID	Genetic ID	% Identity	GenBank accession match
MORPH	HYPO 1	*Hypoderaeum*	*Echinoparyphium* sp. C	95.85	MH369285
MORPH	HYPO 2	*Hypoderaeum*	***Cotylurus* sp. F	97.78	MH369568
MORPH	HYPO 8	*Hypoderaeum*	Sample not kept for barcoding	NA	NA
MORPH	PETA 2	*Petasiger*	Sample not kept for barcoding	NA	NA
MORPH	PETA 9	*Petasiger*	*Petasiger* sp. 4	*94.32	MH369316
BEHAV	HYPO 1	*Hypoderaeum*	No match	NA	NA
BEHAV	HYPO 2	*Hypoderaeum*	No match	NA	NA
BEHAV	HYPO 8	*Hypoderaeum*	*Ribeiroia ondatrae*	98.91	OK210492
BEHAV	PETA 2	*Petasiger*	Sample not kept for barcoding	NA	NA
BEHAV	PETA 9	*Petasiger*	***Echinoparyphium* sp. C	95.85	MH369285
MORPH + BEHAV	HYPO 3	*Hypoderaeum*	*Ribeiroia ondatrae*	100	OK210381
MORPH + BEHAV	HYPO 4	*Hypoderaeum*	*Echinostoma trivolvis*	100	MH369271
MORPH + BEHAV	HYPO 5	*Hypoderaeum*	*Ribeiroia ondatrae*	100	OK210492
MORPH + BEHAV	HYPO 6	*Hypoderaeum*	*Ribeiroia ondatrae*	100	OK210492
MORPH + BEHAV	HYPO 7	*Hypoderaeum*	No match	NA	NA
MORPH + BEHAV	HYPO 9	*Hypoderaeum*	*Ribeiroia ondatrae*	99.74	OK188993
MORPH + BEHAV	HYPO 10	*Hypoderaeum*	*Ribeiroia ondatrae*	100	OK210492
MORPH + BEHAV	PETA 1	*Petasiger*	Sample not kept for barcoding	NA	NA
MORPH + BEHAV	PETA 3	*Petasiger*	*Petasiger* sp. 4	100	MH369317
MORPH + BEHAV	PETA 4	*Petasiger*	***Echinoparyphium* sp. Lineage 3	*91.67	MH369147

Experiment	Colony	Putative ID	Genetic ID	% Identity	GenBank accession match
MORPH + BEHAV	PETA 5	*Petasiger*	***Cotylurus marcogliesei*	*92.45	MH536509
MORPH + BEHAV	PETA 6	*Petasiger*	No match	NA	NA
MORPH + BEHAV	PETA 7	*Petasiger*	*Petasiger* sp. 4	100	MH369318
MORPH + BEHAV	PETA 8	*Petasiger*	*Petasiger* sp. 4	100	MH369318
MORPH + BEHAV	PETA 10	*Petasiger*	*Petasiger* sp. 5	*80.81	OQ543544

Morphology

From morphological observations of trematode colonies in planorbid snails, both *Petasiger* and putative *Hypoderaeum* morphotypes had large reproductive rediae that were actively producing cercariae, whereas I could not see germinal masses or developing cercariae in any of the small rediae used to measure body areas. Small rediae of *Petasiger* were also clear and transparent, more slender than reproductives, had a distinctive collar and relatively large, highly adhesive appendages that were difficult to detach from the bottom of the Petri dish (Fig. 4). However, small rediae from putative *Hypoderaeum* did not differ morphologically from large reproductives in any aspect other than size and apparent lack of germinal masses (Fig. 4). Redia area distributions were right skewed for the pooled data of the 10 colonies of both *Petasiger* and putative *Hypoderaeum* (Fig. S3). However, redia size was very different between years (putative *Hypoderaeum*: $t_{1,8259} = 12.6$, $p < 0.05$; Petasiger: $t_{1,18064} = -33.9$, $p < 0.05$); I thus looked at the area distributions for each colony separately. As seen in Figure 5, redia area distributions from most colonies of both trematode morphospecies followed a bimodal distribution: HYPO3 ($D = 0.08$; $p < 0.001$), HYPO5 ($D = 0.06$; $p < 0.001$), HYPO7 ($D = 0.04$; $p < 0.001$), HYPO8 ($D = 0.05$; $p < 0.001$), HYPO9 ($D = 0.05$; $p < 0.001$); HYPO10 ($D = 0.05$; $p < 0.001$); PETA2 ($D = 0.02$; $p < 0.001$); PETA4 ($D = 0.05$; $p < 0.001$); PETA5 ($D = 0.04$; $p < 0.001$), PETA6 ($D = 0.03$; $p < 0.001$), PETA7 ($D = 0.03$; $p < 0.05$), PETA8 ($D = 0.05$; $p < 0.001$), PETA9 ($D = 0.05$; $p < 0.01$), PETA10 ($D = 0.02$; $p < 0.001$). Bimodal redia size distributions were more common in colonies sampled later in the year (Fig. 5).

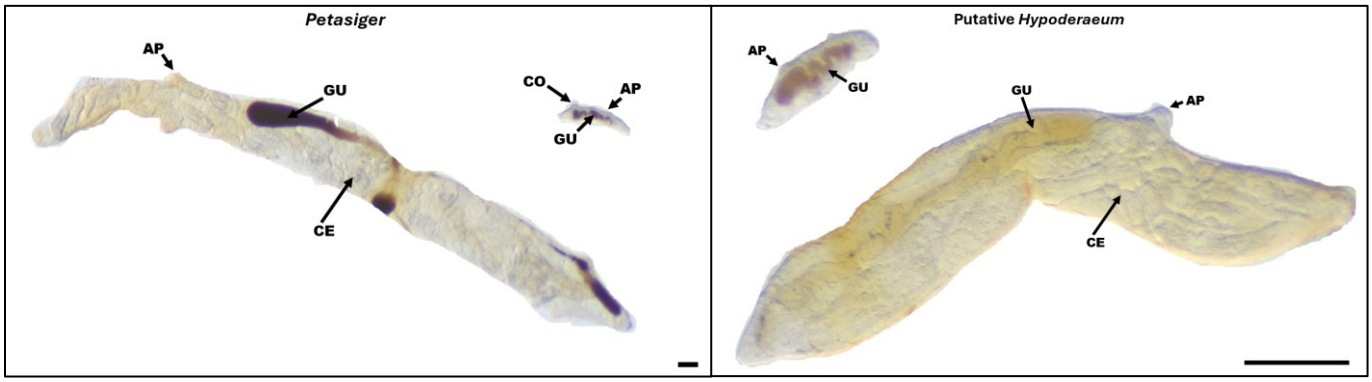


Figure 4. Redia morphology of *Petasiger* (left) and putative *Hypoderaeum* (right). Scale bars = 200 µm. AP = Appendage, GU = Gut, CE = Cercaria, CO = Collar. Rediae were from colonies collected in 2019 from Morinville.

Behavioural trials

In treatments involving only large or only small rediae from the same colony (R4 and S4, Table 3), smaller rediae of putative *Hypoderaeum* were significantly more mobile than reproductives ($F_{1,169}$ = 9.9, $p < 0.005$; Fig. 6 a to c). However, the same difference in mobility was not significant in *Petasiger* (Fig. 6 d to f).

Petasiger reproductives were significantly more mobile in the presence of small rediae ($F_{1,198}$ = 4.4, $p < 0.05$; Fig. 7 d to f) and of sporocysts from a different species ($F_{1,198}$ = 7.8, $p < 0.005$; Fig. 7 d to f), but not in the presence of both (Fig. 7 d to f). There was no significant difference in mobility of reproductives among treatments for putative *Hypoderaeum* (Fig. 7 a to c).

Behaviour of small rediae differed among treatments. Small rediae of putative *Hypoderaeum* moved significantly more in the presence of competitor trematodes than in the presence of conspecific reproductives ($F_{1,142}$ = 5.4, $p < 0.05$; Fig. 8 a to c), but there was no significant difference when both reproductives and competitors were present (Fig. 8, a to c). Contrary to putative *Hypoderaeum*, mobility of small rediae of *Petasiger* did not vary significantly among treatments (Fig. 8 d to f).

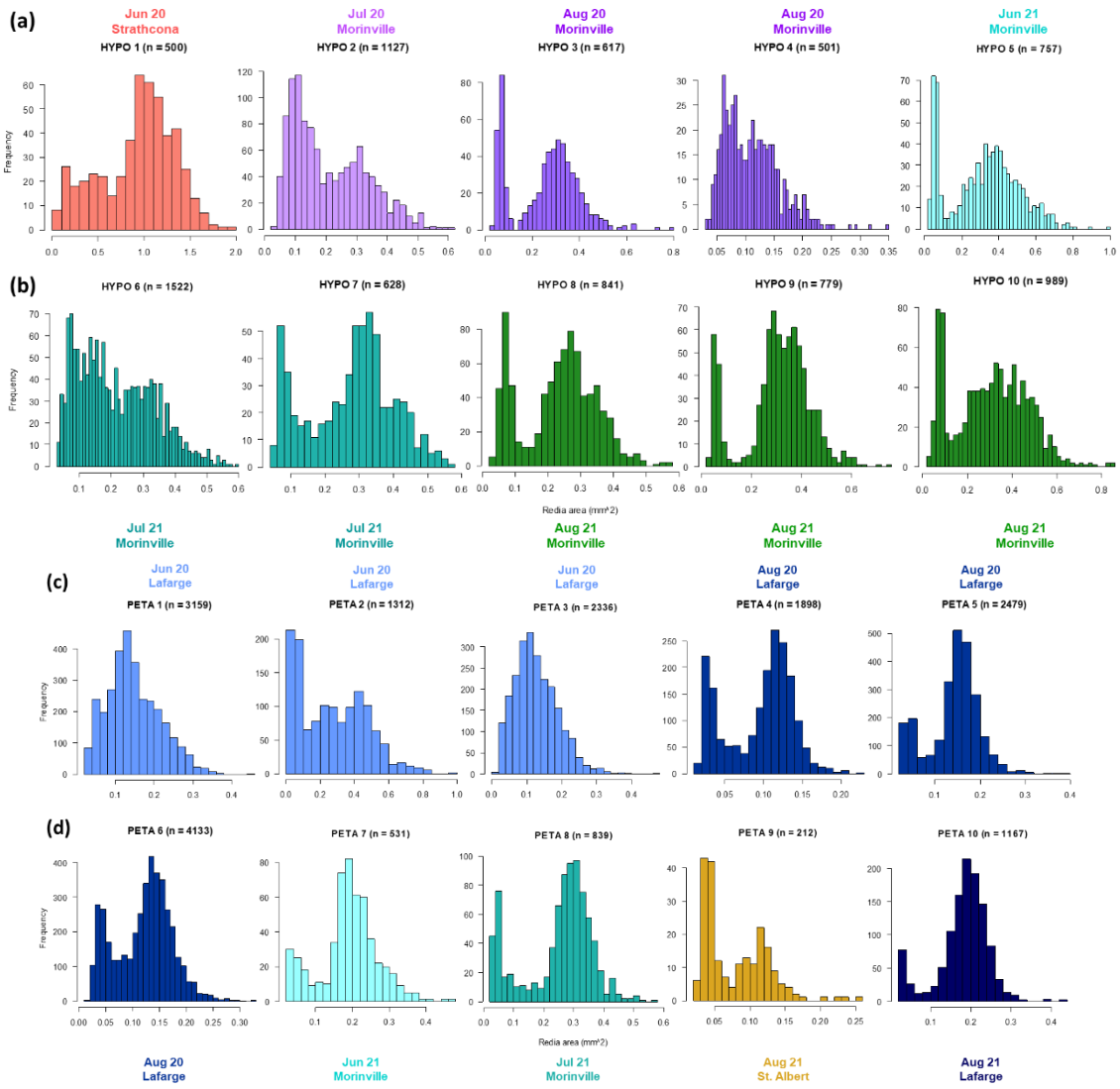


Figure 5. Redia area distributions from putative *Hypoderaeum* (panels a and b) and *Petasiger* (panels c and d) colonies. All colonies were collected in 2020 (a, c) and 2021 (b, d) from Morinville, Lafarge, St. Albert and Strathcona County. Colonies from the same date and location are indicated by the same coloured bars.

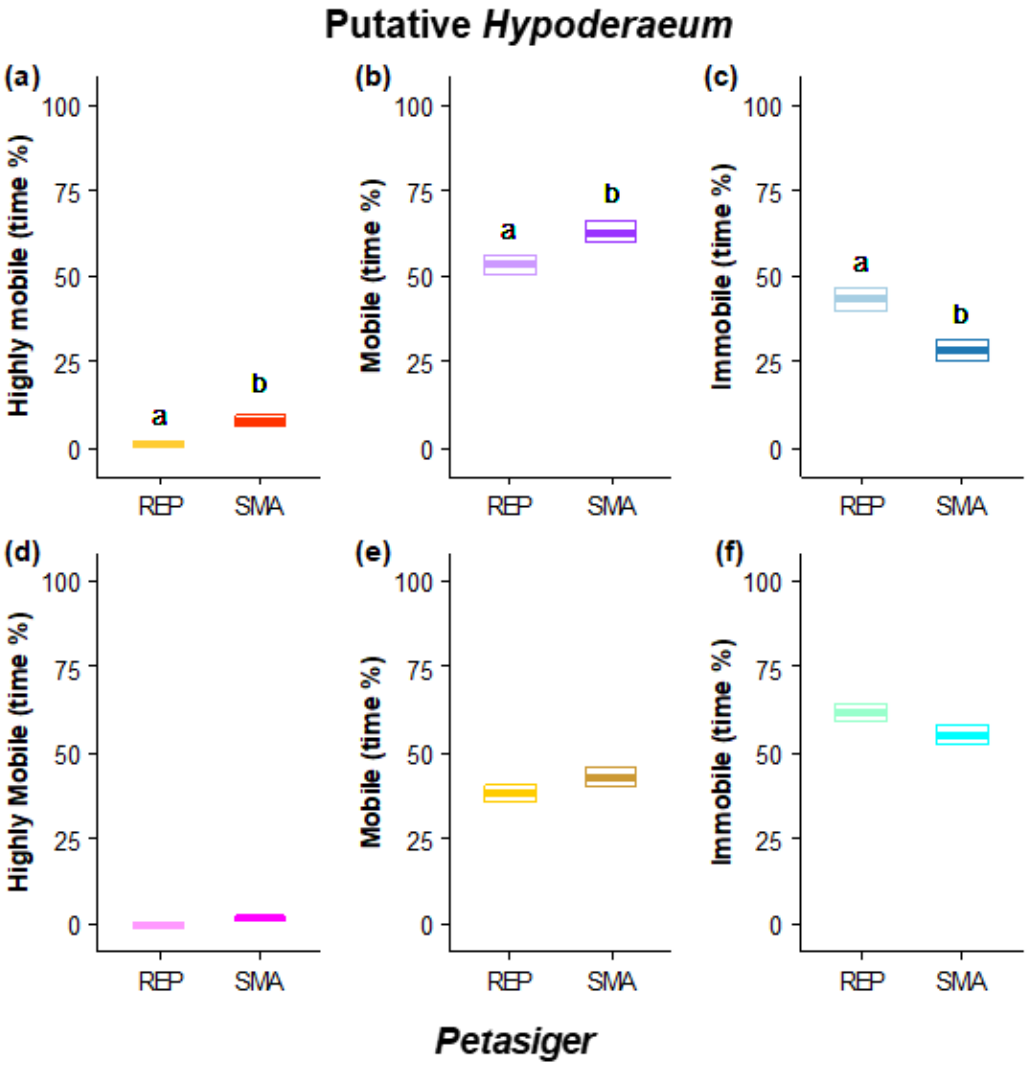


Figure 6. Redia mobility. Mean ± SE of time spent being highly mobile, mobile and immobile (expressed in %). As per Table 3, REP = 4 Reproductives (R4), SMA = 4 Small rediae (S4). Different letters above bars indicate significantly different means ($p < 0.05$). Data from 10 colonies of each trematode morphospecies collected in 2020 and 2021 from Morinville, Lafarge, St. Albert and Strathcona County.

Due to very low attack rates by both putative *Hypoderaeum* and *Petasiger*, I was unable to test statistically whether small rediae would attack members of a competitor trematode species, and if this behaviour would depend on the presence or absence of reproductives in the same treatment. Such low attack rates led to extremely zero-inflated datasets (i.e. over 90% of the data were zeroes) that could not be analyzed, even with packages in R developed specifically for zero-inflated data (e.g, glmmTMB, NBZIMM). Nonetheless, I did witness and record attacking behaviour in small rediae in both species (*Hypoderaeum*: n = 7; *Petasiger*: n = 1), and that behaviour happened only in treatments

that included competitor species or both reproductive rediae and competitors. Surprisingly, reproductive rediae of putative *Hypoderaeum* attacked competitor trematodes in seven trials when they were alone with competitor trematodes. All the attacks were very brief and repeated throughout the duration of the trial. I also recorded small rediae of *Petasiger* attacking discarded tails of cercariae from their own colony. These attacks were more frequent than attacks made by small rediae of putative *Hypoderaeum* against sporocysts of competitor species, and small *Petasiger* rediae that latched to discarded cercaria tails did not release them for the remainder of the trial.

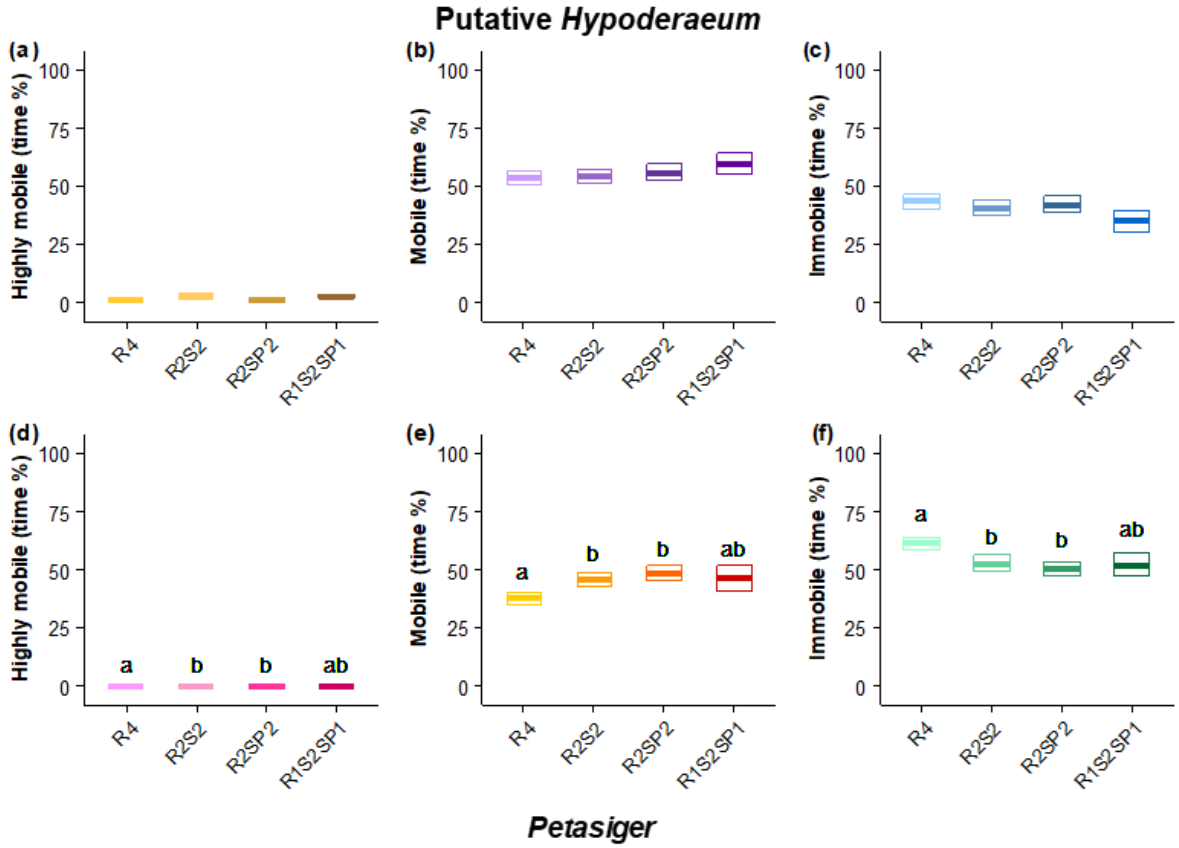


Figure 7. Reproductive redia mobility. Mean ± SE of time spent being highly mobile, mobile and immobile (expressed in %). As per Table 3, R4 = 4 Reproductives alone (10 colonies of each morphospecies), R2S2 = 2 Reproductives and 2 small rediae together (10 colonies of each morphospecies), R2SP2 = 2 Reproductives and 2 competitor sporocysts together (10 colonies of each morphospecies), R1S2SP1 = 1 Reproductive, 2 small rediae and 1 competitor sporocyst together (6 colonies of putative *Hypoderaeum* and 4 colonies of *Petasiger*). Different letters above bars indicate significantly different means ($p < 0.05$).

