BIOL4900 Honours Thesis

An examination of composition and species richness in trematode communities using *Stagnicola elodes*

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Abstract

Human-induced changes to the environment due to climate change, pollution, and loss of habitat have the potential to alter the community of parasites associated with a given host species. This is particularly true for parasites with complex life cycles, such as digenean trematodes, that involve free-living and parasitic stages, and have highly specific relationships with multiple hosts. Based on this, parasites can play a role as bioindicators, where loss of parasite diversity and changes in parasite assemblages can be indicative of degraded environments. In my study, I explored the diversity of digenean trematode parasites associated with a well-studied freshwater snail, *Stagnicola elodes*, in Blacketts Lake, Cape Breton, in relation to another more pristine Cape Breton lake (Lake Ainslie) and to previous studies in North America. *S. elodes* specimens were collected from Blacketts Lake and Lake Ainslie, and infected snails were identified by the emergence of cercarial larval stages. Cercariae were differentiated based on morphotype and behaviour and then preserved for molecular analyses. A DNA barcoding approach was used to assess species boundaries and to determine parasite identities, where possible, through comparisons to other parasite sequences on GenBank using BLAST. Following DNA extraction, a 750 base pair portion of the Cytochrome Oxidase Subunit I gene was amplified using PCR, and then sequenced at The Centre for Applied Genomics in Toronto. Data were also supplemented with results from previous sampling years in Blacketts Lake. My results indicate that a minimum of 13 distinct trematode species channel their life cycles through *S. elodes* in Blacketts Lake, spanning the families Plagiorchiidae, Diplostomidae, Strigeidae, Schistosomatidae, and Echinostomatidae. This assemblage of parasites overlapped

with those reported in previous studies that have focused on *S. elodes*, however, there were notable differences in the family composition between Blacketts Lake and Lake Ainslie. In terms of species richness, that of Blacketts Lake was low compared to the pristine habitats of Douglas and Burt Lakes, Michigan sampled in 1935-1936, but similar to the same habitat following 50 years of human development. Trematode diversity in Blacketts Lake falls within a broad range of values found across other studies in the literature. My study establishes a baseline of the trematode community present in Blacketts Lake that can be used to monitor trematode diversity across years. Having a baseline may be particularly useful for comparing the health of the same aquatic communities over time or for comparing local environments that share similar habitats and climate, but differ in potential human impacts. Further exploration of this trematode-*S. elodes* relationship within a given environmental context should therefore help to provide more insight into the faunal composition of aquatic communities and the integrity of these environments.

Introduction:

Parasitism is the most common way of life among eukaryotic organisms, and most free-living taxa will experience parasitism at some point during their existence (Marcogliese, 2005). Within aquatic environments, parasites can play important roles at all levels of the food chain, yet their effects are often overlooked and understudied (Sures, 2001; Tompkins et al., 2001; Lafferty et al., 2006). At the individual level, parasites can influence the behaviour, physiology, and reproduction of their host species (Marcogliese, 2005; Sures, 2008). At the community and ecosystem level, parasites can be important in regulating the abundance of their host populations, with cascading effects up and down the food web (Marcogliese, 2005).

Parasites are tightly linked to other species in freshwater ecosystems. As such, changes in the species richness and assemblages of these parasites can be indicative of underlying environmental changes (Huspeni et al., 2005; Marcogliese, 2005; Sures, 2008; Vidal-Martinez & Wunderlich, 2017). Consequently, parasites have a potential role as bioindicators – organisms that can signal changes in habitat health (Vidal-Martinez & Wunderlich, 2017). In this context, "good" habitat health is associated with the maintenance of biodiversity along with the perseverance and efficiency of the ecosystem as a whole, and a high diversity of parasite species would be indicative of a healthy community (Hudson et al., 2006).

Parasites have the potential to be good candidates as bioindicators because they are sensitive to both direct and indirect effects of habitat degradation. Direct effects are those that can act on the parasites and affect the survival of the larval stages such as environmental pollutants and toxins (Lafferty, 1997; Huspeni et al., 2005; Marcogliese, 2005; Vidal-Martinez &

Wunderlich, 2017). Parasites can be particularly sensitive to such chemicals (Lafferty, 1997). Indirect effects include those that act on the parasites through the interruption of their life cycles such as those caused by a loss of a host species or through environmental changes such as urbanization, changes in lake chemistry (e.g. pH), nutrient input (eutrophication) and pollution (e.g. from pesticides, sewage, heavy metals, copper sulfate addition) (Lafferty, 1997; Huspeni et al., 2005; Marcogliese, 2005; Vidal-Martinez & Wunderlich, 2017). In this way, changes in lake chemistry have the potential to affect the parasites directly and indirectly. These direct and indirect effects can lead to a decline in parasite diversity and possible changes in the assemblage of parasite species present based on the availability of host taxa (Keas & Blankespoor, 1997; Lafferty, 1997).