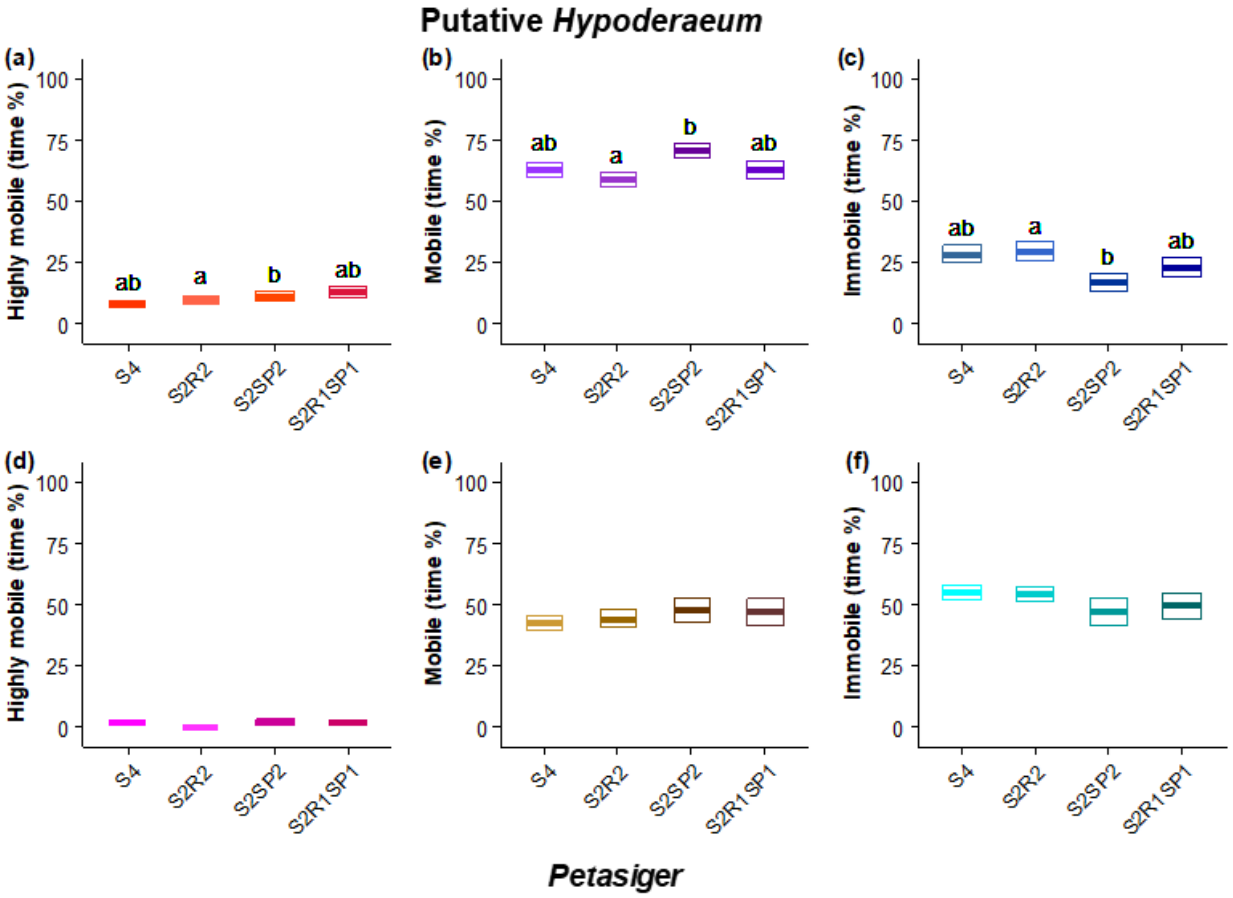


Figure 8. Small redia mobility. Mean ± SE of time spent being highly mobile, mobile and immobile (expressed in %). As per Table 3, S4 = 4 Small rediae alone (10 colonies of each morphospecies), S2R2 = 2 Small and 2 reproductive rediae together (10 colonies of each morphospecies), S2SP2 = 2 Small rediae and 2 competitor sporocysts together (6 colonies of putative *Hypoderaeum* and 4 colonies of *Petasiger*), S2R1SP1 = 2 Small rediae, 1 reproductive and 1 competitor sporocyst together (6 colonies of putative *Hypoderaeum* and 4 colonies of *Petasiger*). Different letters above bars indicate significantly different means ($p < 0.05$). Colonies of each trematode species collected in 2020 and 2021 from Morinville, Lafarge, St. Albert and Strathcona County.

Discussion

Here, I described the occurrence of a smaller redia type that differs morphologically from large reproductive rediae in freshwater trematode colonies from Alberta. I also show that size-frequency distributions in such trematode colonies differ from a unimodal size-frequency distribution, especially in collection dates later in the summer. My results also show a difference in activity levels between reproductive and small rediae. These results support my hypotheses and in general, support my predictions, although behavioural patterns seem to be specific to trematode species and social context.

Similar to results from Hechinger et al. (2011), small rediae from colonies of putative *Hypoderaeum* were more active than reproductive rediae in the absence of competitor trematode species, spending significantly more time mobile or highly mobile relative to reproductive rediae (Fig. 6). Interestingly, when a competitor trematode species is introduced, small putative *Hypoderaeum* rediae are more active when they are alone with competitor species, but not when reproductive rediae are present (Fig. 8). This agrees partially with previous studies showing that small rediae increase activity when presented with heterospecific trematodes (Garcia-Vedrenne et al., 2016), but small rediae have not been reported to lower activity when both competitors and reproductives are present. This behaviour could be explained if reproductives from putative *Hypoderaeum* also assumed a defensive role when competitors are present, increasing their activity levels as a result (Fig. 7). Although attack behaviour could not be analyzed statistically, I did observe in seven trials reproductives of *Hypoderaeum* attacking competitor trematodes when the small morph was absent from the experimental setup, suggesting reproductives can take a defensive role in *Hypoderaeum*. Contrary to my expectations, mobility of small rediae was not significantly different from reproductive rediae in *Petasiger*, with or without competition. Interestingly though, reproductive rediae of *Petasiger* were more active in the presence of small rediae or when competitors were present, but not when both competitors and small rediae were present. These results agree with previous research that showed reproductive rediae are less aggressive when both small rediae and competitors are present (Kamiya and Poulin, 2013). These activity patterns could be explained if the defensive role is shared between small and reproductive rediae, which would be dependent on the immediate proximity from each type of redia to the perceived threat (i.e., competitor species), but more research is necessary to test this.

Size-frequencies for all colonies measured for both putative *Hypoderaeum* and *Petasiger* were either right skewed or bimodal, differing from unimodal size-frequency distributions reported in most freshwater trematodes (Garcia-Vedrenne et al., 2016). Resemblance to a bimodal size-frequency distribution is stronger in colonies sampled later in the year (Fig. 5), suggesting that colonies of both trematode species might start with a unimodal or right skewed size-frequency distribution, when young reproductive rediae are growing fast to increase colony size, and with time, two size classes develop. This type of colony growth has been previously described for marine, but not freshwater, trematode species with division of labour (Garcia-Vedrenne et al., 2017) and for species that form self-sustaining infra-populations (Galaktionov et al., 2015). It is thus necessary to also consider rediae

behaviour when suggesting that division of labour is present in any trematode species. As my results show, both in putative *Hypoderaeum* and *Petasiger*, activity levels differ between small and reproductive rediae, depending on the presence of the other redia type and/or competitor species, supporting thus far the existence of a separate caste in trematode communities in Alberta.

Caste ratio has been proposed to vary depending on whether colonies come from high or low competition environments (Lloyd and Poulin, 2014b). Interestingly, at Strathcona (where trematode prevalence is lowest), the trematode colony sampled also had a bimodal distribution, even at the beginning of sampling season (Fig. 5). Although it is hard to make any assumptions at this point, without more colonies from Strathcona to compare to, it seems that caste ratio in freshwater environments is not correlated to trematode prevalence at each site, as most colonies have two distinct size curves and samples come from different geographical localities with varying trematode prevalence. An alternative explanation for the apparent lack of correlation between a bimodal size-frequency distribution and trematode prevalence in my study system could be that small rediae have a function in the colony other than solely defensive roles against competitor species (e.g., cleaning role), increasing the colony fitness even in the absence of competition (Galaktionov et al., 2015). However, assessing the role of small rediae in *Petasiger* and putative *Hypoderaeum* in their colony would require knowing when each snail contracted the trematode infection, to know exactly how long the infection has been developing for at the time of dissection. To test these hypotheses, it would be necessary to infect snails in the lab, including colonies from sites with low and high competition, and to test the role of small rediae in the colony.

Attack rates were rare/infrequent, although I did notice aggressive behaviour in putative *Hypoderaeum* reproductives in seven individuals when they were alone with competitor trematodes. When attacks did happen, they were brief but repeated throughout the trial. On six occasions, I witnessed putative *Hypoderaeum* small rediae attacking competitor species when reproductives were absent, and on one occasion when reproductives and competitor trematodes were present. Attacks lasted longer in the trial where reproductives and soldiers were exposed to a competitor. Small rediae of *Petasiger* only attacked competitor trematodes once, and only in the absence of reproductives. These observations suggest that attacking behaviour in trematodes from planorbids in Alberta are rare under *in-vitro* conditions, but they do exist and could be triggered by the presence of competitor species. It is also important to bear in mind that, as mentioned above, I did not find evidence of

unimodal size-frequency distribution in any of the colonies I measured, from any of my study sites. Unimodal size-frequency distribution has been previously recorded from freshwater trematode colonies that have been bred for multiple generations and that have potentially lost the small redia morph in absence of competition for generations (Garcia-Vedrenne et al., 2016). The size-frequency distributions I present here are either right-skewed or bimodal, which indicates that small rediae are abundant in trematode colonies from Albertan planorbids. It is therefore plausible to expect small rediae to have a purpose in the colony other than simply to replace reproductives when they die, as it is costly to produce and maintain a large number of small rediae in a colony (Kamiya and Poulin, 2013b). It has been previously proposed that small rediae can potentially protect the snail host (and the colony) against microbial infection, as trematode infections reduce immune response from the snail host and this could potentially make the snail more susceptible to more harmful infections (Lloyd and Poulin, 2012). It is also noteworthy that in *Petasiger*, I recorded small rediae attaching their mouthparts to discarded tails of cercariae from their own species. Unlike the attacks directed to competitor species, the small rediae of *Petasiger* did not release a cercaria tail once they latched onto it. This could indicate a cleaning rather than a defensive behaviour in small *Petasiger* rediae, similar to *Paramonostomum alveatum* that consumes dying and dead mother and daughter rediae (Galaktionov et al., 2015). Cleaning roles have been proposed to reduce negative impacts of trematode colonies on their snail host and to promote the long-term survival of trematode colonies by removing toxic substances formed with decomposing matter (Galaktionov et al., 2015). Thus, more tests are necessary to elucidate the role small rediae play in their colonies at my study sites.

Small and reproductive rediae were morphologically different in colonies of trematodes from planorbid snails. In *Petasiger* colonies, small rediae were clear (almost transparent in some cases), slimmer than reproductives, had a visible collar, relatively large and highly adhesive appendages, and lacked visible germinal masses (Fig. 4). All these characteristics have been previously reported for marine trematode species with division of labour (Garcia-Vedrenne et al., 2016; 2017). The presence of developed appendages in small rediae has been proposed to increase leverage when small rediae are attacking heterospecific trematodes (Garcia-Vedrenne et al., 2016). *Petasiger* small rediae appendages were harder to remove from substrate than appendages from large reproductives (Pers. obs.), and appendages in reproductive rediae were smaller relative to body size than appendages in small rediae (Fig. 4). In putative *Hypoderaeum*, there was a substantial size difference between rediae types and

small rediae lacked visible germinal masses. However, small and reproductive rediae bodies are similar. Their colouration and body shape are similar, they both lack a collar, and both morphs have appendages of proportionally the same size (Fig 3.1). It is therefore conceivable that small rediae of putative *Hypoderaeum* and *Petasiger* have a different role in the colony other than solely grow into reproductives to replace them when reproductives die, as would be the case in self-sustaining infra-populations (Galaktionov et al., 2015).

It was surprising that the majority of samples treated as putative *Hypoderaeum* were genetically identified as *Ribeiroia ondatrae*. However, cercariae from both species have very similar morphology at low magnification. Cercariae of both species have a relatively large oral sucker, distinctive main excretory collecting tubes with excretory granules, and tail fin folds. The main difference between species is the size of cercariae and the lack of a spine collar in *Ribeiroia ondatrae*, which could have been missed when looking at the cercariae under dissecting microscope. It is thus possible that I misidentified some of the cercariae. The same scenario could be applied to the barcode matches for *Echinostoma trivolvis* and *Echinoparyphium* sp. C, as the only significant difference in morphology with *Hypoderaeum* is cercarial size. However, the genetic match with *Cotylurus* sp. F is harder to explain, as cercariae of the genus *Cotylurus* possess morphological characteristics that deviate strongly from cercariae of *Hypoderaeum*. *Cotylurus* cercariae have a thin bifurcated tail without fin folds, their excretory tubes are not visible without staining, they lack spine collar and cercariae bodies are smaller relative to the length of their tail (Fig. S2). Furthermore, *Cotylurus* have sporocysts, while *Hypoderaeum* species produce rediae, and given that I studied rediae taken directly from planorbid snails, it is highly unlikely that the *Cotylurus* identification is correct. Cheng et al. (2023) have highlighted the importance of taking information from morphological features along with DNA barcoding to increase reliability of species identification. Sequences can be linked to erroneously identified organisms in GenBank, and images from organisms are not always provided as metadata to confirm the identity of the original organism where sequences come from (Cheng et al., 2023). With this reasoning, I chose to follow the morphological identification of the original sample over the results from barcoding for *Cotylurus* sp. F.

Molecular identification of samples from *Petasiger* that were matched to either *Echinoparyphium* or *Cotylurus* were also suspect, as morphology of the genus *Petasiger* differs significantly from either genus. *Petasiger* cercariae have a tail that is several orders of magnitude larger than its body and has

an easily identifiable mode of swimming. None of these characteristics could be mistaken for either *Echinoparyphium*, which resembles *Hypoderaeum*, or *Cotylurus*, which has a very slim and bifurcated tail (Fig. S2). Given the morphological differences with every other cercariae type, and that most samples from putative *Petasiger* were matched with *Petasiger* sp. 4 or 5, I am confident all samples used in this chapter belonged in fact to the genus *Petasiger*.

In this chapter, I described the existence of two morphologically different redia types that form colonies with a bimodal size-frequency distribution. This size-frequency distribution seems to be independent of trematode prevalence in the environment, suggesting the small redia type may have a role other than defending the colony against competitor species. My results also show behavioural differences between reproductive and small rediae, depending on trematode species and the presence of conspecific rediae and/or competitor species, suggesting that trematode species in my study system have the capacity to attack members of a competitor species, but could also perform cleaning services for the colony. Even though the role of small rediae in planorbid snails studied here is not currently clear, this study provides, for the first time in Canada, compelling and thorough evidence that division of labour is present in freshwater trematode species in Alberta.

Chapter 4: Trematode metacercarial infection and snail survival

Introduction

Trematodes are a diverse group of obligate parasitic flatworms with complex life cycles that use snails as first intermediate hosts, a variety of vertebrates and invertebrates as second intermediate hosts and vertebrates as final hosts (see Chapter 1 for details). Trematodes are well known for altering the behaviour of their intermediate hosts in a variety of ways (see review in Moore, 2012). For example, trematode metacercariae can change activity patterns and distribution of their mosquito larva and fish second intermediate hosts (Webber et al., 1987a; Gopko et al., 2017), or mechanically impede normal protective behaviours in cockles (Mouritsen and Poulin, 2003a). These behavioural changes render infected second intermediate hosts more susceptible to predation, increasing transmission success to the definitive host (Webber et al., 1987b; Mouritsen and Poulin, 2003b). One of the best-known examples are the severe malformations induced in frogs and other vertebrate second intermediate hosts (Blaustein and Johnson, 2003; Cunningham et al., 2005; Kelly et al., 2010). Other morphological alterations, such as stunted host growth (Thieltges, 2006), or changes in the host's physiological functions, including heart rate and neurological processes (Bakhmet et al., 2017), are less spectacular but also related to trematode infection. These can have important effects on host populations, making their study important for conservation, biodiversity, community structure and food web dynamics (Huxham et al., 1995; Lafferty et al., 2008; Byers, 2009; Kelly et al., 2010).

Metacercarial infection in second intermediate hosts can affect host population dynamics and ultimately, the entire community structure. Cockles that are unable to burrow due to metacercarial infection increase the complexity of mudflat environments by creating hard substrate for benthic invertebrates, resulting in communities with higher species richness than in sites where trematodes are absent (Mouritsen and Poulin, 2003a). By promoting predation on its amphipod host, metacercariae of the trematode *Maritrema poulini* Presswell, Blasco-Costa and Kostadinova (Microphallidae) reduce the population density of their host and affect the structure of crustacean communities in New Zealand lakes (Friesen et al., 2020). In some cases, the effects of trematode infection on individual host

survival are not immediate but can still have important repercussions for host population dynamics. Newts infected with metacercariae reach sexual maturity earlier and have shorter lifespans than uninfected newts, potentially causing host populations to decline over multiple generations (Sinsch et al., 2018).

Surprisingly, studies focusing on direct effects (i.e., not as a result of host manipulation by parasites) of trematode infections on second intermediate host survival are scarce. Some of these studies have found no significant effect, while others have reported severe repercussions on second intermediate host survival. For instance, survival of leeches and cockles does not seem to be directly affected by trematode metacercarial cysts (de Montaudoin et al., 2012; Calhoun et al., 2020). In contrast, cercarial penetration and subsequent cyst formation can lower survival in tadpoles (Marino et al., 2017), especially when tadpoles are young (Pathirana et al., 2019a). In cases where trematode infection is deleterious for tadpole survival, trematode coinfections and, most importantly, the sequence of infection have opposite effects (Pathirana et al., 2019b). Metacercarial infection can have additive effects with environmental stressors such as temperature. For example, when amphipods are exposed to higher water temperatures, survival of individuals infected with trematode cysts is lower than survival of uninfected amphipods (Mouritsen et al., 2018). However, trematode effects combined with temperature can depend on intensity of infection. At high temperatures, mussels with low cyst counts have lower survival than uninfected mussels, but mussels infected with a large number of cysts tend to have higher survival than uninfected mussels (Selbach et al., 2020). Overall, metacercarial infection can affect survival of second intermediate hosts in multiple ways, and these alterations can have important effects at the community, population and ecosystem level.

Alberta lakes and ponds, both natural and artificial, harbour a great diversity of invertebrates that provide food for resident fish and mammals, and serve as important migratory stops for many bird species. These bodies of water are often inhabited by dense populations of multiple snail species, some considered to be endangered (Prescott and Curteanu, 2004). These snails are also hosts to numerous trematode species (Gordy et al., 2016, 2017; Gordy and Hanington, 2019). Individual freshwater snails in Alberta can be infected with a single to over 1600 trematode metacercarial cysts (pers. obs.). In this chapter, I evaluate the effects of metacercarial infection on snail survival. Trematodes in Albertan snails encyst mainly in the snail's gut, where they form large cysts, and kidney, where they form small cysts. Due to size differences between the two types of cysts, they are likely to represent at least two

different trematode species. Due to the importance of kidneys as waste disposal organs in metazoans and given that coinfections usually have additive negative effects on host survival (Pathirana et al., 2019b), I hypothesize that (I) cyst location (gut vs kidney), (II) number of cysts per infected host (from here on, referred to as infection intensity), and (III) infection status (i.e. single infection, coinfection or uninfected) will affect snail survival. I predict that (i) survival of snails with cysts in the kidney will be lower than that of snails with cysts only in the gut, (ii) snail survival should decrease as infection intensity increases, and (iii) snails with coinfections should die sooner than uninfected snails and snails with single infections. Given the scarcity of research on direct effects of metacercarial infection in second intermediate hosts, investigating potential effects of trematode cysts in snails used as second intermediate hosts is necessary and novel. Assessing the effects of cyst infections on snail survival could help understand freshwater snail community composition in Albertan wetlands.

Methods

To evaluate potential effects of trematode metacercarial cysts on snail survival, I collected 362 freshwater snails (>3 mm shell size). Snails were collected between June 2nd and August 28th, 2020, from Narrow Lake, Wabamun Lake, Morinville, Lafarge, Strathcona County, St. Albert (see Chapter 1 for more information on location) and water bodies on a farm just north of Ardrossan, AB. Snails were captured using a dip net (2 mm mesh opening) to scoop up macrophytes growing close to the sediment. Snails were identified to genus level based on morphology, using Prescott and Curteanu (2004). I collected snails from four genera: *Lymnaea* (n = 62), *Physa* (n = 23), *Planorbella* (n = 206) and *Stagnicola* (n = 71). Snails were transported to the laboratory, and isolated in plastic specimen cups filled with 60 mL of natural pond water. Specimen cups were maintained in an incubator at 15°C, and small pieces of commercial Hikari© algae wafers were provided as food *ad libitum*. I changed half the water in the container weekly with fresh water from the original source lake or pond to maintain the water relatively clean without shocking the snails with potential changes in salinity or pH. I monitored snail survival daily for 7 months and dissected the snails as they died to record the number of cysts in each snail. Snails were assumed dead when they did not retract into their shell after being prodded with a dissecting needle. Prior to dissection, I measured snail shells from the bottom of the aperture to the apex of the spire for lymnaeids and physids. For planorbid snails, I measured width of

the outermost shell whorl (i.e., from the top of the aperture to the widest part of the shell) as a proxy for snail size.

After dissection, I preserved some trematode cysts in 95% ethanol and stored them at -20°C for DNA barcoding. As metacercariae from dead snails may have had degraded mitochondrial DNA (mtDNA), I also dissected live snails to preserve cysts from fresh individuals as comparison. For barcoding, I followed genomic DNA (gDNA) extraction protocols from DNeasy® Blood & Tissue kits (Quiagen, cat. no. 69506). Amplification of the mitochondrial *cox1* gene fragment from each sample was performed according to Van Steenkiste et al. (2015), using the primers Dice1F and Dice11R as described in Van Steenkiste et al. (2015). PCR was done in 20 µl volumes using 10 µl of the AccuStart II GelTrack® PCR SuperMix (Quanta Bio, cat. no. 95136-500), 0.5 µl of each primer (concentration = 10 µM), and 9 µl of gDNA. PCR products (i.e., amplicons) were then electrophoresed in 1% agarose and purified using Truin Science PCR cleanup kits (Truin Science, model: KTS1115). Purified amplicons were sent to Macrogen Inc. (Korea) for Sanger sequencing, using the same PCR primers to amplify both strands. Resulting sequences were trimmed and analyzed for quality with SnapGene© Viewer (Dotmatics) software, and subsequently MUSCLE aligned and BLASTed using Geneious Prime® (version 2023.2.1) software. Library sequences with highest percent match rates were then selected and a cut-off of 5% nucleotide divergence of *cox1* sequences was used to determine identity of cysts species. This threshold was selected as a conservative standard of maximum intraspecific mtDNA divergence (Gordy et al., 2016).

To analyse snail survival, I constructed Cox Proportional Hazards (CPH) regression models using the coxph function in the Survival package in R (Therneau, 2022). CPH models give accurate survival estimates while incorporating information of covariates that could potentially reduce survival over time. Given inherent size differences among snail species, and that models with an interaction term between snail size and snail species did not perform correctly, I built CPH models for each snail genus separately. Thus, CPH models for each snail genus included survival over time as response variable, location of trematode cysts, snail size, and number of cysts in kidney, gut or in kidney and gut as explanatory variables. Data from uninfected snails was kept in the models to provide a comparison point for snail survival. Data from snails without size information were removed from all models.

Results

All four genera of snails included uninfected individuals, individuals with gut-only, kidney-only and both types of metacercarial infections (i.e., gut and kidney). The number of gut cysts per snail ranged from one to 500, and number of kidney cysts from one to 1669. Trematode cysts extracted from snails used in this chapter proved difficult to sequence and most of the samples taken from both frozen and live snails did not produce usable sequences. The gut cyst sample that was successfully extracted, amplified and sequenced was obtained from a live planorbid snail and the nine cysts were identified as *Cotylurus cornutus* (Rudolphi, 1809) Szidat, 1928 (Strigeidae) (99.2% sequence match; GenBank accession: MH369500). The kidney cyst sample that produced usable sequences was also obtained from a live planorbid and contained 30 cysts that were a match with the genus *Echinoparyphium* (Echinostomatidae) (96.4% sequence match; GenBank accession: MH369270).

Overall, snail survival showed a steep decline in the first 20 days and less than 10% of snails survived longer than 100 days in the lab, regardless of infection status. Different factors had variable effects on snail survival depending on snail species (Table 5). In *Planorbella*, the location of cysts (i.e. kidney, gut or coinfections), intensity of infection in gut (number of cysts), and snail size significantly affected snail survival (Table 5). Planorbids with cysts in the kidney survived significantly longer than uninfected snails and snails with coinfections (i.e. with cysts in both kidney and gut; $z = -2.8$; $p < 0.005$; Table 5; Fig. 9). Survival of snails with cysts in the gut only was significantly lower than survival of uninfected snails and snails with coinfections ($z = 3.4$; $p < 0.001$; Table 5; Fig. 9). In planorbids, coinfections were not associated with lower survival than for uninfected snails (Table 5; Fig. 9). *Planorbella* survival had a positive correlation with intensity of infection (i.e. number of cysts) in the gut ($z = -2.4$, $p < 0.05$; Table 5; Fig. 10b). However, there was no effect of intensity of infection in the kidney nor in both kidney and gut on planorbid survival (Table 5; Fig. 10c). Finally, smaller planorbid snails survived significantly longer than larger snails in the lab ($z = 4.7$, $p < 0.001$; Table 5; Fig. 11).

In *Lymnaea*, intensity of infection in the kidney ($z = 2.2$, $p < 0.05$; Table 5; Fig. 10a) and in both kidney and gut together ($z = 2.2$, $p < 0.05$; Table 5; Fig. 10c) had a significant negative effect on snail survival, but neither intensity of infection in the gut (Table 5; Fig. 10b) nor location of cysts (Table 5) affected lymnaeid survival. Smaller lymnaeids survived significantly longer than smaller snails ($z = 4.0$, $p < 0.001$; Table 5; Fig. 11).

In *Physa*, there was a significant negative effect of intensity of infection in the kidney ($z = 2.2$, $p < 0.05$; Table 5; Fig. 10a) and in both kidney and gut together ($z = 2.6$, $p < 0.05$; Table 5; Fig. 10c) on snail survival, but intensity of infection in the snail's gut had no effect on survival of physids (Table 5; Fig. 10b). Neither location of infection nor snail size (Table 5; Fig. 11) had a detectable effect on survival of *Physa*.

Lastly, survival in *Stagnicola* was not affected by location of cysts (Table 5), intensity of infection in any organ (Table 5; Fig. 10), or snail size (Table 5; Fig. 11).

Table 5. Trematode infection factors and effects on survival of four genera of freshwater snails. Results from Cox Proportional Hazards (CPH) regression models. CPH regressions models were run independently for each snail genus. Snail survival compared to survival of uninfected snails. N = No significant effect on snail survival. + = Significant positive effect on snail survival. - = Significant negative effect on snail survival.

Snail genus	Coinfections	Cysts in gut	Cysts in kidney	# Cysts in kidney	# Cysts in gut	# Cysts in kidney + gut	Snail size
Lymnaea	N	N	N	-	N	-	-
Physa	N	N	N	-	N	-	N
Planorbella	N	-	+	N	+	N	-
Stagnicola	N	N	N	N	N	N	N

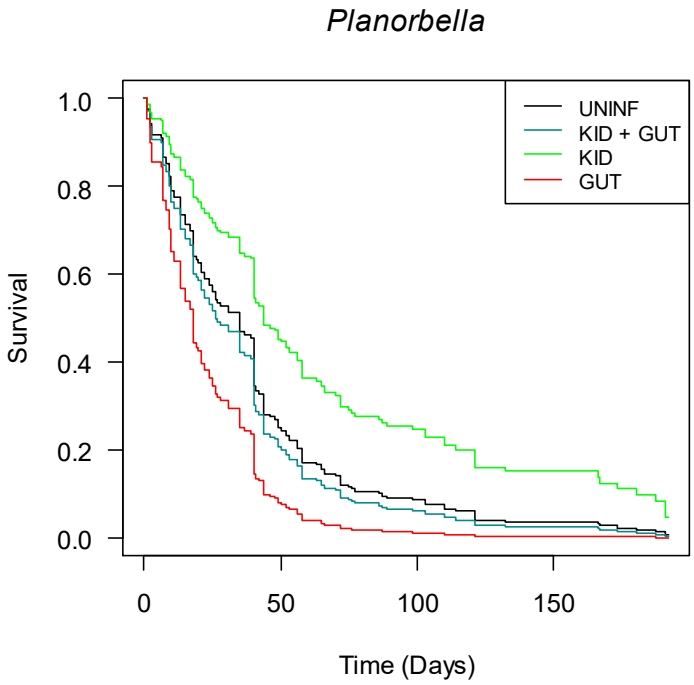
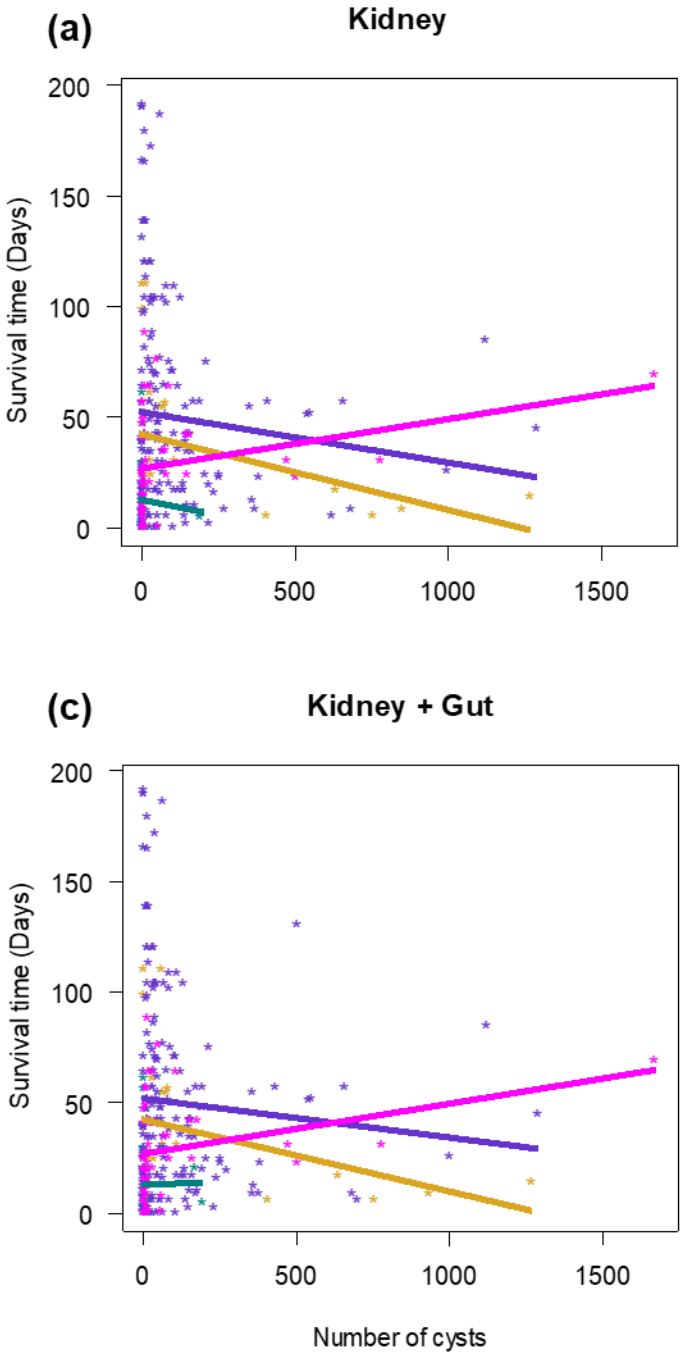


Figure 9. Cox Proportional Hazards (CPH) Survival estimates for *Planorbella* sp. (n = 178) snails kept in isolation in lab conditions. Data from CPH model (Survival ~ Location of metacercariae + Snail size). Legend labels: UNINF = Uninfected snails, KID + GUT = Snails with cysts in kidney and gut, KID = Snails with cysts in kidney, GUT = Snails with cysts in gut.



(d)

Snail genus	# Cysts in Kidney	# Cysts in gut	# Cysts in kidney + gut
Lymnaea	0.003*	0.007	0.0002*
Physa	0.126*	0.004	0.120*
Planorbella	0.009	0.005*	0.006
Stagnicola	0.063	0.005	0.065

Figure 10. Correlation between survival (in days) and the number of cysts (i.e. infection intensity) separated by genus of freshwater snails kept in isolation in lab conditions. Panel (a) infection intensity in kidney; panel (b) infection intensity in gut; panel (c) infection intensity in kidney and gut together; panel (d) r^2 values for each regression line. Asterisks indicate r^2 values are associated with p values < 0.05 from Cox Proportional Hazards (CPH) model outputs.

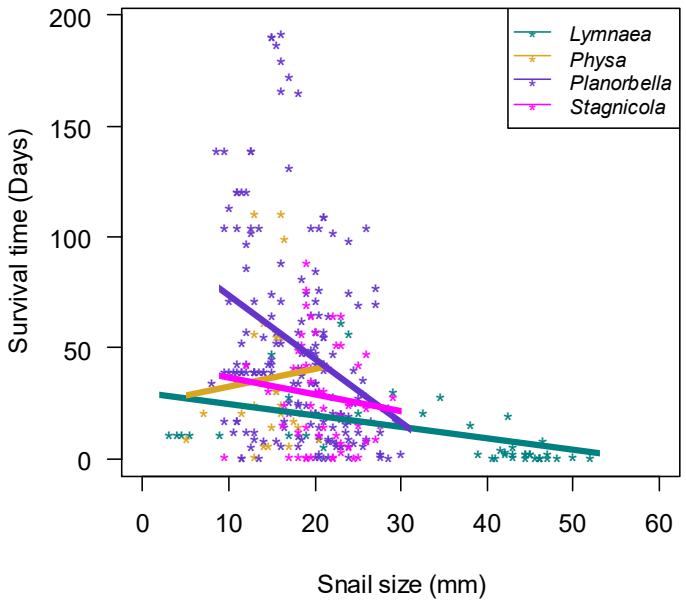


Figure 11. Correlation between survival (in days) and snail size separated by genus of freshwater snails kept in isolation in lab conditions. r^2 values for regression lines: *Lymnaea* = 0.26*; *Physa* = 0.007; *Planorbella* = 0.111*; *Stagnicola* = 0.019. Asterisks indicate r^2 values are associated with p values < 0.05 from Cox Proportional Hazards (CPH) model outputs. Data for all infection categories pooled.

Discussion

Independent of infection status, snail survival in the lab showed a steep decline over time, with less than 10% of snails surviving longer than 100 days. It was noteworthy that different factors affected survival differently according to snail genus. Out of the four snail genera used in this study, location of cysts affected survival of planorbid snails only. *Planorbella* individuals with cysts in the kidney survived longer than uninfected snails and individuals in all other infection categories. In contrast, the lowest survival was shown by planorbids with cysts in the gut. Despite this seemingly negative effect of gut cysts in this categorical approach, infection intensity in the gut had a positive effect on survival of *Planorbella*. Further, intensity of infection had significant effects on two of the other snail genera in my study system. In *Lymnaea* and *Physa*, survival of snails decreased with higher infection intensity in the kidney and in co-infections (both kidney and gut together), but there was no effect of infection intensity in gut only. Finally, snail survival was negatively correlated with snail size in *Lymnaea* and *Planorbella*, but not in *Physa* or *Stagnicola*.

These results are consistent with my hypotheses that snail survival would be affected by cyst location and infection intensity, but they contradict my hypothesis that infection status (single infection, coinfection or uninfected) would affect snail survival. Data also partially support my

prediction that snail survival would decrease with intensity of infection in the kidney, which was true for *Lymnaea* and *Physa*. However, they partially contradict my prediction that snails with cysts in the kidney would have lower survival than uninfected snails and snails in any other infection category (which was not the case for *Planorbella*). The latter was the most unexpected observation. Positive correlation between snail survival and the presence of trematode cysts in the kidney is counterintuitive. Due to the importance of kidneys in metazoan excretory systems, we would expect kidney functions to be impaired by the presence of *Echinoparyphium* cysts, with potentially negative effects on snail health and survival. One potential explanation is that *Echinoparyphium* cysts in planorbids are able to perform some sort of waste disposal service for the snail by nutrient exchange through the cyst wall. It has been documented that during early development, metacercariae of the genus *Diplostomum* have a folded outer plasma membrane that increases surface area for active transport of glucose with surrounding brain tissue of their fish host (Bibby and Rees, 1971a, b). In invertebrate hosts, microphallid metacercariae also display nutrient absorption, but this nutrient exchange stops when the second cyst layer is formed (i.e., when the cysts is fully matured); the final cyst is a capsule that is resistant to chemical transport and prevents host-derived metabolites from harming the metacercariae (Galaktionov et al., 1996). Further, lymnaeid snails infected with *Cryptocotyle lingua* (Creplin, 1825) Fischoeder, 1903 (Opisthorchiidae) or *Renicola roscovitus* (Stunkard, 1932) Werding, 1969 (Renicolidae) show lower levels of copper and iron concentrations than uninfected snails in polluted bodies of water (Cross et al., 2003), suggesting trematodes can absorb pollutants from the body of their snail host. Similarly, parasites can accumulate heavy metals at much higher concentrations than the surrounding host tissue and than the host's environment (Sures et al., 1999).

This evidence indicates that trematodes can absorb potentially harmful chemicals from their snail host body, but whether *Echinoparyphium* cysts can act as supplementary or surrogate kidneys in planorbid snails in my study system remains to be tested, especially given the contradictory result of intensity of infection in the kidney not having a significant effect on survival of planorbid snails. An alternative explanation could be that the presence of *Echinoparyphium* cysts creates a cross-signaling effect (independent from intensity of infection) that triggers an immune response from the snail, and this response makes the snail more resilient to some environmental stressors. Similar patterns have been documented from protozoan and viral infection that promote increased thermal tolerance in oysters and aphid vectors by triggering the expression of heat shock proteins (Encomio and Chu, 2007;

Porras et al., 2020). Further, it has been suggested that immune responses triggered by *Echinoparyphium* cysts lower replication of a ranavirus and increase frog survival to viral infection when frogs are exposed to trematode infection 10 days prior to viral infection (Wuerthner et al., 2017). Whether any of these apply in my study system needs to be further tested.

The apparent absence of infection intensity effects on survival of *Stagnicola* snails was also surprising. Previous research suggests that metacercariae of *Uvulifer ambloplitis* (Hughes, 1927) Dubois, 1938 (Diplostomidae) can be very virulent, killing their fish host if they reach infection intensities higher than 50 cysts per fish (Esch et al., 2002). It is especially astounding that *Stagnicola* in my study system can carry several hundreds of cysts without apparent negative impacts on host survival. However, it has been noted that hundreds of *Posthodiplostomum minimum* (MacCallum, 1921) Dubois, 1936 (Diplostomidae) encyst in the heart, liver and spleen of bluegill sunfish without affecting body condition or survival of their fish host (Esch et al., 2002). It is thus plausible that *Echinoparyphium* and *Cotylurus* found in my study system have low virulence once encysted. Because they rely on trophic transmission and reach their definitive host when second intermediate hosts are eaten by a bird, low virulence and increased snail survival may be beneficial to the parasite (Campbell, 1973; Huffman and Fried, 2012). It is noteworthy however, that at the categorical level (uninfected/metacercariae in gut/metacercariae in kidney), trematode infection in the gut of planorbids had a negative effect on *Planorbella* survival, but high intensity of infection in the gut was correlated with increased survival. These results might be a combination of the metacercarial effects discussed above. Low infection intensity of *Cotylurus* cysts may harm their planorbid host by disrupting digestive functions in the snail, but there might be an infection threshold above which either cysts become less virulent, high numbers of cysts trigger immune response in the snail host, or cysts absorbs hazardous chemicals from the body of the host. As with effects of location of infection, however, these explanations remain to be tested.

The lack of effect of coinfections on snail survival also contradicts my expectations and previous research (e.g., Pathirana et al., 2019b) reporting increased deleterious effects on host survival from trematode coinfections. Potential explanation for the results observed here might be less straightforward. The survival curve for planorbid snails coinfected with *Echinoparyphium* and *Cotylurus* resembles more the survival curve for uninfected snails than the survival curve of snails with single infections from *Cotylurus* or *Echinoparyphium*. A plausible explanation for this might be

that the beneficial effects of *Echinoparyphium* are thwarted by *Cotylurus* infection. However, both *Cotylurus* and *Echinoparyphium* share the same bird definitive hosts (e.g., Black-billed Magpie, Common Goldeneye, Mallards) and potential differences observed here cannot be attributed to intra-host conflicts over transmission pathways. Previous research suggests that interactions among trematode species are complex and depend on the species of trematodes that inhabit a second intermediate host. The outcome of coinfections and parasite interactions also depend on the sequence of events that leads to such interactions (Esch et al., 2001). Metacercarial coinfections in frogs infected with mildly virulent trematode species can replace or reduce the effects of more virulent trematode species and increase survival of their frog host (Johnson and Hoverman, 2012). *Cotylurus* may use a different transmission strategy to increase the probability of predation of its snail host in the wild (e.g., behavioural manipulation of the snail host) with increased virulence being a trade-off from it. *Echinoparyphium* may not use such a transmission strategy, and simply benefit from increased survival of the snail host, passively increasing probability of predation by birds. Whether any of these explanations apply in my study system remains to be tested, and further research on trematode competition and effects on snail second intermediate host behaviour and survival is necessary. Independent of coinfections, metacercarial infection had no negative effects on survival of *Stagnicola* individuals. These results are consistent with previous research on the effects of metacercarial infection in invertebrate second intermediate hosts. Microphallid cysts do not appear to have a negative effect on amphipod host survival, as larger (i.e., older) amphipod hosts have higher number of cysts, suggesting amphipods survive to accumulate cysts over time (Thomas et al., 1995). Similarly, leech survival is not correlated with exposure to *Australapatemon* cercariae (Calhoun et al., 2020), and cockle survival is not affected by metacercarial infection with *Himasthla interrupta* (de Montaudoin et al., 2012).

Survival of smaller snails was higher than larger snails in *Lymnaea* and *Planorbella*. These results make intuitive sense, as larger snails are likely older and thus are more likely to be closer to death than smaller conspecifics irrespective of infection status. Larger snails may also be more resilient to cercarial penetration and encystment than smaller snails (Kuris and Warren, 1980), which might make isolation of trematode effects on snail survival more difficult when dealing with snail size.

However unexpected my results were, this is the first study to identify snails as second intermediate hosts for *Cotylurus cornutus* and *Echinoparyphium* in Canada, and is one of the very few studies to

look into direct effects of metacercarial infection on survival of second intermediate hosts worldwide. My results provide key information on the effects of trematode cyst infection on freshwater snail survival, which will help advance our understanding of snail population dynamics and community structure of ecologically important herbivore species. Future research of metacercarial effects on snail survival should focus on controlled cercarial infections of lab raised snails to explore differences in effects of trematode species, intensity of infection and sequence of infection on snails used as second intermediate hosts by trematodes.