Because of the complexity of their life cycles, digenean trematodes can be especially useful indicators of environmental changes. Increased complexity is associated with more host linkages, each of which is vulnerable to environmental change (Huspeni et al., 2005). Digenean trematodes are parasitic members of the Neodermata (Phylum Platyhelminthes) and are characterized as having complex life cycles with a minimum of one intermediate host, and a definitive host in which the parasite develops into sexual maturity (Cribb et al., 2003). The first intermediate host is usually a mollusc, and most frequently, a gastropod (Zakikhani & Rau, 1999). In aquatic communities, the typical life cycle of digenean trematodes begins when a fertilized egg is released in the urine or feces of its definitive host and matures into a free-living, swimming larval stage, known as a miracidium. The miracidium then enters the gastropod intermediate host and undergoes asexual amplification, increasing in number and transitioning through additional larval stages, typically castrating their host in the process (Niewiadomska &

Pojmańska, 2011; Blakeslee et al., 2012). Evidence of infection by digenean trematodes is most often determined through the emergence of cercarial larval stages from the snail (Lockyer et al., 2004). Cercariae can be free swimming, or they can encyst on aquatic vegetation as metacercariae and wait to be eaten by another intermediate host or a definitive host. Some can remain in the mollusc until it is preyed upon by a definitive host (Niewiadomska & Pojmańska, 2011).

The relationship between digeneans and their molluscan intermediate hosts is typically a highly specific one, often more so than the relationship between a trematode and its definitive host (Lockyer et al., 2004). Of the 25 families of digenean trematodes, 23 are known to exploit gastropods as the first intermediate host (Cribb et al., 2003). This suggests that trematodes first exploited snails early in the 200 million year evolutionary history of these parasites (Esch & Fernandez, 1994; Blair et al., 2001). Trematode-snail relationships can be so specific that trematodes of a given species may only be able to infect snails within a family, genus, species, or even population (Sapp & Loker, 2000; Southgate et al., 2001). Given this specificity, particular species of snail intermediate hosts are often essential for these parasites to complete their life cycles (Blakeslee et al., 2012), and, consequently, these snails can be used to assess the presence and prevalence of trematode species (Huspeni et al., 2005). And by confirming the presence of trematode species, one can assess habitat integrity (Hudson et al., 2006).

Past studies of aquatic environments over time have demonstrated that trematodes can indeed be effective bioindicators of habitat quality. For instance, in Douglas Lake, Michigan, in the summers of 1935 and 1936, 16 species of trematodes were found to be exploiting the

freshwater snail *Stagnicola emarginata* as an intermediate host (Cort et al., 1937). This same site was studied more than 50 years later using the same protocols, and only 8 species of trematodes were associated with the same snail (Keas & Blankespoor, 1997). This decline in trematode species richness was interpreted to result from an increase in shoreline development around the lake and recreational activities, such as boating, that resulted in a decline in the presence of vertebrate hosts (Keas & Blankespoor, 1997). In California, Huspeni and Lafferty (2004) used trematode species richness as a metric for validating the effectiveness of restoring a wetland saltmarsh habitat using the California horn snail, *Cerithidea californica*. The mean species richness of trematodes rose from 4.5 species before restoration, to 9 species six years after the restoration (Huspeni & Lafferty, 2004). Such studies suggest that the presence of trematode species represent key links in the local environment, and thus provide insight into the integrity of these communities (Marcogliese, 2005). In a similar way, benchmarks of the current snail-trematode relationships in an environment can help to monitor variation in habitat health over time (Keas & Blankespoor, 1997; Huspeni et al., 2005).

The freshwater snail *Stagnicola elodes* represents an ideal candidate to explore the use of trematode diversity within a gastropod intermediate host as an indicator of habitat health. *S. elodes* is a large pulmonate snail that is abundant, widespread and easy to collect and maintain in the lab (Clarke, 1981) and is commonly exploited by a wide range of trematode species (Table 1). In Canada, this snail is present in all ten provinces, including local lakes in Cape Breton, Nova Scotia (Rawlings, personal communication), and in the southern regions of the three territories (Clarke, 1981). In the United States, it is present primarily in the northern states, but reaches as far south as New Mexico (Johnson et al., 2013). Lymnaeid snails, such as

S. elodes, demonstrate a high plasticity of shell morphology, which has lead to confusion over species' identities and an over inflation of species diversity based on traditional taxonomic methods using shell morphology (Correa et al., 2010). Recent molecular evidence suggests that *S. elodes* is conspecific with the species *S. catascopium*, *S. emarginata*, *S. elrodi* and *S. bonnevillensis* (Remigio, 2002; Correa et al., 2010). And, as such, data from these species can also be grouped under *S. elodes.*

S. elodes is exploited by a wide variety of trematode species based on previous parasite-based studies that have focused on this snail (Table 1). These studies began with the early work of Cort et al. (1937) followed by Keas and Blankespoor (1997), both of which studied *S. elodes* in the same lake in Michigan. In Nova Scotia, this snail (reported as *Lymnaea emarginata*) was also the focus of a study of trematode parasites in Lake Ainslie, Cape Breton (Farley, 1967b); more recently, *S. elodes* has been found to host a number of trematode species in Blacketts Lake, Cape Breton (Rawlings lab, unpublished data). This snail is still present in these lakes today (Rawlings, personal communication). Gordy and colleagues also included *S. elodes* as part of an intensive study of parasites of freshwater snails in six lakes in central Alberta (Gordy et al., 2016; 2017).