Chapter 5: Trematode infection in relation to other snail symbionts

Introduction

The term symbiosis encompasses a variety of relationships among two or more different species that live together and share resources (Paracer and Ahmadjian, 2000). Symbioses can be obligate, if the individuals involved depend on each other for survival, or they can be facultative, if the individuals can survive without each other. The most widely accepted types of symbioses are mutualism, commensalism and parasitism. In mutualistic organisms, both partners benefit from the association. In commensalism, only one of the partners benefits while the other neither benefits nor is harmed. Parasitism encompasses associations where one partner benefits from the association at the detriment of the other. Finally, in competitive interactions, both partners incur costs. Symbioses can be further divided into endosymbiosis, when the symbiont lives inside the body of their host, and ectosymbiosis, when the symbiont lives outside of their host's body. Most organisms are hosts to at least one symbiont species; for example, freshwater snails can host up to 23 different taxa of symbionts, including aquatic insect larvae, nematodes, leeches, oligochaete worms, temnocephalans, and trematodes (Damborenea et al., 2006; McCaffrey and Johnson, 2017).

Trematodes are obligate (endo)parasites with complex life cycles that use snails both as first and second intermediate hosts (see details in Chapter 1). Snails acquire trematode infections as first intermediate hosts by accidentally eating trematode eggs while grazing, or when free-living trematode miracidia infect snail hosts by direct penetration (Esch et al., 2002). Snails can be further infected by trematodes as second intermediate hosts when free-living cercariae penetrates the skin of the snail, or by swimming directly into the snail's pneumostome (Esch et al., 2002; Huffman and Fried, 2012).

Other organisms use freshwater snails as symbionts, but the nature of the relationships often remains unclear. In some circumstances, leeches (Annelida: Hirudinea) act as ectosymbionts of freshwater snails, inhabiting the space between the snail's mantle and shell (El-Shimy and Davies, 1991f). It has been suggested that leeches are commensals of planorbid snails, as gut contents of leeches consist mainly of diatoms (Brooks and Welch, 1977), while snails seem to be unharmed by the

presence of multiple leeches of different species (Damborenea and Gullo, 1996). But other studies indicate that the relationship between snails and different species of leech range from obligate to opportunistic parasites (Damborenea and Gullo, 1996; McCaffrey and Johnson, 2017; Gullo and Lopretto, 2018), facultative predators (Brooks and Welch, 1977), predators, or scavengers (Klemm, 1976). Similarly, another group of annelids, the oligochaete worm species-complex *Chaetogaster* '*limnaei*' s.l. (Naididae) (hereafter '*Chaetogaster*') are ectosymbionts found on the mantle, head and foot of dozens of species of freshwater snails around the world (Mack et al., 2023). *Chaetogaster* has been proposed to be a commensal of freshwater snails, using the snail mainly as a shelter while feeding on rotifers and ostracods (Fashuyi and Williams, 1977). However, *Chaetogaster* may also be a mutualist of freshwater snails, reducing trematode infection by feeding on miracidia or cercariae before they can infect the snail (Sankurathri and Holmes, 1976; Muñiz-Pareja and Iturbe-Espinoza, 2018). To assess the type of symbiotic relationship between snails and these ectosymbionts, I tested for potential correlations between trematode infection and ectosymbionts in freshwater snails.

Albertan lakes and ponds harbour a high diversity of invertebrates, with freshwater snails being some of the most abundant. I observed that these snails have two annelid ectosymbionts that inhabit the space between the mantle and shell (pers. obs.). Leeches (Glossiphoniidae: *Helobdella* sp.) can be found in *Planorbella* sp. and *Lymnaea* sp., and oligochaete worms (*Chaetogaster* sp.) are present in lymnaeid, physid and planorbid snails. The influence of these ectosymbionts on trematode infection in snail hosts is currently unclear. Leech species in the genus *Helobdella* can be predators of snails (Saglam et al., 2023) or in some cases as ectoparasites that feed on the haemolymph of living snails without killing their host (Damborenea and Gullo, 1996; Kutschera et al., 2013). If *Helobdella* sp. is an ectoparasite of freshwater snails in Alberta, these leeches might weaken snails and make them more susceptible to infection by trematodes. On the other hand, in the laboratory, *Chaetogaster* sp. can lower infection by trematode miracidia in lymnaeid (Muñiz-Pareja and Iturbe-Espinoza, 2018) and planorbid snails (Zimmermann et al., 2011), and cercarial infection in physids (Sankurathri and Holmes, 1976). Whether *Chaetogaster* sp. protects snails in the wild remains unknown. More recently, *Chaetogaster* was suggested to reduce miracidial infection by consuming active trematode larvae, but may not be effective protectors if snails acquire their infection by ingesting trematode eggs (Hobart et al., 2022). *Chaetogaster* might also be attracted to snails already infected and shedding cercariae, thus feeding on exiting cercariae instead of protecting the snail against incoming trematode miracidia

(McKoy et al., 2011); cercariae may represent an important food source for *Chaetogaster*, mouth gape being the only limiting factor for cercariae selection and consumption (Fernandez et al., 1991). It is thus important to consider the way snails acquire trematode infections and trematode infection stage while studying potential protective effects of *Chaetogaster*.

Here, I evaluated the effects of *Chaetogaster* sp. and *Helobdella* sp. on trematode infection in freshwater snails. Infection pathway (i.e. egg ingestion or miracidia penetration) to the snail host and trematode larval stage (egg/miracidium or cercaria) are important when testing for *Chaetogaster* sp. effects on trematode infection. I thus separate my predictions about effects of these annelids depending on whether snails are first intermediate hosts (i.e., egg or miracidial successful infection, which results in an infected snail shedding cercariae) or second intermediate hosts (i.e., infection by cercariae, which results in metacercarial infection in snails) in the rest of the chapter.

Predictions for snails as first intermediate hosts

I first test whether abundance of *Chaetogaster* sp. per snail differs depending on trematode infection pathway. If *Chaetogaster* sp. receives nutrients from and has a protective role against miracidia but not against egg infection, I hypothesized that *Chaetogaster* sp. would affect trematode infection differently based on the mode of transmission: passive transmission (i.e., via ingestion of eggs) vs. active transmission (i.e., via perforation by miracidia). I predicted that, as miracidia would have had to pass the protective *Chaetogaster* sp. barrier to infect the snail successfully, (i) snails infected with trematode species with active transmission would have lower *Chaetogaster* sp. loads than snails infected by trematode species with passive transmission and uninfected snails. Secondly, if snails shedding cercariae are a food source for *Chaetogaster* sp. and attract the annelid, I hypothesized that *Chaetogaster* sp. load would differ between infected and uninfected snails. I thus predicted that (ii) uninfected snails would have fewer *Chaetogaster* than snails infected with sporocysts or rediae, as would be expected if *Chaetogaster* sp. is attracted by snails shedding cercariae. Finally, I test whether abundance of leeches (*Helobdella* sp.) is correlated with trematode infection in their snail hosts. If leeches weaken the snail and make them more susceptible to trematode infection, I hypothesized that *Helobdella* sp. loads would vary between infected and uninfected snails. I predicted that (iii) snails with relatively high *Helobdella* sp. loads are more likely to be infected with trematodes than snails with low leech loads.

Predictions for snails as second intermediate hosts

First, I assess whether presence of metacercariae in snails varies with number of *Chaetogaster* sp. If *Chaetogaster* protects the snail against cercarial penetration, I predict that (iv) snails without metacercariae would have higher *Chaetogaster* loads. Secondly, I test whether leeches of the genus *Helobdella* would influence metacercarial infection in snails. I hypothesized that *Helobdella* sp. leech is an ectoparasite of freshwater snails, making snails more susceptible to cercarial infection. I predicted that (v) snails with higher *Helobdella* sp. loads would be more likely to have metacercariae than snails with lower *Helobdella* sp. loads.

Methods

Snail collections and husbandry

To evaluate the effects of *Helobdella* sp. and *Chaetogaster* sp. on trematode infections in snails, I collected 2,276 snails (>8 mm shell height or width, depending on snail genus) between May 12th and September 4th, 2019, June 2nd and August 31, 2020, and June 21st and September 23rd, 2021. Snails were sampled in Lafarge, Lac Ste. Anne, Morinville, Pigeon Lake, St. Albert, Strathcona County, and Wabamun Lake (Fig. 1). Freshwater snails were collected using an aquatic dip net (2 mm mesh opening) by scooping and sorting vegetation growing close to the sediment. I identified snails morphologically to genus based on Prescott and Curteanu (2004). Four genera were sampled: *Lymnaea* (n = 650), *Physa* (n = 295), *Planorbella* (n = 882), and *Stagnicola* (n = 449). Snails were transported to the lab, where they were isolated in plastic specimen cups with 60 mL of pond water from the same sites where snails were collected, and kept in an incubator at 15°C. Small pieces of store-bought Hikari© algae wafers (2019) or organic lettuce (2020 and 2021) were provided as food *ad libitum*. I changed the food type after 2019, because snails preferred lettuce as food source and because without algae wafers, the water where snails were kept remained clean for longer. Half the water in the container was changed weekly with fresh pond water to maintain the water and salinity or pH close to natural conditions.

Snail dissections and symbiont surveys

For lymnaeids and physids, I measured each snail's shell height from the bottom of the aperture to the apex of the spire. For planorbid snails, I measured maximum width of the shell in lateral view, from the anterior rim of the aperture to the widest part of the shell. I then dissected the snails by gently crushing their shells with a small hammer, separated the columellar muscles from the axis of the shell, and rinsed the shell with tap water using a squirt bottle. I examined shell fragments under a dissecting microscope for any ectosymbiont attached to the shell. I counted the number of leeches and/or *Chaetogaster* from each snail. *Chaetogaster* sp. were identified morphologically by Prof. Heather Proctor of the University of Alberta. Leeches were identified to genus level using the identification keys within Klemm (1972; 1982), Meyer and Moore (1954), and Moore (1964). After counting the ectosymbionts, I dissected each snail under a dissecting microscope by separating the gonad/digestive gland from the head/foot tissue using a sharp pair of dissecting scissors, and submerged the head/foot tissue in 95% ethanol immediately after. Gonad/digestive glands were then dissected under the dissecting microscope by teasing apart the tissue membrane along the ventral side of the gonad/digestive gland to look for trematode larvae. Lastly, I recorded if sporocysts, rediae, cercariae and/or metacercariae were present in each snail.

Molecular identification

Morphological identification of leeches can be difficult and my keying initially led to the identification of leeches as *Marvinmeyeria* sp. I consulted with a leech systematist, Dr. Sebastian Kvist (then at the Royal Ontario Museum in Toronto), who suggested the leeches might belong to the genus *Helobdella* instead. As for members of the genus *Chaetogaster* (Mack et al., 2023), morphospecies of *Helobdella* can have high cryptic diversity (Bely and Weisblat, 2006). Thus, to increase identification accuracy of ectosymbiont leeches from snails sampled in 2021, I preserved and stored some leeches in 95% ethanol at -20°C for barcoding. Snails often had more than one leech on their body. When this was the case, I selected one leech from each snail sample for molecular analysis. To reduce contamination potential from other DNA sources that might be present in the leech body (e.g., snail DNA from haemolymph in gut, trematode cysts), I used only the hind sucker from each leech for DNA extractions. From each leech tissue sample, I extracted genomic DNA (gDNA) following extraction protocols from DNeasy® Blood & Tissue kits (Quiagen, cat. no. 69506). I then amplified the

mitochondrial *cox1* gene fragment from each gDNA sample according to Folmer et al. (1994), using the primers LCO1490 and HC02198 as described in Folmer et al. (1994). PCR was done in 20 µl volumes using 10 µl of the AccuStart II GelTrack® PCR SuperMix (Quanta Bio, cat. no. 95136-500), 0.5 µl of each primer (concentration = 10 µM), and 9 µl of gDNA. PCR products (i.e. amplicons) were electrophoresed in 1% agarose and purified following the instructions from Truin Science PCR cleanup kits (Truin Science, model: KTS1115). Purified amplicons were sent to Macrogen Inc. (Korea) for Sanger sequencing, using the same PCR primers to amplify both strands. I trimmed and analyzed resulting sequences for quality with SnapGene© Viewer (Dotmatics) software, and subsequently MUSCLE aligned and BLASTed to GenBank records using Geneious Prime® (version 2023.2.1) software. Library sequences with the highest pairwise comparison percent match rates were then selected and a cutoff of 10% nucleotide divergence of *cox1* sequences was used to determine identity of leech species. This threshold was selected because it is a conservative standard of maximum intraspecific *cox1* gene divergence for leeches (Bely and Weisblat, 2006).

Statistical analysis

In line with treating datasets separately for snails used as first intermediate hosts and snails used as second intermediate hosts for trematodes, I separated data according to trematode life stages to evaluate potential effects of *Helobdella* sp. and *Chaetogaster* sp. on trematode infection. The first dataset contained information from snails infected with sporocysts or rediae only (i.e. snails used as first intermediate hosts and infected successfully by miracidia or eggs), while the second dataset contained information from snails with metacercariae only (i.e. snails used as second intermediate hosts and infected successfully by cercariae). I removed data from snails that were simultaneously infected with sporocysts/rediae and metacercariae, and from snails missing information on shell size, from both datasets. Due to inherent differences in size among snail species, I used the interaction term between snail species and snail size as fixed effect in all models.

Snails as first intermediate hosts for trematodes

To test if *Chaetogaster* affects trematode infection depending on transmission strategy (i.e. eggs *vs.* miracidia), I fitted a cumulative link mixed model (CLMM) using the function clmm in the ordinal package in R (Christensen, 2023). These models allow testing for linear relationships of fixed and

random effects on response variables that are categorical with more than two levels by using the probit link function and equidistant as threshold structure. In this CLMM, I used transmission strategy as response variable (i.e. egg, miracidia or uninfected), *Chaetogaster* load, interaction between snail species and size as fixed effects, and site as random effect. Transmission strategy was determined from life-history literature associated with each trematode species. Snails infected with trematode species that could not be identified (and thus I was unable to identify transmission strategies) were removed from the database prior to running the model. Model fitness was checked using the function performance in the R package performance (Lüdecke et al., 2022).

To test if *Chaetogaster* is attracted to snails shedding cercariae, I fitted a generalized linear mixed-effects model (GLMM) using the function glmm.zinb in the NBZIMM package in R (Yi, 2020). This function is specific for zero-inflated count data that has a negative binomial error distribution. In this GLMM, I used *Chaetogaster* count as response variable, trematode infection status (i.e. with or without reproducing rediae/sporocysts), interaction term between snail species and snail size as fixed effects, and site as random effect. Model fitness was checked using the function performance in the R package performance (Lüdecke et al., 2022).

To test the prediction that *Helobdella* sp. leeches weaken their snail host and could potentially make them more susceptible to trematode infection via eggs or miracidia, I fitted a generalized linear mixed-effects model (GLMM) using the function glmer in the R package lme4 (Bates et al., 2015). In the model, I used trematode infection status as response variable with binomial error distribution, number of leeches, interaction between snail species and snail size as fixed effects, and site as random effect. Model fitness was checked using the functions simulateResiduals, plotQQunif and plotResiduals in the R package DHARMa (Hartig, 2022).

Snails as second intermediate hosts for trematodes

To test if *Chaetogaster* sp. protects their snail host from cercarial infection, and/or if *Helobdella* sp. makes snails more susceptible to cercarial infection, I fitted a Generalized linear mixed-effects model (GLMM) using the function glmer in the R package lme4 (Bates et al., 2015). In the model, I used metacercarial infection status as response variable with binomial error distribution, number of *Chaetogaster* sp., number of *Helobdella* sp., interaction between snail species and snail size as fixed

effects, and site as random effect. Model fitness was checked using the functions simulateResiduals, plotQQunif and plotResiduals in the R package DHARMa (Hartig, 2022).

Results

Molecular identification

Taxonomy based on morphology of leeches of the genus *Helobdella* has resulted in great confusion (Siddall and Borda, 2003). Based on BLAST results for COI, the ectosymbiotic leeches I sequenced had the highest % identity matches with *Helobdella lineata* (Verrill, 1874) with one relatively distant match with *Helobdella robusta* (Shankland, Bissen & Weisblat, 1992) (Table 6). According to Bely and Weisblat (2006), these two species of leeches are sister taxa and thus share most external morphological characters, promoting further confusion. As these two sister taxa of *Helobdella* leeches share most morphological characters, their ecology is likely similar, and they could use freshwater snails in the same way. I thus refer to leeches from my study system in general as *Helobdella* sp. throughout this chapter.

Table 6. Species identification results based on sequencing of fragments from mitochondrial gene *cox1* from symbiotic leeches of freshwater snails. Each leech sample was taken from a different individual snail.

Host ID	Barcoding ID	% identity	GenBank accession match
Lymnaea	*Helobdella lineata*	100	MN071352
Lymnaea	*Helobdella lineata*	97.89	AF329039
Lymnaea	*Helobdella lineata*	97.42	MN071352
Lymnaea	*Helobdella lineata*	95.2	MN071352
Planorbella	*Helobdella lineata*	100	MN071352
Planorbella	*Helobdella lineata*	99.88	MN071352
Planorbella	*Helobdella lineata*	99.88	MN071352
Planorbella	*Helobdella lineata*	99.7	OM935746
Planorbella	*Helobdella lineata*	99.12	MN071352
Planorbella	*Helobdella lineata*	98.57	AF329039
Planorbella	*Helobdella lineata*	98.41	OM935746
Planorbella	*Helobdella lineata*	98.14	AF329039
Planorbella	*Helobdella lineata*	96.27	AF329039
Planorbella	*Helobdella lineata*	96.2	MN071348
Planorbella	*Helobdella lineata*	95.92	OM935746
Planorbella	*Helobdella lineata*	95.26	AF329039
Planorbella	*Helobdella lineata*	95.24	MN071352

Planorbella	*Helobdella robusta*	92.02	MN071350
Planorbella	*Helobdella lineata*	91.7	MN071352

Snails as first intermediate hosts

Chaetogaster loads relative to trematode infection were significantly different between transmission pathways of the different species of trematode (i.e., egg vs miracidium; $z = 5.4$, $p < 0.001$; Fig. 12); snails infected with trematode species with an active transmission strategy (penetration by miracidia) had more *Chaetogaster* than snails infected with trematodes that use a passive transmission strategy (egg ingestion) and uninfected snails (Fig. 12). There was no difference in *Chaetogaster* loads between uninfected snails and snails infected with trematode species that use a passive transmission strategy (Fig. 12). The interaction between snail species and snail size had a significant effect on trematode infection strategy (for details, see Fig. S4 in the appendix).

The GLMM used to test if *Chaetogaster* are more common on infected snails (independent of trematode transmission strategy) suggested that there are significantly more *Chaetogaster* on snails infected with trematodes, and thus shedding cercariae, than on uninfected snails ($t = 5.2$, $p < 0.001$; Fig. 13). The interaction between snail species and snail size did not have a significant effect on *Chaetogaster* load.

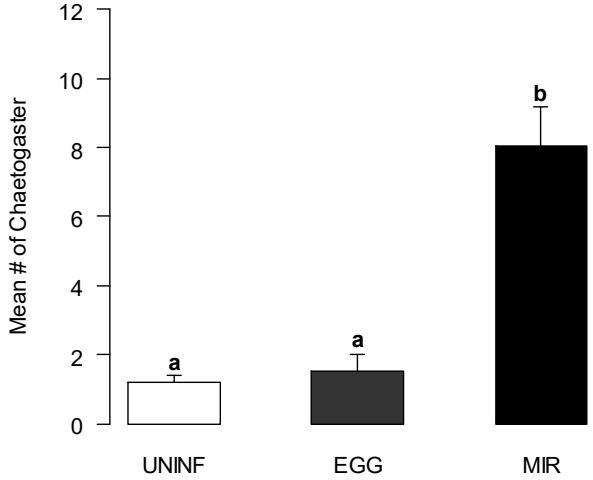


Figure 12. Infection status of snails used as first intermediate hosts in relation to mean *Chaetogaster* sp. load (± SE). UNINF = Uninfected snails (n = 663), EGG = Snails with trematode species that infect snails passively via egg ingestion (n = 71), MIR = Snails with trematode species that infect snails actively with miracidia (n = 217). Lower case letters above error bars indicate significant differences between infection groups ($p < 0.05$). Data from symbionts and trematodes collected in 2019-2021. Data from uninfected snails and snails infected with sporocysts or rediae only.

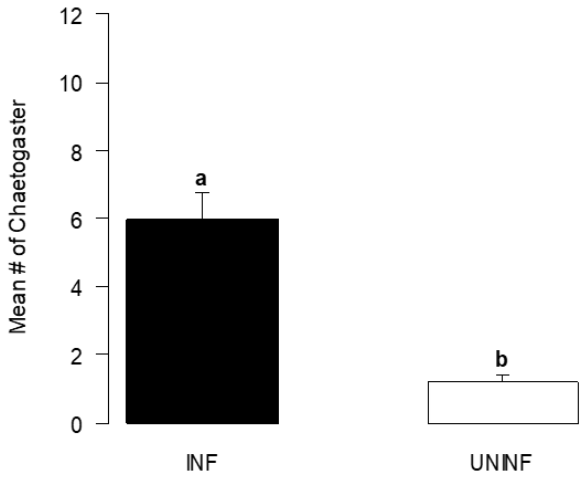


Figure 13. Mean *Chaetogaster* sp. load (± SE) in relation to trematode infection of snails used as first intermediate hosts. INF = Snails infected with trematodes and shedding cercariae (n = 312), UNINF = Uninfected snails (n = 663). Lower case letters above error bars indicate significant differences between infection groups ($p < 0.05$). Data from symbionts and trematodes collected in 2019-2021. Data from uninfected snails and snails infected with sporocysts or rediae only.

The model used to test the prediction that *Helobdella* sp. leeches weaken their snail host and could potentially make them more susceptible to trematode infection as first intermediate hosts shows no significant difference between mean number of leeches on snails with and without trematode infection ($z = 1.4$, $p = 0.17$; Fig. 14). The interaction between snail species and snail size had a significant effect on trematode infection. Snails infected with trematodes are significantly larger than uninfected snails in *Lymnaea* ($z = 10.1$, $p < 0.001$; Fig. 15), *Planorbella* ($z = 9.8$, $p < 0.001$; Fig. 15) and *Stagnicola* ($z = 9.1$, $p < 0.001$; Fig. 15). However, physids infected with trematodes are significantly smaller than uninfected snails ($z = 4.62$, $p < 0.001$; Fig. 15).

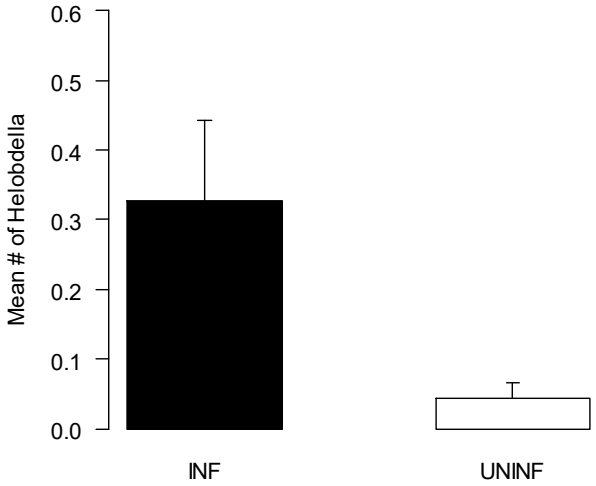


Figure 14. Infection status of snails used as first intermediate hosts in relation to mean *Helobdella* sp. load (± SE). INF = Infected snails (n = 312), UNINF = Uninfected snails (n = 663). There was no significant difference in number of *Helobdella* between infection categories. Data from symbionts and trematodes collected in 2019-2021. Data from uninfected snails and snails infected with sporocysts or rediae only.

Snails as second intermediate hosts

The model used to test if *Chaetogaster* protects their snail host from cercarial infection, and if *Helobdella* sp. makes their snail host more susceptible to cercarial infection, shows no significant effect of number of *Chaetogaster* ($z = 0.3$, $p = 0.74$; Fig. 16), nor number of *Helobdella* sp. ($z = -0.2$, $p = 0.83$; Fig. 17) on whether their hosts were infected with metacercariae. However, the interaction between snail species and snail size had a significant effect on trematode infection. In *Physa*, snails infected with metacercariae were significantly larger than uninfected snails ($z = -2.7$, $p < 0.01$; Fig. 18). In contrast, snails infected with metacercariae were significantly smaller than uninfected snails in *Planorbella* ($z = -2.9$, $p < 0.01$; Fig. 18) and *Stagnicola* ($z = 3.3$, $p < 0.01$; Fig. 18). In *Lymnaea,* size was not significantly different between uninfected snails and snails infected with trematodes (Fig. 18).

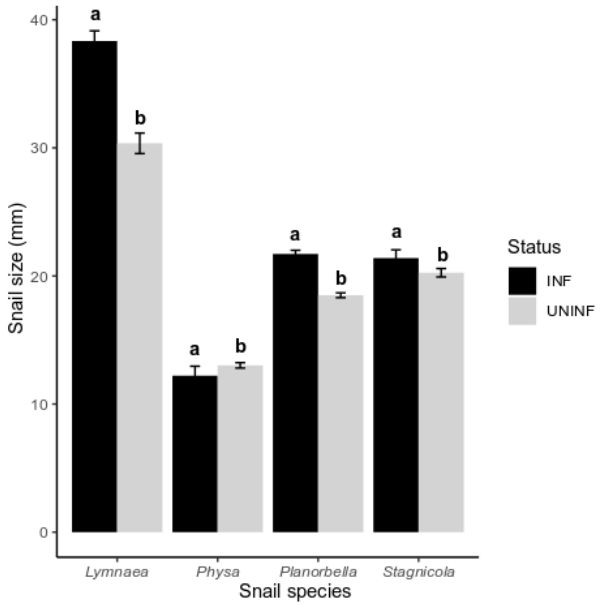


Figure 15. Interaction between snail species and mean snail size (mm ± SE)for trematode infection in snails used as first intermediate hosts. INF = Infected snails (*Lymnaea* n = 84; *Physa* n = 7; *Planorbella* n = 183; *Stagnicola* n = 38), UNINF = Uninfected snails (*Lymnaea* n = 124; *Physa* n = 104; *Planorbella* n = 329; *Stagnicola* n = 106). Lower case letters above error bars indicate significant differences between infection groups ($p < 0.05$). Data from snails and trematodes collected in 2019-2021. Data from uninfected snails and snails infected with sporocysts or rediae only.

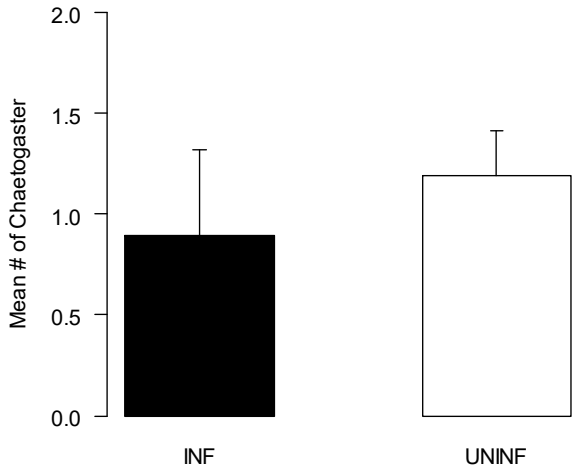


Figure 16. Metacercarial infection status of snails used as second intermediate hosts in relation to mean *Chaetogaster* load (± SE). INF = Infected snails (n = 501), UNINF = Uninfected snails (n = 663). There was no significant difference in number of *Chaetogaster* between infection categories. Data from symbionts and trematodes collected in 2019-2021. Data from uninfected snails and snails infected with metacercariae only.

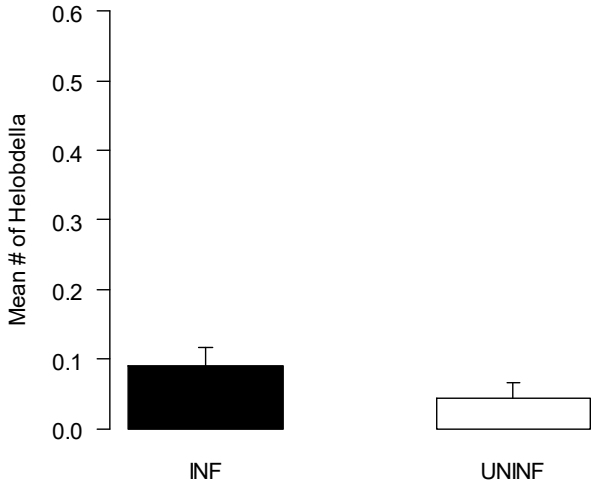


Figure 17. Metacercarial infection status of snails used as second intermediate hosts in relation to mean *Helobdella* sp. load (± SE). INF = Infected snails (n = 501), UNINF = Uninfected snails (n = 663). There was no significant difference in number of *Helobdella* between infection categories. Data from symbionts and trematodes collected in 2019-2021. Data from uninfected snails and snails infected with metacercariae only.

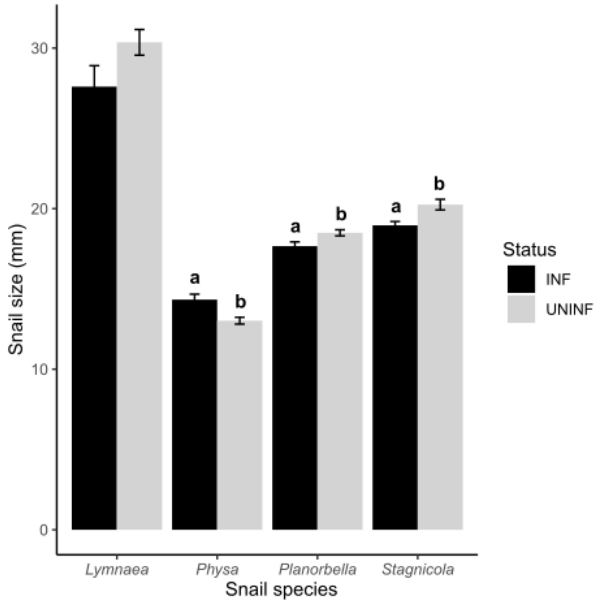


Figure 18. Interaction between snail species and mean snail size (mm ± SE) for trematode infection in snails used as second intermediate hosts. INF = Infected snails (*Lymnaea* n = 87; *Physa* n = 24; *Planorbella* n = 180; *Stagnicola* n = 210), UNINF = Uninfected snails (*Lymnaea* n = 124; *Physa* n = 104; *Planorbella* n = 329; *Stagnicola* n= 106). Lower case letters above error bars indicate significant differences between infection groups ($p < 0.05$). Data from snails and trematodes collected in 2019-2021. Data from uninfected snails and snails infected with metacercariae only.

Discussion

I found that *Chaetogaster* loads were significantly higher in infected snails used as first intermediate hosts by trematodes (i.e., infected with sporocysts or rediae, and shedding cercariae) (Fig. 13). My results also suggest that on snail first intermediate hosts, *Chaetogaster* presence does not lower trematode transmission success in species that infect snails via miracidia penetration. Mean intensity of *Helobdella* was higher in infected snails, but not significantly so (Fig. 14). The interaction between snail species and snail size was an important factor in snails used as first intermediate and second intermediate hosts. Finally, I showed that neither *Chaetogaster* nor *Helobdella* seem to influence trematode infection in snails used as second intermediate hosts.

These results were partially in agreement with my non-directional hypotheses, but mostly contradicted my directional predictions. The hypothesis that *Chaetogaster* load would differ between snails infected with sporocysts or rediae and uninfected snails was supported, as well as my prediction that uninfected snails would have less *Chaetogaster* than infected snails. *Chaetogaster* load was significantly higher on snails infected with sporocysts or rediae (i.e., shedding cercariae) than on uninfected snails. These results contrast with previous studies suggesting that *Chaetogaster* protects their snail host against trematode miracidial infection (Rodgers et al., 2005; Muñiz-Pareja and Iturbe-Espinoza, 2018). Similarly, my results contrast with studies suggesting that *Chaetogaster* does not show a preference for snails shedding cercariae (Fashuyi and Williams, 1977). However, my results are in line with previous research that showed *Chaetogaster* were more abundant on snails shedding trematode cercariae than on uninfected snails (Fernandez et al., 1991). *Chaetogaster* might be attracted to infected snails, as trematode cercariae are an abundant and continually accessible food source for *Chaetogaster* (McKoy et al., 2011; Hobart et al., 2022). Additionally, this abundance of food may promote asexual reproduction of *Chaetogaster* by fission, increasing the number of individuals on infected snail hosts (Fernandez et al., 1991). I thus propose the latter explanation for the large numbers of *Chaetogaster* on snails infected with sporocysts or rediae observed in my study system, as opposed to the idea that *Chaetogaster* defends their snail host from miracidial infection.

My observation that infection pathway in snails used as first intermediate hosts is related to *Chaetogaster* load agree with my hypothesis. However, they contradict my prediction that uninfected snails and snails infected by trematode species with a passive infection strategy (via snail consumption of eggs) would have higher *Chaetogaster* loads than snails infected by miracidia. These results also

contrast with previous research showing that *Chaetogaster* is effective against miracidial infection (Hobart et al., 2022). These discrepancies are unexpected and difficult to explain. The only trematode species with a passive transmission strategy in my study system is *Plagiorchis* sp., whose cercariae are very small compared to most of the trematode species that use miracidia to infect the snail host (See Fig. S2 for cercaria size reference). Mouth gape of *Chaetogaster* thus cannot be a limiting factor and fails to explain the low number of *Chaetogaster* on snails shedding *Plagiorchis* sp. cercariae. It is noteworthy that research using field-collected snails (as in my study and Hobart et al., 2022) to observe *Chaetogaster* effects on miracidial vs. egg infection risk makes it impossible to know the relative timing of trematode infection vs *Chaetogaster* establishment. It is therefore necessary to interpret these field-based results with caution, as relative timing of establishment along with several other factors (e.g. host preference, host immune system, etc.) cannot be accounted for in the statistical models. However, my results support my proposal that *Chaetogaster* are attracted to snails shedding cercariae rather than protecting snails against trematode infection. Most trematode species in this study are transmitted by miracidia, and snails infected by these species had significantly more *Chaetogaster* than uninfected individuals and snails infected via trematode egg ingestion.

The significant interaction between host species and size on trematode infection in snails used as first intermediate hosts makes biological sense. In most snail species that I examined, individuals infected with sporocysts or rediae were larger than uninfected snails. The most parsimonious explanation for this is that larger snails are older and have thus been exposed to trematode miracidia or eggs for longer, increasing their probability of becoming infected over time. However, physids infected with sporocysts or rediae were smaller than their uninfected counterparts, suggesting that length of exposure to potential infection might not be the only explanation. These interactions are likely host-species specific. In *Physa*, trematode infection might induce a higher energetic cost than in other snail species, resulting in stunted growth (Mouritsen et al., 1999; Sorensen and Minchella, 2001). Alternatively, physid immune system may be compromised by infection (Kryukova et al., 2014), making infected snails more susceptible to other pathogens, thus reducing snail survival. These hypotheses require further testing, however.

The interaction between snail species and host size for trematode infection in snails used as second intermediate hosts was almost exactly contrary to their relationship in snails used as first intermediate hosts. Physids infected with metacercariae were significantly larger than uninfected individuals, which

could also be explained by time of exposure to cercarial infection. However, infected *Planorbella* and *Stagnicola* were significantly smaller than uninfected individuals. Unfortunately, the cause for this difference remains unclear, as metacercariae do not seem to have deleterious effects on snail survival in the lab (see Chapter 4 and references therein). It seems unlikely that cercariae would prefer to target smaller snails, as larger snails have more tissue exposed for penetration. Larger snails are also more mobile and therefore easier to locate and infect successfully than smaller snails (McKoy et al., 2011). It is plausible, however, that larger infected snails are more susceptible to predation in the field, and thus removed from the population.

My results suggest there is no effect of *Chaetogaster* on trematode infection in snails used as second intermediate hosts, contradicting my hypothesis and prediction as well as previous research describing protective roles of *Chaetogaster* against cercarial infection in freshwater snails (Sankurathri and Holmes, 1976; Hopkins et al., 2016). The most logical explanation for this pattern is that *Chaetogaster* consumption of incoming cercariae is insufficient to prevent them penetrating the host snail. *Chaetogaster* crawls or swims near the substrate when not inhabiting a snail, eventually dying if they cannot find a snail of their preferred species (Hopkins et al., 2022). Furthermore, *Chaetogaster* can feed on almost anything small enough to fit in its mouth (Fernandez et al., 1991), and stomach contents are often dominated by rotifers, protozoans, diatoms, and ostracods (Fashuyi and Williams, 1977; Fernandez et al., 1991; H. Proctor, pers. comm.). In this study, when dissecting snails, I witnessed *Chaetogaster* ingesting trematode rediae, which supports the hypothesis that *Chaetogaster* can feed on any organism available and small enough to be swallowed by the oligochaete. I thus propose that *Chaetogaster* is a commensal of freshwater snails and that they feed opportunistically on cercariae being shed from infected snails (as discussed above), but that *Chaetogaster* does not protect snails from cercarial infection. Incoming cercariae are likely not abundant enough to support *Chaetogaster* on an uninfected host relative to the concentrations of outgoing cercariae produced by infected snails, and *Chaetogaster* is not abundant enough on uninfected snails (Fig. 12) for *Chaetogaster* to be able to protect the snail against incoming cercarial infection efficiently.

The results that *Helobdella* sp. leeches had no significant effect on trematode infection in snails used as either first or second intermediate hosts contrasted with previous research proposing that leeches protect their host from trematode infection (Brooks and Welch, 1977; Elkhodary et al., 2018). These results also contradict my hypotheses and predictions that leeches would make their snail host

more susceptible to trematode infection. Leeches in my system seem to neither weaken the host, making them more susceptible to trematode infection, nor provide protection against it. *Helobdella lineata* has been previously reported to be harmless to planorbid snails, even in relatively large numbers (Sarah, 1971), suggesting *Helobdella* is a commensal of planorbid snails. However, in my study system, leech gut contents are visually identical to the red haemolymph of their planorbid snail hosts, whereas leeches that infect lymnaeid snails have almost transparent gut contents; this suggests that leeches are in fact feeding from their snail host. Young glossiphonid leeches feed on snail haemolymph without killing their host (Klemm, 1976; Kutschera et al., 2013). Further, in another glossiphonid leech, *Alboglossiphonia polypompholyx* Oosthuisen, Hussein and El-Shimy, young individuals feed on haemolymph from their snail host while they are small and vulnerable, and leave the snail when they reach a certain size or sexual maturity, becoming free-living and predatory (El-Shimy and Davies, 1991), suggesting they are parasitic on snails only when they are young. All *Helobdella* sp. specimens I observed inhabiting snail's shells were smaller than 7 mm long and none were carrying brood, suggesting they are indeed all juveniles. Furthermore, I have collected larger (> 9 mm long) free-living, reproductive (i.e., carrying brood) *Helobdella lineata* at my study sites (Chapter 6, Table 7), suggesting leeches leave their snail host once they become reproductive. I thus propose that *Helobdella* sp. in Alberta have a similar life cycle to that of *Alboglossiphonia polypompholyx* and when young are facultative parasites of freshwater snails.

As Albertan trematode assemblages have been so recently described (Gordy et al., 2016), the relationships between trematode larvae and other snail symbionts remain understudied. Similarly, the relationship between both annelids and their snail hosts remains debatable. Here, I show that *Chaetogaster* prefer snails shedding cercariae, but they do not appear to protect their snail host against infection via either miracidial/egg or cercarial routes. Thus, the relationship between *Chaetogaster* and their snail host can be classified as commensalism. In this chapter, I also provide evidence that leeches of the genus *Helobdella* are facultative parasites of freshwater snails in Alberta, feeding on snail haemolymph when they are young and leaving the snails when they attain sexual maturity. This role has not been previously reported for *Helobdella* spp., making the present study the first to provide a likely explanation of the symbiotic relationship between the *Helobdella* sp. in my study system and its snail hosts. This is also the first study to provide a plausible description of the life cycle of *Helobdella* spp. sampled in this project (i.e., *H. lineata* and *H. robusta*) in Alberta. To my knowledge, the present

project is the first one to address these questions and to provide reasonable descriptions of the ecological relationships between *Chaetogaster*, leeches and freshwater snails coexisting in such close proximity. Knowing if trematode infection of snail hosts could potentially be lowered or enhanced by other symbionts present on snails broadens our knowledge of community ecology and energy flow in wetlands as a whole. Even though *Chaetogaster* does not seem to protect their snail host against trematode infection, *Chaetogaster* could potentially reduce transmission success to second intermediate hosts by feeding preferentially on cercariae being shed from snails (McKoy et al., 2011). This, however, remains to be tested, as it has been proposed that this is not the case for tadpoles (Hopkins et al., 2016).

Chapter 6: Trematode life cycles: closing the gaps in central Alberta

Introduction

Parasites are the most abundant organisms in the planet and all living multicellular organisms have at minimum, one parasitic species living in or on them at some point in their lifetime (Bush et al., 2001; Kuris et al., 2008). Parasites can alter population dynamics and fitness of their hosts and drive community composition in their ecosystem (Huxham et al., 1993; Lafferty, 1993; Fredensborg et al., 2005; Granovitch and Maximovich, 2013). Further, parasite biomass can exceed the biomass of top predators in their ecosystem and can affect host populations in similar ways to predators when hosts invest resources in parasite-avoidance behaviours and immune response to infection (Kuris et al., 2008; McLaughlin et al., 2020; Koprivnikar et al., 2021). When incorporated in food web energy flow, parasites can increase complexity and dominate structure, forming part of 75% of trophic interactions in food webs (Lafferty et al., 2006; McLaughlin et al., 2020). However, parasites are often overlooked in ecology, animal behaviour and food web studies, likely rendering incomplete results (Marcogliese and Cone, 1997; Lane et al., 2022). Given the ubiquity and importance of parasites in ecosystems and across food webs, it is necessary to study the life histories of parasites to fully understand food web dynamics and ecosystem functioning.

Trematodes are obligately parasitic flatworms that, depending on species, need from one to four hosts to complete their life cycle. The typical trematode life cycle requires three different hosts and is the ancestral form from which shorter or longer life cycles evolved (Poulin, 1998; Bush et al., 2001, Esch et al., 2001). In this three-host life cycle, adult trematodes live and reproduce sexually in a vertebrate definitive host, from which eggs pass into the environment. In some trematode species, eggs develop and hatch into free-living larvae called miracidia that infect the first intermediate host by direct penetration. In other species, eggs need to be eaten by the first intermediate host within which eggs hatch into miracidia. The first intermediate host for most trematode species is a snail. In the snail host, again depending on the trematode species, the miracidia develop into a clonal colony of either sporocysts or rediae. Rediae and sporocysts asexually produce free-living larvae called cercariae.

Cercariae then leave the snail to infect the second intermediate host, which can be another snail, other invertebrates or vertebrates such as fishes or amphibians. Within the second intermediate host, cercariae develop into encysted metacercariae. At that stage, some trematode species are known to alter the behaviour and morphology of their second intermediate host (see review in Moore, 2012). Trematode-induced changes in behaviour and morphology can render second intermediate hosts more susceptible to predation, increasing the probability of transmission to definitive hosts (Webber et al., 1987a; Mouritsen and Poulin, 2003a).

Because trematodes depend on trophic transmission to reach their definitive host, all hosts involved need to be present in the environment for the parasite to complete each generation. This has led trematodes to be proposed as bioindicators of ecosystem health (Campião et al., 2012; Shea et al., 2012; Shah et al., 2013). Trematode cercariae can also be an important food source for non-host organisms in their environment (Morley, 2012; Koprivnikar et al., 2023). Overall, trematodes have important effects on food web dynamics in their ecosystem (e.g., McLaughlin et al., 2020). Parasites with complex life cycles, such as trematodes, can also be used to elucidate predator-prey interactions (Bennet et al., 2023). However, even in well-studied marine environments, the majority of trematode life cycles remain unknown (Blasco-Costa and Poulin, 2017). This is an important gap in our current knowledge. Trematode assemblages in snails from Albertan lakes have been recently documented (Gordy et al., 2016; Gordy, 2018), but complete life cycles for most of these trematode species have not yet been elucidated.

It is also important to document trematode life cycles to better understand and reduce threats to human health (e.g. swimmer's itch) or to detect vulnerable areas in conservation efforts (e.g., more diverse trematode communities as indicators of ecosystem health). With few exceptions (Vermeer, 1969; Hair and Holmes, 1970; Palmieri, 1973; Ramalingam and Samuel, 1978; Bush and Holmes, 1986; Stock and Holmes, 1988), definitive hosts remain unknown for trematode species in Alberta. Furthermore, to my knowledge, second intermediate hosts for trematodes in Alberta have never been studied. In this chapter, I aim to (i) describe invertebrate species used as second intermediate hosts by trematodes in my study sites, (ii) describe the species of birds and mammals present at my study sites that are likely used as definitive hosts by these trematodes, and (iii) propose complete life cycles for trematode species found in central Alberta.

Methods

To identify freshwater invertebrates used as second intermediate hosts by trematodes at my study sites (Fig. 1), I collected invertebrates from Lafarge, Morinville, St. Albert and Strathcona County in July 2019, July and August 2020, and June, July and August 2021. Invertebrates were collected using a dip net (2 mm mesh opening) to scoop vegetation growing close to the sediment. I froze potential intermediate hosts for future dissection. I chose freezing over ethanol to preserve second intermediate hosts to facilitate dissections and trematode metacercariae identification. Snails were identified morphologically to genus level using Prescott and Curteanu (2004). I dissected the following: snails (*Lymnaea* sp., n = 54; *Physa* sp., n = 90; *Planorbella* sp., n = 178; *Stagnicola* sp., n = 45), amphipods (*Gammarus lacustris* G.O. Sars, 1983, n = 170), leeches (Hirudinea, n = 48), backswimmers (Notonectidae, n = 114), water boatmen (Corixidae, n = 14), caddisfly larvae (Limnephilidae, n = 10), nymphal damselflies (Coenagrionidae, n = 79), nymphal dragonflies (Aeshnidae, n = 5), and adult water beetles (Dytiscidae, n = 17). These organisms have been documented as second intermediate hosts for trematodes (Palmieri, 1973; Yamaguti, 1975; Aksenova et al., 2016; Shaw et al., 2020). Samples of metacercariae found in invertebrates and tissue samples from non-snail invertebrates were preserved in 95% ethanol and stored at -20°C for DNA barcoding. I calculated prevalence of metacercarial infection as described in Bush et al. (1997) for comparative purposes only.

For barcoding, I followed genomic DNA (gDNA) extraction protocols from DNeasy® Blood & Tissue kits (Quiagen, cat. no. 69506). Amplification of the mitochondrial *cox1* gene fragment from each invertebrate tissue sample was performed according to Folmer et al. (1994), using the primers LCO1490 and HC02198 described in Folmer et al. (1994). For trematode metacercariae, I amplified the *cox1* gene fragment from each sample according to Van Steenkiste et al. (2015), using the primers Dice1F and Dice11R as described in Van Steenkiste et al. (2015). PCR reactions were done in 20 µl volumes using 10 µl of the AccuStart II GelTrack® PCR SuperMix (Quanta Bio, cat. no. 95136-500), 0.5 µl of each primer (concentration = 10 µM), and 9 µl of gDNA. PCR products (i.e. amplicons) were then electrophoresed in 1% agarose and purified following the instructions from Truin Science PCR cleanup kits (Truin Science, model: KTS1115). Purified amplicons were sent to Macrogen Inc. (Korea) for Sanger sequencing, using the same PCR primers to amplify both strands. Resulting sequences were trimmed and analyzed for quality with SnapGene© Viewer (Dotmatics) software, and subsequently MUSCLE aligned and BLASTed using Geneious Prime® (version 2023.2.1) software.

Library sequences with highest percent match rates were then selected and a cut-off of 5% nucleotide divergence of *cox1* sequences was used to infer identity of invertebrate and metacercariae species. This threshold was chosen because it is a conservative standard of maximum intraspecific mitochondrial DNA (mtDNA) divergence (Gordy et al., 2016). However, in some cases, the highest pairwise comparison percent match rates exceeded the 5% ideal divergence threshold. Thus, those identifications in particular should be interpreted with caution, as should all matches determined only via GenBank sequence data, as it has been suggested that these are not always fully reliable (Cheng et al., 2023).

To obtain a list of suitable definitive hosts for trematodes in my study system, birds and mammals were surveyed by placing Reconyx Hyperfire 2™ motion activated outdoor video cameras and Wildlife Acoustics Song Meter Mini ™ birdcall recorders at Morinville and St. Albert between May and September in 2020, 2021 and 2022. I chose those two sites because they had intermediate trematode prevalence and are relatively distant from each other, and might thus provide a more comprehensive, yet conservative list of potential definitive hosts for trematodes in central Alberta than sites close together. My research assistant, Kayley Burke, identified the bird species captured by the field cameras at both sites and performed live bird surveys for an hour every two weeks to supplement camera surveys. I analyzed outputs from the birdcall recorders using BirdNet analyzer from Cornell University to identify birds present at both sites. Bird species suggested by BirdNet analyzer that are unlikely to be present in Alberta (e.g., Eurasian Scops-Owl, Egyptian Goose) were removed from the list. I combined information from visual surveys, video and audio equipment for the final list of potential vertebrate hosts. I did not collect fishes or amphibians in this project, due to the added complications of animal care requirements and of obtaining wildlife permits required to sample and euthanize vertebrates.

To suggest plausible complete life cycles for trematodes in central Alberta, I used information from morphological identification of cercariae from Chapter 2 of this thesis, barcoding identification of metacercariae in snails used as second intermediate hosts from Chapter 4 of this thesis, and barcoding information of metacercariae from snails and other invertebrate hosts within the present chapter. To complement information from bird and mammal surveys, I did a literature search for trematodes that have been reported in birds and mammals that can be found at my study sites. I used information from the Host-parasite Database of the Natural History Museum in London (Gibson et al., 2005

https://www.nhm.ac.uk/research-curation/scientific-resources/taxonomy-systematics/host-parasites/database/search.jsp), and from published scientific articles obtained through Web of Science and Google Scholar search engine.

Results

Trematode metacercariae were found mostly in snails (prevalence = *Lymnaea*: 22.22%, *Physa*: 71.11%, *Planorbella*: 76.04%, *Stagnicola*: 75.56%) and leeches (prevalence = 77.08%). Only one of the 170 amphipods dissected was infected with one tail-less cercaria (prevalence = 0.59%). All other invertebrates from my study sites were free from metacercarial infection. Five species of leeches were infected with trematodes and the amphipod infected with one cercaria was identified as *Gammarus lacustris* (Table 7). Eight tissue samples from uninfected leeches failed to amplify, and thus their identities remain unknown. One tissue sample from a leech infected with metacercariae rendered a low quality sequence that could not be matched with sequences stored in GenBank. This leech was thus identified based on morphology only (Table 8).

Table 7. Species identification results based on sequencing of fragments from mitochondrial gene *cox1* from aquatic invertebrates used as second intermediate hosts by trematodes. Only successful identifications provided.

	Barcoding ID	**% identity**	**GenBank accession match**
Amphipod			
	Gammarus lacustris	99.85	KM611657
Leeches			
Erpobdellidae			
	Erpobdella obscura	99.8	HQ961503
	Erpobdella obscura	100.0	HQ961503
	Erpobdella obscura	99.8	GU679474
	Erpobdella obscura	98.9	HQ961503
	Erpobdella obscura	99.5	MN612972
	Erpobdella obscura	99.9	HQ961503
	Erpobdella obscura	100.0	HQ961503
	Erpobdella punctata	99.9	MN612985
	Erpobdella punctata	99.9	MN612928
	Erpobdella punctata	99.9	MN612985
	Erpobdella punctata	100.0	MN612959
Glossiphoniidae			

Barcoding ID	% identity	GenBank accession match
Glossiphonia elegans	100.0	HQ961446
Glossiphonia elegans	99.6	JQ073859
Glossiphonia elegans	100.0	HQ961488
Glossiphonia elegans	99.8	MG421521
Glossiphonia elegans	100.0	MK479237
Glossiphonia elegans	96.6	MK479230
Glossiphonia elegans	100.0	MK479265
Glossiphonia elegans	100.0	JQ073859
Helobdella lineata	100.0	MN071352
Helobdella lineata	100.0	MN071352
Helobdella lineata	100.0	MN071352
Helobdella lineata	100.0	MN071352
Helobdella lineata	100.0	MN071352
Helobdella lineata	100.0	MN071352
Helobdella lineata	100.0	MN071352
Helobdella lineata	100.0	MN071352
Helobdella lineata	99.0	AF329039
Helobdella lineata	99.0	AF329039
Helobdella lineata	99.0	AF329039
Helobdella lineata	99.0	AF329039
Helobdella lineata	99.0	AF329039
Helobdella lineata	99.0	AF329039
Helobdella lineata	100.0	MN071352
Placobdella akahkway	100.0	OL743154
Placobdella akahkway	100.0	OL743148
Placobdella akahkway	99.9	OL743149
Placobdella akahkway	100.0	OL743148
Theromyzon trizonare	99.7	OK586852

Through barcoding, I could identify 11 different species of trematodes that use snails and leeches as second intermediate hosts (Table 8). A large proportion of metacercarial samples (47.8%) collected from snails and 24.3% of the cyst samples from leeches failed to amplify or to produce usable sequences, or could not be matched with sequences in GenBank due to poor quality of sequences. Unfortunately, barcoding to identify the tail-less cercaria from its amphipod host failed, thus, the trematode identity remains unknown (Table 8).

Table 8. Species identification results based on sequencing of fragments from mitochondrial gene *cox1* from trematode metacercariae found in invertebrate second intermediate hosts. Letters after host names refer to particular individuals. * = closest match to GenBank, but below 5% divergence ideal threshold. M = Identified morphologically, low quality sequence that could not be matched in GenBank.

Host ID	Barcoding ID	% identity	GenBank accession match
Snails			
Lymnaeidae			
Lymnaea A	*Cotylurus cornutus*	98.0	MH369489
Lymnaea A	*Cotylurus cornutus*	*90.7	KY513232
Lymnaea B	*Cotylurus* sp. C	98.2	MH369576
Lymnaea B	*Cotylurus* sp. C	*90.7	MH369576
Lymnaea C	*Cotylurus* sp. F	98.4	MH369568
Lymnaea C	*Cotylurus* sp. F	*94.3	MH369512
Lymnaea D	*Cotylurus* sp. C	99.7	MH369576
Lymnaea D	*Cotylurus* sp. F	98.4	MH369512
Lymnaea E	*Cotylurus* sp. F	99.7	MH369512
Lymnaea F	*Cotylurus* sp. F	99.3	MH369570
Lymnaea G	*Cotylurus* sp. B	97.7	LC599709
Lymnaea H	*Cotylurus* sp. C	97.5	MH369576
Lymnaea I	*Cotylurus* sp. C	*93.1	MH369576
Stagnicola A	*Cotylurus* sp. E	100.0	OM102969
Stagnicola B	*Cotylurus cornutus*	98.6	MH369544
Stagnicola C	*Cotylurus cornutus*	96.4	MH369597
Stagnicola D	*Cotylurus cornutus*	98.9	MH369597
Stagnicola E	*Cotylurus cornutus*	96.7	MH369500
Stagnicola E	*Cotylurus* sp. C	*91.2	MH369576
Stagnicola E	*Cotylurus marcogliesei*	*79.4	MH369479
Stagnicola F	*Cotylurus cornutus*	95.6	MH369480
Stagnicola G	*Cotylurus* sp. C	95.5	MH369576
Stagnicola G	*Cotylurus cornutus*	*91.9	MH369557
Stagnicola H	*Cotylurus* sp. F	*88.4	MH369570
Stagnicola I	*Cotylurus* sp. F	96.3	MH369568
Physidae			
Physa A	*Cotylurus strigeoides*	99.3	MH369599
Physa B	*Cotylurus strigeoides*	97.6	MH369599
Physa B	*Cotylurus strigeoides*	98.2	MH369518
Physa B	*Echinoparyphium* sp. A	*93.9	MH369247
Physa C	*Echinoparyphium* sp. A	*93.6	MH369308
Physa C	*Cotylurus strigeoides*	*92.1	MH369525

Host ID	Barcoding ID	% identity	GenBank accession match
Physa D	*Cotylurus strigeoides*	96.7	MH581280
Physa E	*Cotylurus cornutus*	97.2	MH369516
Physa F	*Cotylurus* sp. F	95.3	MH369514
Physa G	*Echinoparyphium* sp. A	97.7	MH369290
Physa H	*Echinoparyphium* sp. A	99.3	MH369307
Physa I	*Cotylurus cornutus*	96.0	MH369480
Physa J	*Echinoparyphium* sp. A	99.8	MH369308
Physa K	*Cotylurus strigeoides*	*84.0	MH369588
Physa L	*Cotylurus strigeoides*	*90.7	MH369572
Physa M	*Diplostomum* sp.	*78.0	KJ726489
Planorbidae			
Planorbella A	*Cotylurus cornutus*	99.2	MH369500
Planorbella A	*Cotylurus cornutus*	98.3	MH369500
Planorbella B	*Echinoparyphium* sp. Lineage 3	96.4	MH369270
Leeches			
Erpobdellidae			
Erpobdella obscura A	*Australapatemon* sp.	*92.5	MH369767
Erpobdella obscura A	*Cotylurus strigeoides*	99.7	MH369575
Erpobdella obscura B	*Cotylurus strigeoides*	95.9	MH369572
Erpobdella obscura B	*Cotylurus strigeoides*	99.3	MH369517
Erpobdella obscura C	*Cotylurus strigeoides*	99.3	MH369599
Erpobdella punctata	*Cotylurus strigeoides*	99.7	MH369599
Glossiphoniidae			
Glossiphonia elegans A	*Cotylurus strigeoides*	96.7	MH369572
Glossiphonia elegans B	*Cotylurus strigeoides*	100.0	MH369529
Glossiphonia elegans C	*Cotylurus strigeoides*	99.1	MH369599
Glossiphonia elegans D	*Cotylurus strigeoides*	99.7	MH369529
Helobdella lineata A	*Cotylurus strigeoides*	97.7	MH369571
Helobdella lineata A	*Cotylurus strigeoides*	*93.6	MH369572
Helobdella lineata B	*Cotylurus strigeoides*	98.3	MH369596
Helobdella lineata B	*Cotylurus strigeoides*	99.7	MH369575
Helobdella lineata C	*Cotylurus strigeoides*	95.8	MH581282
Helobdella lineata D	*Cotylurus strigeoides*	*92.0	MH581282
Helobdella lineata D	*Cotylurus strigeoides*	100.0	MH581281
Helobdella lineata E	*Cotylurus strigeoides*	99.6	MH581280
Helobdella lineata E	*Cotylurus strigeoides*	95.4	MH369517
Helobdella lineata F	*Cotylurus strigeoides*	*92.3	MH581280

Host ID	Barcoding ID	% identity	GenBank accession match
Helobdella lineata G	*Cotylurus strigeoides*	98.8	MH369572
Helobdella lineata G	*Cotylurus strigeoides*	99.1	MH369525
Helobdella lineata H	*Cotylurus strigeoides*	98.0	MH369584
Helobdella lineata I	*Cotylurus strigeoides*	99.2	MH369599
Helobdella lineata J	*Cotylurus strigeoides*	*91.0	MH369572
Helobdella lineata J	*Cotylurus strigeoides*	*91.2	MH369572
Helobdella lineata K^{M}	*Cotylurus strigeoides*	99.1	MH369525
Placobdella akahkway	*Cotylurus strigeoides*	*92.3	MH369572

With information from snail first-intermediate hosts obtained in previous chapters, information on second intermediate hosts gathered in this section, and vertebrate surveys (supported by existing reports of trematodes in birds or mammals obtained through a literature search) of potential definitive hosts, I was able to suggest complete life cycles for 20 trematode species that inhabit wetlands in central Alberta. I suggest that most trematode taxa use birds as definitive hosts, but muskrats and foxes are likely also used by trematodes in my study system (Table 9).

Table 9. Suggested complete life cycles for trematode taxa found in central Alberta. Information based on results within this thesis and literature research. M = Trematode taxa collected from intermediate host identified morphologically; B = Trematode taxa collected from intermediate host identified through barcoding. Fish and frog second intermediate hosts were observed at the sites, but not collected. All definitive hosts were observed or surveyed with audio and birdsong recorders during this project and subsequently matched with reported trematode taxa through literature review.

Trematode species	Hosts			Sources
	First	Second	Definitive	
Apharyngostrigea sp.	*Stagnicola*^{M}	Brook Stickleback[1] Minnow[1] Frogs[1]	American Bittern[1]	**1.** Locke et al., 2021
Australapatemon sp.	*Lymnaea*^{M} *Planorbella*^{M} *Stagnicola*^{M}	*Erpobdella obscura*^{B}	American Wigeon[1], Cinnamon Teal[1], Mallard[1], Ruddy Duck[1]	**1.** Aksenova et al., 2016
Cotylurus cornutus	*Lymnaea*^{1} *Physa*^{1}	*Lymnaea*^{B} *Physa*^{B} *Planorbella*^{B} *Stagnicola*^{B}	Black-billed Magpie[2], Common Goldeneye[2], Mallard[3]	**1.** Gibson et al., 2005 **2.** Bychovskaja, 1962 **3.** Bychkova, 1998

Trematode species	Hosts			Sources
	First	Second	Definitive	
Cotylurus sp.	*Lymnaea*[B,M] *Physa*[M] *Planorbella*[B,M] *Stagnicola*[M]	*Lymnaea*[B] *Physa*[B] *Planorbella*[B] *Stagnicola*[B]	Black-billed Magpie[1], Black Tern[2], Common Goldeneye[1], Common Loon[3], Mallard[4,5], Osprey[6], Red-necked Grebe[7]	**1.** Bychovskaja, 1962 **2.** Kiskaroly and Tafro, 1988 **3.** Kinsella and Forrester, 1999 **4.** Bychkova, 1998 **5.** Pyrka et al., 2022 **6.** Filimonova and Zinov'yeva, 1998 **7.** Stock and Holmes, 1988
Cotylurus strigeoides	*Physa*[1]	*Erpobdella obscura*[B] *Erpobdella punctata*[B] *Glossiphonia elegans*[B] *Helobdella lineata*[B] *Lymnaea*[B] *Physa*[B] *Placobdella akahkway*[B]	Mallard[2]	**1.** Zazornova, 1993 **2.** Pyrka et al., 2022
Diplostomum sp.	*Lymnaea*[B,M] *Planorbella*[M] *Stagnicola*[M]	Brook Stickleback[1,2] Minnow[1,2]	American White Pelican[3], Black Tern[4], Common Goldeneye[5], Common Loon[6,7], Common Tern[8,9,10], Great Blue Heron[12], Greater Yellowlegs[13], Horned Grebe[14], Mallard[11], Muskrat[15], Red-necked Grebe[14], Ring-billed Gull[16]	**1.** Lester and Huizinga, 1977 **2.** Field and Irwin, 1995 **3.** Dronen et al., 2003 **4.** Sulgostowska and Czaplinska, 1987 **5.** Sitko and Rzad, 2014 **6.** Kinsella and Forrester, 1999 **7.** Storer, 2002 **8.** Hoglund and Thulin, 1992 **9.** Sitko, 1993 **10.** Reimer, 2002 **11.** Gray et al., 1989 **12.** Forrester and Spalding, 2003 **13.** McNeil et al., 1995 **14.** Storer, 2000 **15.** Rigby and Threlfall, 1981 **16.** Levy et al., 1995
Drepanocephallus spathans	*Planorbella*[B]	Brook Stickleback[1] Minnow[1]	Double-crested cormorant[2]	**1.** Pinto et al., 2016 **2.** Alberson et al., 2022

Trematode species	Hosts			Sources
	First	Second	Definitive	
Echinoparyphium sp.	*Planorbella*[B,M] *Stagnicola*[B,M]	*Lymnaea*[B] *Physa*[B] *Planorbella*[B] *Stagnicola*[B]	American Avocet[1], Black-billed Magpie[2], Black Tern[2], Broad-winged Hawk[3], Bufflehead[4], Common Goldeneye[2], Common Tern[5], Red Fox[7], Gray Partridge[8], Great Horned Owl[3], Horned Grebe[9], Mallard[6], Muskrat[10], Red-tailed Hawk[8], Ring-necked Duck[11]	**1.** Edwards and Bush, 1989 **2.** Sulgostowska and Czaplinska, 1987 **3.** Taft et al., 1993 **4.** Ewart and McLaughlin, 1990 **5.** Bychovskaja, 1962 **6.** Hoeve and Scott, 1988 **7.** Kennedy, 1988 **8.** Kanev et al., 1994 **9.** Storer, 2000 **10.** Detwiler et al., 2012 **11.** Forrester and Spalding, 2003
Echinostoma trivolvis	*Planorbella*[B]	*Dugesia tigrina*[1] Freshwater snails[2]	American Kestrel[3], Broad-winged Hawk[4], Great Horned Owl[4], Muskrat[5], Red-tailed Hawk[4]	**1.** Fried and Rosa-Brunet, 1991 **2.** Fried et al., 1997 **3.** Forrester and Spalding, 2003 **4.** Taft et al., 1993 **5.** Detwiler et al., 2012
Hypoderaeum sp.	*Lymnaea*[M] *Physa*[M] *Planorbella*[M] *Stagnicola*[M]	Freshwater snails[1]	Common Goldeneye[2], Mallard[3]	**1.** Muñoz-Antolí et al., 2000 **2.** Bychovskaja, 1962 **3.** Hoeve and Scott, 1988
Ichthyocotylurus sp.	*Lymnaea*[M] *Stagnicola*[M]	Brook Stickleback[1] Minnow[1]	Common Loon[2], Common Tern[3,4], Horned Grebe[5], Red-necked Grebe[5]	**1.** Faltýnková et al., 2011 **2.** Storer, 2002 **3.** Sitko, 1993 **4.** Pojmańska et al., 2012 **5.** Storer, 2000

Trematode species	Hosts			Sources
	First	Second	Definitive	
Notocotylus sp.	*Physa*[M] *Planorbella*[M]	None, encysts on substrate[1]	American Avocet[2,3], American Coot[4], Bufflehead[5], Common Goldeneye[6,7], Common Tern[6], Horned Grebe[9], Mallard[8], Muskrat[1], Northern Shoveler[10], Red-necked Grebe[9], Red-necked Phalarope[11], Snow Goose[12]	**1.** Herber, 1955 **2.** Edwards and Bush, 1989 **3.** Garcia and Canaris, 1987 **4.** Lamothe-Argumedo et al., 1997 **5.** Ewart and McLaughlin, 1990 **6.** Bychovskaja, 1962 **7.** Iskova et al., 1995 **8.** Graczyk and Shiff, 1993 **9.** Storer, 2000 **10.** Mhaisen, 1994 **11.** Belopol'skaya, 1971 **12.** Clinchy and Barker, 1994
Petasiger sp.	*Planorbella*[B,M]	Brook Stickleback[1] Minnow[2]	Horned Grebe[4,5], Mallard[3], Pied-billed Grebe[6], Red-necked Grebe[5]	**1.** Selbach et al., 2014 **2.** Beaver, 1939 **3.** Bychovskaja, 1962 **4.** Storer, 2000 **5.** Stock and Holmes, 1988 **6.** Leon-Regagnon, 1992

Trematode species	Hosts			Sources
	First	Second	Definitive	
Plagiorchis sp.	*Lymnaea*[M] *Physa*[M] *Planorbella*[M] *Stagnicola*[M]	Mosquito larvae[1]	American Avocet[2], Barn Swallow[3], Black-billed Magpie[4], Black Tern[5], Bufflehead[6], Common Raven[7], European Starling[9,10], Red Fox[11], Gray Partridge[7], Greater Yellowlegs[12], House Sparrow[13], Mallard[8], Muskrat[14], Pied-billed Grebe[15], Red-necked Phalarope[4,16], Red-necked Grebe[15], Short-eared Owl[7]	**1.** Zahiri et al., 1997 **2.** Edwards and Bush, 1989 **3.** Janssen and Bock, 1990 **4.** Sharpilo and Iskova, 1989 **5.** Sulgostowska and Czaplinska, 1987 **6.** Ewart and McLaughlin, 1990 **7.** Bychovskaja, 1962 **8.** Bychkova, 1998 **9.** Bernard, 1987 **10.** Borgsteede et al., 2000 **11.** Kennedy, 1988 12. Secord and Canaris, 1993 **13.** Ivanov et al., 2002 **14.** Gibson et al., 2005 **15.** Storer, 2000 **16.** Canaris and Kinsella, 2000
Posthodiplostomum sp.	*Physa*[M]	Brook Stickleback[1] Minnow[1]	American Bittern[2], American White Pelican[3]	**1.** Ritossa et al., 2013 **2.** Forrester and Spalding, 2003 **3.** Kinsella et al., 2004
Ribeiroia ondatrae	*Planorbella*[B]	Frogs[1]	American White Pelican[2], Common Loon[3], Great Blue Heron[4], Horned Grebe[5], Osprey[6], Pied-billed Grebe[5], Red-necked Grebe[5]	**1.** Kelly et al., 2010 **2.** Kinsella et al., 2004 **3.** Storer, 2002 **4.** Johnson and Lunde, 2005 **5.** Storer, 2000 **6.** Kinsella et al., 1996
Schistosomatidae gen.	*Lymnaea*[M] *Physa*[M] *Planorbella*[M] *Stagnicola*[M]	None, cercariae penetrate skin of definitive host[1]	Canada Goose[2], Double-crested Cormorant[2], Humans[1], Muskrat[3], Ring-billed Gull[4]	**1.** Davis, 2006 **2.** Barber and Caira, 1995 **3.** Loker et al., 2022 **4.** Lockyer et al., 2003
Schistosomatium sp.	*Lymnaea*[M]	None, cercariae penetrate skin of definitive host[1]	Muskrat[1]	**1.** Loker et al., 2022

Trematode species	Hosts			Sources
	First	Second	Definitive	
Strigeidae gen.	*Lymnaea*^{M} *Planorbella*^{M} *Stagnicola*^{M}	Leeches[1]	American Kestrel[2], Broad-winged Hawk[3], Common Goldeneye[4], Common Tern[4], Cooper's Hawk[3], Great Horned Owl[3], Sandhill Crane[2]	**1.** Aksenova et al., 2016 **2.** Forrester and Spalding, 2003 **3.** Taft et al., 1993 **4.** Iskova et al., 1995
Trichobilharzia sp.	*Lymnaea*^{M} *Physa*^{M} *Planorbella*^{M}	None, cercariae penetrate skin of definitive host[1,2]	Common Goldeneye[2], Mallard[3]	**1.** Loker et al., 2022 **2.** Soldánová et al., 2022 **3.** Fedynich and Pence, 1994

Discussion

In this chapter, I used molecular methods to identify trematodes that use invertebrates as second intermediate hosts at my research sites in central Alberta (Table 8), and the identity of these second intermediate hosts (Table 7). Through wildlife surveys, literature review and data within this thesis, I was able to produce plausible complete life cycles for the trematode species identified from my study sites (Table 9).

As seen in Chapter 2 of this thesis, trematodes use freshwater snails from the genera *Lymnaea*, *Physa*, *Planorbella* and *Stagnicola* as first intermediate hosts in my study system. My results suggest that most trematodes mainly use the same four genera of snails together with leeches as second intermediate hosts rather than other invertebrates in the same ecosystem. However, I did not find metacercariae of *Plagiorchis* in any of the invertebrates sampled, suggesting I missed sampling the preferred second intermediate hosts for *Plagiorchis*, which are mosquito larvae (Zahiri et al., 1997). Prevalence of metacercarial infection is much higher in leeches and snails than in any other invertebrate group studied here. These differences are unlikely to be simply a product of uneven sample sizes among invertebrate groups. Leeches harboured high metacercarial prevalence whereas the second most sampled organisms were amphipods, and only one individual had a tail-less cercaria inside. It is noteworthy that finding a tail-less cercaria does not constitute proof that trematodes are able to encyst in amphipods at my study sites, as cercariae could be melanized and killed after shedding their tail instead of developing fully into metacercariae (Kostadinova and Mavrodieva, 2005). It is also noteworthy that 24 of the 170 amphipods dissected (i.e., 14.12%) were infected with acanthocephalans. The lack of trematode infection in backswimmers, water boatmen, caddisfly larvae,

damselfly and dragonfly nymphs, and adult water beetles was surprising, as all of these invertebrates have been previously documented as second intermediate hosts for trematodes (Yamaguti, 1975). However, I found one cysticercoid infecting one of the 79 nymphal damselflies dissected. Trematode species that infect these invertebrates may not be present in my study area, or trematodes simply prefer to infect snails and leeches, which are probably easier for free-living cercariae to bore into due to their lack of exoskeleton.

My results showing that snails are used as both first and second intermediate hosts for trematodes is in line with previous research that suggests trematode cercariae frequently transfer to suitable organs within their snail first intermediate host, where they encyst as metacercariae (Evans and Gordon, 1983; Fried et al., 1997; Esch et al., 2002; Zimmerman et al., 2015). Alternatively, cercariae can leave their snail intermediate host and infect other snails by swimming into the pneumostome of a different individual (Huffman and Fried, 2012). Regardless of the pathway used to infect their snail second intermediate host, metacercariae from *Cotylurus cornutus* (Rudolphi, 1809) Szidat, 1928 (Strigeidae) have been reported previously in *Lymnaea*, *Stagnicola* and *Physa* (Barragán-Sáenz et al., 2009), but at the time of writing this thesis, this is the first record of *C. cornutus* metacercariae in *Planorbella*. Likewise, this is the first record of *Cotylurus strigeoides* Dubois, 1958 (Strigeidae) using *Lymnaea* and *Physa* as second intermediate hosts. *Echinoparyphium* sp. metacercariae have been previously reported in *Lymnaea*, *Physa* and *Planorbella* (Detwiler and Minchella, 2009), which is confirmed by my results. However, this is the first record of *Echinoparyphium* sp. metacercariae in *Stagnicola* in parasitology literature.

Leeches have been previously described as second intermediate hosts for different trematode species, but in some cases, leech species identity was not provided (e.g., Aksenova et al., 2016). Here, I provide molecular identification of five species of leeches used as second intermediate hosts by trematodes. *Australapatemon* sp. has previously been reported from *Erpobdella microstoma* (Moore, 1901) (Calhoun et al., 2020) and *E. octoculata* (Linnaeus, 1758) (Pyrka et al., 2021), but at the time of writing this thesis, there was no previous record of *Erpobdella obscura* (Verrill, 1872) as second intermediate host for this trematode genus. Information on second intermediate hosts for *Cotylurus strigeoides* is even more scarce, making this study the first to describe *Erpobdella obscura*, *E. punctata* (Leidy, 1870), *Glossiphonia elegans* (Verrill, 1872), *Helobdella lineata* (Verrill, 1874) and *Placobdella akahkway* Fan, De Carle & Kvist, 2022 as hosts for *C. strigeoides* metacercariae.

In my study system, I found six trematode taxa with literature records of using fish as second intermediate hosts, and two taxa that use frogs. While I did not dissect vertebrates from any of my study sites, I did observe minnows (Cyprinidae), Brook Sticklebacks and frogs while collecting invertebrates. Nine-spine Sticklebacks have been previously described as second intermediate hosts for *Diplostomum* in Canada (Curtis, 1981), and Yamaguti (1975) described how minnows are often used by both *Diplostomum* and *Petasiger*. Lebedeva et al. (2022) later proposed that *Diplostomum* spp. have the flexibility to use several unrelated species of fish, suggesting trematodes in general can use a wide variety of fish hosts to complete their life cycles. It is therefore plausible that Brook Sticklebacks and minnows are used as second intermediate hosts by the six trematode species described here. While I did not catch frogs to identify them, some trematode species can use a wide variety of frogs as intermediate hosts (Szuroczki and Richardson, 2009). From my study system, the most widely known trematode species likely to have this type of life cycle is *Ribeiroia ondatrae* (Price, 1931) Price, 1942 (Psilostomidae), which can cause severe malformations of their frog hosts, making them more susceptible to predation by birds. Particularly at Morinville, *R. ondatrae* was highly prevalent, suggesting that frog populations might be at risk of increased mortality caused by this trematode (Szuroczki and Richardson, 2009).

Lastly, by using more than one method to obtain bird and mammal surveys combined with literature research, I was able to get a large diversity of highly plausible potential definitive hosts for the 20 trematode species found at my study sites. This is the first study to attempt to provide full life cycles for trematodes in Alberta, and by focusing on definitive hosts actually present at the study sites, the complete life cycles proposed here are very likely to be accurate, even though I did not dissect any vertebrates during my research. Knowing full life cycles for trematodes in a region is very important not only for ecological reasons, but to have better information to inform conservation efforts, and to increase the knowledge on vertebrate fauna that are likely to harbour trematode species that are responsible for causing swimmer's itch in the province.

To my knowledge, this study is the first to provide a verified and plausible set of second intermediate and definitive hosts for the trematode species present in Alberta, and the first to attempt to close the current gap in the information on trematode life cycles after they leave their freshwater snail first intermediate host. This study is the first to report *R. ondatrae* in Alberta, and while trematode populations are very dynamic, this first report of *R. ondatrae* in this province should be

considered in amphibian conservation studies in the region. There are at least three trematode taxa that can cause swimmer's itch in Albertan lakes: Schistosomatidae gen., *Schistosomatium* sp. and *Trichobilharzia* sp. (Loker et al., 2022). My results suggest that these three taxa use lymnaeid, physid and planorbid snails as first intermediate hosts, five species of birds, and muskrats as definitive hosts. This is valuable information for human health authorities and for provincial government, as these three trematode taxa have a reduced life cycle that requires only two hosts to be completed.

Chapter 7: Conclusions and future research

In this thesis, I studied several aspects of trematode ecology in central Alberta. I surveyed trematode prevalence and species richness across multiple freshwater ponds and lakes, gathering data from four snail genera. I also assessed, for the first time in Canada, evidence for the occurrence of division of labour in freshwater trematode colonies. Further, I researched direct effects of trematode metacercarial infection on the survival of their snail second intermediate hosts. I additionally studied the relationship between trematode infection and the presence of other symbionts that use freshwater snails as hosts. Lastly, I provided for the first time, plausible complete life cycles for trematode species surveyed in this thesis.

Trematode community composition

In Chapter 2, I surveyed trematode prevalence and species richness at seven locations in central Alberta. Pigeon and Wabamun lakes were previously surveyed by Gordy et al. (2016), Lafarge and Strathcona sites were simultaneously sampled for the first time with the Hanington lab at the University of Alberta, and Morinville (i.e. Heritage Lake), St. Albert and Lac Ste. Anne were surveyed for the first time. I identified 21 different trematode taxa morphologically, of which members of the Echinostomatidae were the most prevalent, occurring at four sites. Trematode prevalence and species richness differed among sites. Trematode prevalence also differed across snail genera. When all trematode taxa were pooled, St. Albert had the highest trematode prevalence amongst all sites, while Pigeon Lake had the lowest prevalence. Snails of the genus *Stagnicola* had the highest pooled trematode prevalence, in agreement with Gordy et al. (2016) who reported highest infection prevalence in lymnaeid snails. However, at my study sites, high infection prevalence in snails was driven by metacercarial infection (i.e., snails used as second intermediate hosts). Results in Gordy et al. (2016) are based solely on sporocyst or redia infections (i.e., snails used as first intermediate hosts). This distinction is important in overall trematode prevalence estimates. When determining infection prevalence based only on the proportion of snails shedding cercariae, metacercarial infections as well as undeveloped redia and sporocyst infections are missed. This leads to underestimates of trematode prevalence and likely of species richness.

Using systematic trematode surveys, I reported for the first time the presence of *Ribeiroia ondatrae* and *Drepanocephalus spathans* in Alberta. These two trematode species are significant to wildlife conservation in the province, and to aquaculture in Canada. *R. ondatrae* causes malformations in frogs when cercariae encyst in tadpole tails, disrupting hind limb formation and causing adult frogs to develop multiple hind legs. These malformations impede movements of the frog and make it more susceptible to predation by birds (Szuroczki and Richardson, 2009; Roberts and Dickinson, 2012). Especially at St. Albert, *R. ondatrae* was highly prevalent, suggesting that frog populations at this site might be at risk of high mortality caused by *R. ondatrae* infections (Szuroczki and Richardson, 2009). Similarly, *D. spathans* can cause high mortality in fish second intermediate hosts, especially when the host acquires the trematode infection as a juvenile (Griffin et al., 2012). As with *R. ondatrae*, the new record of *D. spathans* in Alberta is relevant for wildlife conservation and fisheries management in the province.

I recorded the presence of *Trichobilharzia* at six sites, and *Diplostomum* at five sites out of the seven surveyed. These trematode genera have been previously reported by Gordy et al. (2016), and are responsible for swimmer's itch in central Alberta. Given their presence at most of my study sites, they are likely very common across the province. This information is of importance to the health sector, public and provincial government, as it can inform better policies to mitigate the contraction of swimmer's itch in Alberta. Overall, the new knowledge provided by this survey adds important information to the novel research of trematode ecology in central Alberta, but more research is necessary to get better estimates of trematode prevalence and species richness in the province.

Division of labour in trematodes

This is the first study in Canada, and one of the very few worldwide, to attempt to find division of labour in freshwater trematodes. In Chapter 3, I described a smaller redia morph in two trematode types: putative *Hypoderaeum* and *Petasiger*. The size-frequency distribution in all colonies of both trematode taxa was bimodal, especially if colonies were collected in late summer. This suggests that smaller redia morphs are highly frequent in these trematode colonies, and likely have a specific function, as producing small non-reproductive rediae is potentially costly for the colony (Kamiya and Poulin, 2013b). Similar to soldier morphs previously described from marine trematodes (Garcia-Vedrenne et al., 2016; 2017), small rediae of *Petasiger* were almost transparent and slimmer, had

visible collars and highly adhesive appendages, and proportionately larger pharynges than their large conspecific reproductive rediae of the same colony. All *Petasiger* reproductive rediae were actively releasing cercariae, or had cercariae inside, unlike small redia morphs, which lacked germinal masses. In putative *Hypoderaeum*, the size difference between redia types was substantial, and smaller redia morphs lacked visible germinal masses. However, other than lacking germinal masses, small rediae in putative *Hypoderaeum* shared colouration and body shape with reproductives. Both small and reproductive rediae lacked a collar, and both morphs had appendages that were proportional in size.

Activity patterns also differed between redia morphs in both trematode taxa. In *Hypoderaeum*, small redia morphs were more active than reproductives in the absence of competition (i.e., presence of sporocysts of a different taxon). The small redia type in this species was also more active when competitor trematodes were present. However, their activity levels decreased when both conspecific reproductive rediae and competitor trematodes were present at the same time. This behaviour partially agrees with previously described marine trematode behaviour in that small redia types are more active than reproductives (Garcia-Vedrenne et al., 2016). However, my results are novel in that the small redia morph seems to lower activity levels when both competitors and conspecific reproductives are present at the same time. In *Petasiger*, activity patterns were also different between reproductive and small rediae, but were unlike what I observed in putative *Hypoderaeum*. Reproductive *Petasiger* rediae were more active when small conspecific rediae were present and when exposed to competitors, but not when both conspecific small rediae and competitors were present at the same time. Results from activity patterns of *Petasiger* agree with the observations of Kamiya and Poulin (2013a) that reproductive rediae are less aggressive when both competitors and small rediae are present. It is thus possible that the role of defense is shared between small and reproductive morphs in my study system. To tease apart the exact roles of both redia morphs, further *in vitro* experiments are necessary to measure activity patterns of large and small redia types in *Petasiger* and *R. ondatrae*, which was the trematode species that composed the majority of samples of putative *Hypoderaeum* studied here.

Attacks towards competing trematode species occurred but were very rare in both trematode taxa. Both reproductive and small rediae of putative *Hypoderaeum* occasionally attacked by latching their mouthparts to competitor sporocysts. Attacks from small rediae on competitor trematodes lasted longer when conspecific reproductives were present at the same time. Attacks on competitor trematodes were observed only once in small rediae of *Petasiger*, and this attack happened in the

absence of conspecific reproductives. Furthermore, small rediae of *Petasiger* attached their mouthparts to discarded tails of conspecific cercariae. These attacks lasted for prolonged periods, indicating the possibility that the small redia morph in this genus may also have a cleaning role in the colony. Overall, my observations of bimodal size distributions of rediae and attack behaviour suggest that division of labour exists in trematodes in freshwater environments. Additional in-vitro experiments exposing small redia morphs to competitor trematodes of different species and to discarded cercaria tails from their own colony would help to evaluate the likelihood of defensive *vs* cleaning roles of small redia morphs. It is also necessary to perform in-vivo experiments to test if putative *Hypoderaeum* or *Petasiger* can outcompete other trematode species inside snail hosts.

Trematode infection and snail survival

Chapter 4 described snail survival over time of four genera of freshwater snails found in central Alberta. In general, there was a steep decline in snail survival in laboratory conditions, with less than 10% of sampled individuals surviving longer than 100 days. The four snail genera carried metacercarial infection in kidneys and in the gut, with some snails having cysts in both organs. Metacercariae found in the kidney were identified as *Echinoparyphium* sp., while cysts in the gut belonged to *Cotylurus cornutus*. Factors that affected snail survival varied among snail genera. Neither location of metacercarial infection, infection intensity nor coinfections had any effect on survival over time in *Stagnicola*. However, location of metacercarial infection significantly affected snail survival in *Planorbella* only. In this genus, the presence of cysts in the kidney was associated with longer survival, while cysts in the gut had the opposite effect. Interestingly, *Planorbella* with cysts present in both kidney and gut survived a similar time to uninfected congeners.

Intensity of metacercarial infection had different effects on survival depending on snail genus and location of infection. In *Planorbella*, snail survival increased with increasing intensity of infection in the gut. In *Lymnaea* and *Physa*, an increase in number of cysts in the kidney and the presence of coinfections reduced duration of snail survival. These results suggest that in *Planorbella*, *Echinoparyphium* cysts might have a waste disposal function and act as an accessory kidney, similar to *Cryptocotyle lingua*, a trematode that seemingly absorbs pollutants from snail tissues (Cross et al., 2003). Alternatively, *Echinoparyphium* might create a cross-signalling effect that, independent of intensity of infection, triggers an immune response rendering the snail more resilient to environmental

stressors. Similar effects have been observed from protozoan and viral infections in oysters and aphid vectors (Encomio and Chu, 2007; Porras et al., 2020). *Echinoparyphium* cysts in frogs reduce replication of a ranavirus and increase survival of virus-infected frogs (Wuerthner et al., 2017). Future studies should aim to expose snails infected with *Echinoparyphium* cysts and uninfected snails to pollutants, and test the levels of these pollutants in both snail tissue and *Echinoparyphium* cysts to test if *Echinoparyphium* can in fact function as an accessory kidney to its snail host. It would also be worthwhile to expose snails with and without *Echinoparyphium* cysts to infectious fungi or other microbes to test if cyst infections with this trematode genus increase immune response in their snail host.

Trematode infection in relation to other snail symbionts

In Chapter 5, I described correlations between three taxa symbiotically associated with freshwater snails in Alberta: members of the oligochaete species-complex *Chaetogaster 'limnaei'* s.l., leeches of the genus *Helobdella*, and trematodes. My results suggest that *Chaetogaster* loads are significantly higher in snails shedding cercariae (i.e., those acting as first intermediate hosts for trematodes), and that this pattern is independent of the infection pathway of the different trematode species (i.e., penetration by miracidia *vs* ingestion of eggs by snails). I found no evidence that *Chaetogaster* reduces infection of snails by cercariae (i.e., when snails are used as second intermediate hosts by trematodes). My results suggest that infected snails attract *Chaetogaster* because they shed cercariae, an abundant high-quality food source for *Chaetogaster* (McKoy et al, 2011; Hobart et al, 2022). These resources may then promote *Chaetogaster* reproduction by fission, increasing the number of individuals on the snail host (Fernandez et al., 1991).

With regard to *Chaetogaster* potentially reducing the likelihood of snails being used as first intermediate hosts, I provide evidence that this may be the case for *Plagiorchis* sp., a trematode genus that infects snails when its eggs are ingested by snails while grazing. *Chaetogaster* in my study system did not reduce infection of trematode taxa that infect snail hosts via miracidia. These results were opposite to the findings in Hobart et al. (2022) who proposed that *Chaetogaster* protects its snail host against miracidial infection, but not against trematode egg infections. Both Hobbart et al. (2022) and results in this chapter must be interpreted with care. Data were collected from snails naturally infected by trematodes in the wild, and exact timing of *Chaetogaster* infestation in relation to trematode

infection could not be determined. Timing of infection or infestation would greatly affect the outcome of interactions between trematode miracidia and *Chaetogaster*. In future research, it is thus necessary to conduct experiments exposing lab-raised snails known to be free of trematode infection to *Chaetogaster* infestation first. Once *Chaetogaster* is established, snails with and without *Chaetogaster* should be exposed to miracidial, egg and cercarial infection to test the defensive role of *Chaetogaster* against the three types of trematode infection. To test if *Chaetogaster* is attracted to snails shedding cercariae over uninfected snails, it would be necessary to expose lab-reared snails to trematode infection first, and once the trematode colonies are producing cercariae, conduct choice experiments with *Chaetogaster*.

In my study system, leeches that infected planorbid snails did not seem to have an effect on trematode infection, independent of whether snails were used as first or second intermediate hosts by trematodes. These results were contrary to my hypotheses that leeches would make snails more susceptible to trematode infection by feeding on snail haemolymph, and to previous research proposing that leeches protect their host against trematode infection (Brooks and Welch, 1977; Elkhodary et al., 2018). My results partially agree with previous observations on *Helobdella lineata* indicating that the leech is a harmless commensal of planorbid snails (Sarah, 1971). However, *H. lineata* in my study system had visible stomach contents that resembled planorbid haemolymph. I thus proposed that *H. lineata* has a life cycle similar to *Alboglossiphonia polypompholyx*, which is parasitic on snail hosts as juveniles, and leave the snail when they reach certain size or sexual maturity, becoming free-living and predatory on snails (El-Shimy and Davies, 1991). Future research on this relationship should include infection of planorbid snails with juvenile *H. lineata* and follow their development to test if the life cycle I propose here is correct.

Trematode life cycles: closing the gaps in central Alberta

In Chapter 6, I used information from morphological identification of cercariae, together with molecular genotyping of rediae, sporocysts and metacercariae gathered in previous chapters to get a list of trematode taxa that use snails as first intermediate hosts in central Alberta. I also genetically identified trematode species that use invertebrates as second intermediate hosts. I then used information gathered through an extensive literature search to propose plausible complete life cycles for 21 taxa of trematodes found in central Alberta.

I documented that leeches and snails are the preferred second intermediate hosts for the trematode taxa at my study sites. This preference may be driven by the availability of these hosts and/or because it might be easier for cercariae to infect soft-bodied invertebrates than insect larvae or crustaceans that have an exoskeleton. I observed that trematodes in the province frequently use snails as both first and second intermediate hosts, in agreement with previous research by Evans and Gordon (1983), Fried et al. (1997), Esch et al. (2002) and Zimmerman et al. (2015). I proposed that trematodes that encyst as metacercariae in snails have two alternative infection pathways. Either cercariae that are released from rediae or sporocysts within the snail first intermediate host transfer to suitable organs within the snail of origin and encyst as metacercariae, or cercariae leave the snail of origin and actively infect other snail individuals by swimming through their pneumostome, as has been previously reported elsewhere (Huffman and Fried, 2012).

Regardless of infection pathway to snail second intermediate hosts, this thesis provides the first records of *Cotylurus cornutus* using *Planorbella*, *Cotylurus strigeoides* using *Lymnaea* and *Physa*, and *Echinoparyphium* using *Stagnicola* as second intermediate hosts. I also provide the first records of *Australapatemon* using *Erpobdella obscura*, and of *Cotylurus strigeoides* using *Erpobdella punctata*, *Glossiphonia elegans*, *Helobdella lineata* and *Placobdella akahkway* as second intermediate hosts.

My thesis also provides the first record of *Ribeiroia ondatrae* in Alberta. Given that this trematode species uses fish and amphibians as second intermediate hosts, increasing host mortality (Szuroczki and Richardson, 2009), this information is vital for wildlife conservation strategies. I suggest that especially at St. Albert, fish and frog populations might be at highest risk, as prevalence of *R. ondatrae* was high during my sampling season. Through other genetic identifications, I provide additional locality records for Schistosomatidae gen., *Schistosomatium* sp. and *Trichobilharzia* sp., all responsible for swimmer's itch in central Alberta. These trematode taxa have a reduced life cycle, which I proposed here to involve lymnaeid, physid and planorbid snails as first intermediate hosts, and five species of birds and muskrats as definitive hosts. This information adds to the current knowledge of trematode ecology from Gordy et al. (2016), and can be used by human health authorities and the provincial government to produce better-informed health and recreation policies in Alberta. Even though this thesis adds important information from sites that had not been surveyed before in Alberta, it is necessary to describe trematode communities from more localities to broaden our knowledge of

potential risks to wildlife and humans in the province, and to understand ecological dynamics that drive trematode communities in central Alberta as a whole.

Concluding thoughts and future prospects

In this thesis, I studied several aspects of the ecology of trematode parasites present in freshwater snails in Alberta. By surveying multiple localities, I was able to find new sites that have high trematode prevalence and species richness, and was able to attract attention to new records of trematode species that have the potential to have negative effects on aquaculture and amphibian populations in the province. However, as this survey was done in my first sampling season and my project spanned multiple aspects of trematode ecology, I was not able to visit sites that were farther from Edmonton more than once. Because trematode communities of freshwater snails are very dynamic, I probably missed some trematode species present at the sites that were sampled only once. Future research on trematode community composition should ideally encompass several localities around Alberta, as well as several sampling occasions over more than one year. Those studies would provide more information on how much trematode prevalence varies within a year and amongst years, and would likely gather information of more trematode species that can be found in the region.

This project was the first to use EthoVision, powerful video tracking software, to analyze redia behaviour in different scenarios. This approach is free from observer bias and results are very accurate compared to manual scoring (Hédou et al., 2001; Evans et al., 2015; Laursen et al., 2021). I initially recorded rediae videos for 20 minutes, but I later realized that redia behaviour does not vary noticeably after a few minutes, and thus 10-minute trials would be long enough in future projects. Recording shorter videos would reduce significantly the time necessary to process all trematode colonies, the size of video files, and the video analysis time with EthoVision. This would in turn leave more time to either gather behavioural information from more colonies, or to focus on other experiments to elucidate the role of small rediae in trematode colonies. While researching division of labour in trematodes, I used CBSS culture media to maintain rediae alive long enough to record all the necessary video trials, with great success. Future research could use culture media to try to elucidate the role of small redia morphs, but in vivo techniques could also be helpful to study trematode interactions in a more natural manner.

I also proposed a plausible life cycle for leeches of the genus *Helobdella* that use lymnaeid and planorbid snails as hosts. Because none of the leeches that inhabited the space between the mantle and the shell of snails had brood or were larger than 7 mm long, I proposed that they were all juveniles. I also collected several free-living individuals of *H. lineata* from the same ponds that were carrying brood and were longer than 9 mm, leading me to conclude that *Helobdella* uses snails for food and protection when they are young and vulnerable, feeding on haemolymph of the snails, and that they leave the snail once they reach sexual maturity. However, I based my categorization of leeches as parasitic on planorbid snails when young by visually comparing gut content of leeches to the haemolymph of planorbid snails. Future research on life cycles of *Helobdella* should include analysis of stomach contents of leeches while they are on the snail and should track the growth of *Helobdella* to observe if small individuals leave the snail to reproduce, if leeches become predatory on snails when they are free-living, or if they feed on other organisms after they leave the snail.

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Appendix

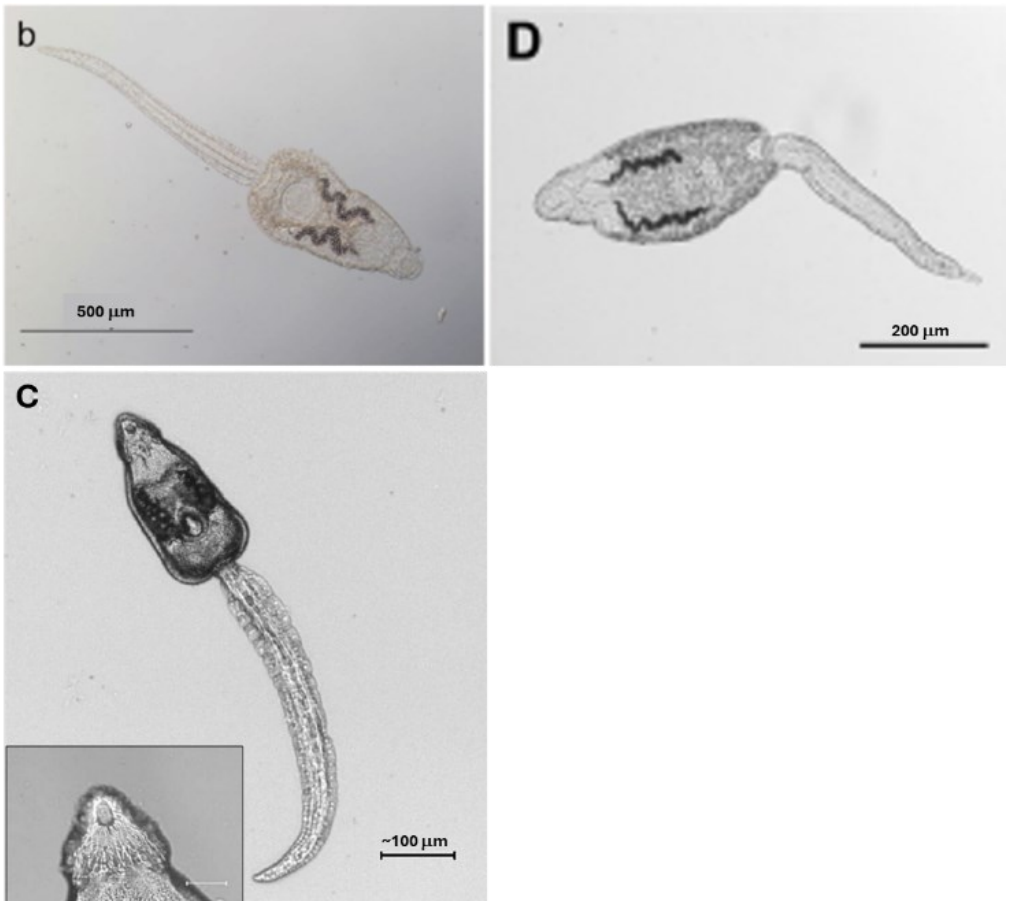

Figure S1. Cercariae images excerpt from previous research. b *Ribeiroia ondatrae* (Orlofske et al., 2015; Figure 1), d *Hypoderaeum* sp. (Gordy et al., 2016; Figure 2), c *Drepanocephalus spathans* (Griffin et al., 2012; Figure 1)

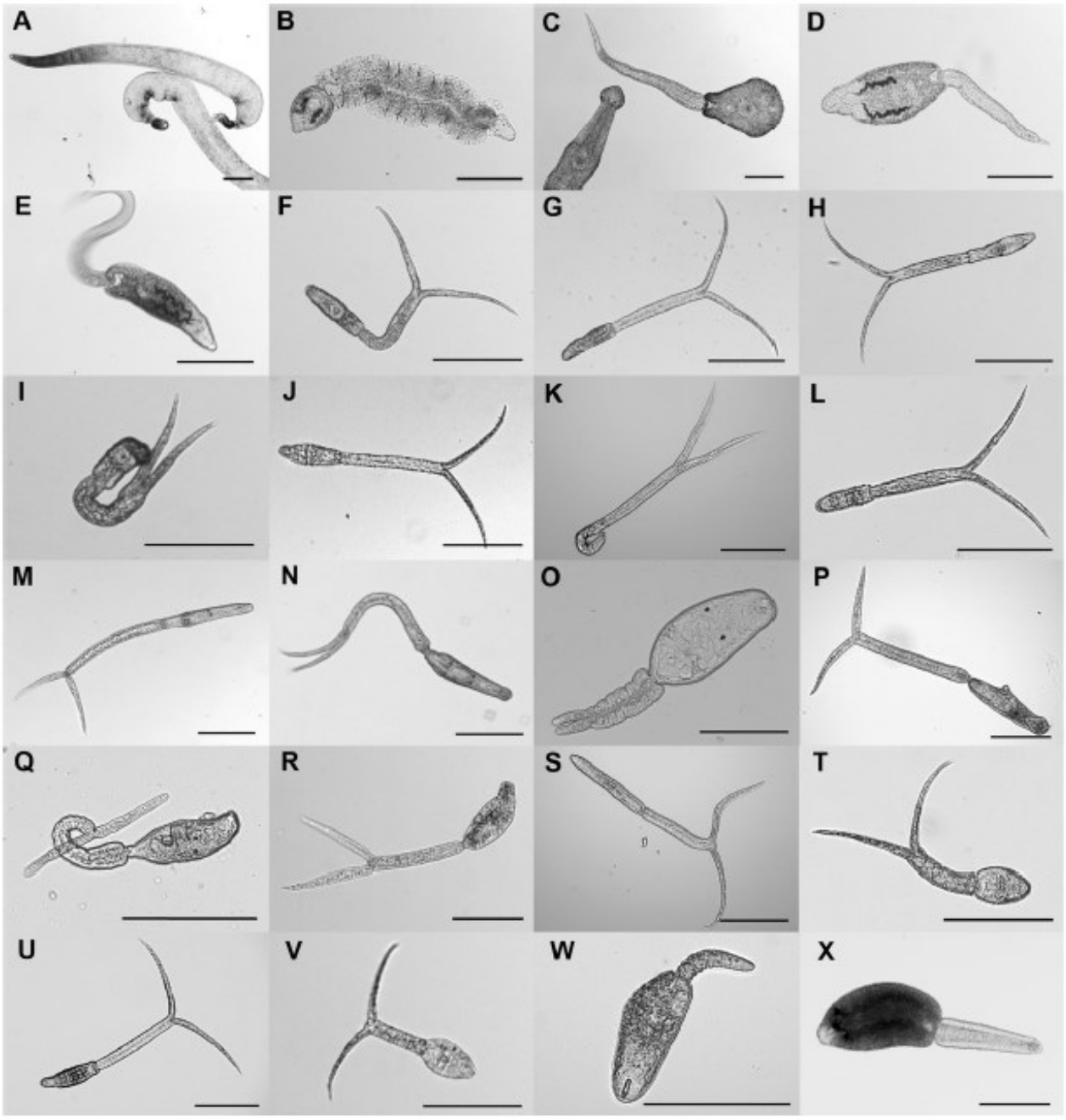

Figure S2. Cercariae images used as reference for morphological identification of *Petasiger* and putative *Hypoderaeum* from field collections. Excerpt from Gordy et al., 2016 (Figure 2). Photographs of digenean trematode cercariae from field collections. Scale bars 200µm. Echinostomatidae: **a** *Neopetasiger islandicus*, **b** *Petasiger* sp. 4, **c** *Echinostoma trivolvis**, **d** *Hypoderaeum* sp., **e** *Echinoparyphium* sp. Diplostomatidae: **f** *Diplostomum* sp. 4, **g** *Diplostomum baeri*, **h** *Tylodelphys scheuringi**, **i** *Diplostomum* sp. 2*, **j** *Fibricola* sp., **k** *Bolbophorus* sp., **l** *Ornithodiplostomum* sp. Schistosomatidae: **m** *Trichobilharzia stagnicolae*, **n** Schistosomatidae gen., **o** *Schistosomatium douthitti**, **p** *Trichobilharzia* sp., **q** *Schistosomatidae* gen. Strigeidae: **r** *Icthyocotylurus* sp. 3*, **s** *Cotylurus gallinulae**, **t** *Australapatemon burti**, **u** *Apharyngostrigea pipientis**, **v** Strigeidae gen. Plagiorchiidae: **w** *Plagiorchis* sp. Gorgoderidae: **x** *Gorgoderina* sp.

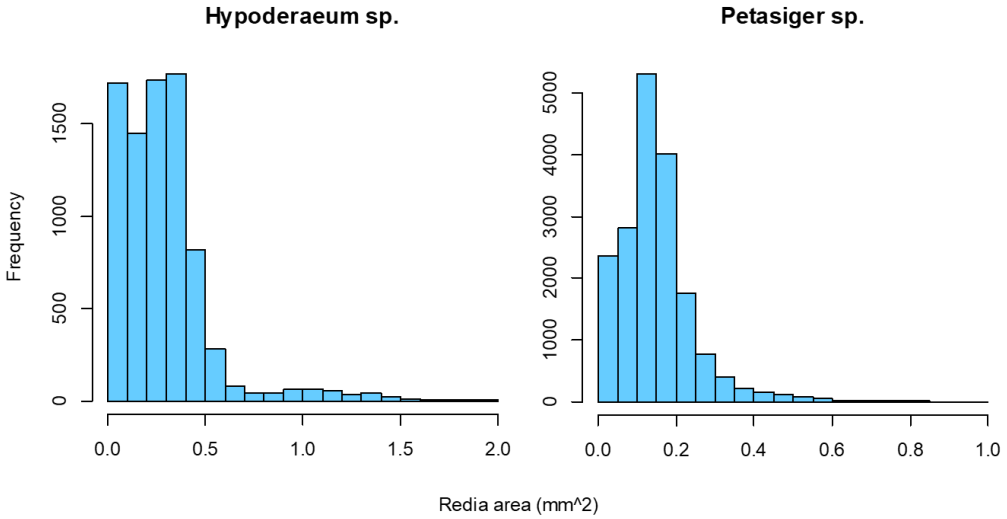

Figure S3. Redia area distributions from putative *Hypoderaeum* and *Petasiger* colonies. All colonies were collected in 2020 and 2021 from Morinville, Lafarge, St. Albert and Strathcona County. Data from both collection years and 10 colonies of each species pooled.

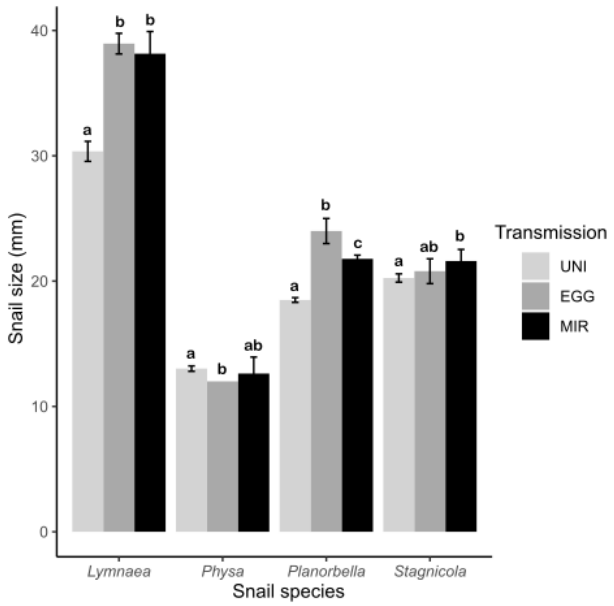

Figure S4. Interaction between snail species and snail size (mm ± SE) for trematode infection in snails used as first intermediate hosts. UNI = Uninfected snails, EGG = Snails with trematode species that infect snails passively as eggs, MIR = Snails with trematode species that infect snails actively with miracidia. Different letters above error bars indicate significantly different means ($p < 0.05$). Data from snails and trematodes collected in 2019-2021. Data from snails infected with sporocysts or rediae only.