Data from studies focusing on *S. elodes* in North America have revealed that 27 species of trematodes from 8 families use *S. elodes* as a host (Table 1). These data sets included Farley (1967b), new datasets by Gordy *et al*. (2016 & 2017) from six lakes in central Alberta, as well as records spanning 1988-2003 as summarized in the Natural History Museum (NHM) host-parasite database (London, UK; www.nhm.ac.uk/research-curation/scientific-resources/taxonomy-systematics/host-parasites/) (Gibson et al., 2005). Together, these records

provide an impressive inventory of digenean trematode parasites that one might expect to find in *S. elodes* within a given aquatic community in North America.

Objectives:

My honours research examined the species richness and composition of trematodes exploiting one species of snail in a well-studied Cape Breton lake. The main objectives of my honours thesis were:

1. To determine the species richness and composition of digenean trematodes exploiting *Stagnicola elodes* in Blacketts Lake using collection data from 2017 and archived data from 2011-2016.

2. To compare the results of *S. elodes* collections in Blacketts Lake to a second Cape Breton lake, Lake Ainslie, based on field sampling in the fall, 2017.

3. To compare the species richness and assemblage of trematodes exploiting *S. elodes* in Blacketts Lake to previous studies undertaken in North America.

These objectives were undertaken to evaluate the potential for using this snail-trematode relationship as an indicator of habitat quality across space and time.

Methods:

Study Sites

S. elodes specimens were collected from a rocky area on the Coxheath side of Blacketts Lake in Cape Breton County, Nova Scotia (46° 04 N/ 60° 18 W) from September to November 2017 (Figure 1a, b). Snails were collected by hand off of the rocks, or by moving a dip net though the muddy sediment. Other snail genera found in Blacketts Lake include *Amnicola*, *Gyraulus*, *Helisoma*, *Physa*, *Pseudosuccinea*, and *Ferrissia* (Wright, 1990; Lawrence, 2011; Novorolsky, 2017; Rawlings, personal communication). Blacketts Lake is a small suburban lake with a surface area of 172 hectares and an average water depth of 9.5 meters and maximum depth of 30 meters (Nova Scotia Department of Fisheries and Oceans, 2010; Table 2). This lake has been characterized in past studies as relatively neutral, with pH range of 5.9 to 7.5 as determined from three samples taken by the Department of Fisheries and Oceans (Nova Scotia Department of Fisheries and Oceans, 2010) in 1975, 1984 and 2000, and Alexander et al. (1986) (Table 2). Plant life and anecdotal evidence suggest that this is not a highly eutrophied lake. Blacketts Lake has also experienced species introductions in the last several years, including the chain pickerel and the smallmouth bass (LeBlanc, 2010).

For comparative purposes, *S. elodes* were also collected at two sites (A and B) in Lake Ainslie in Inverness County, Cape Breton (46° 08 N/ 61° 11W) over the same time period (Figure 2a, b). Lake Ainslie is situated in a rural area, approximately 110 kilometers from Blacketts Lake, and is a much larger lake with a surface area of 5735.8 hectares (Table 2). This lake has an average water depth of 5.7 meters and a maximum depth of 18 meters (Table 2), and is a more pristine environment relative to Blacketts Lake. Collection Site A was located on the windswept

eastern side of the lake (Rawlings, personal communication). This site was Farley's (1967b) collection site "C". Snails were collected on small rocks and coarse sediment in a shallow weedy area, either by hand or dip net. Collection Site B was located on the western side of the lake, in a more protected sandy area, and is the same site as Farley's (1967b) collection site "1". Snails were collected there by dragging a dip net through the sediment. Lake Ainslie as a whole has been identified as oligotrophic based on concentrations of phosphorus and chlorophyll (Taylor, 1994). The pH of water in Lake Ainslie has ranged from 6.60 to 8.00 based on six samples taken by the Nova Scotia Department of Fisheries and Oceans (2010) in 1978, three in 1982, 1989 and 2001, and Alexander et al. (1986) (Table 2). Lake Ainslie experienced the introduction of the smallmouth bass approximately 18 years ago (LeBlanc, 2010; Madden et al., 2010), and experiences rare algal blooms that are likely due to natural weather events that bring an influx of nutrients into the lake (Taylor, 1994).

Collection and Processing

In both lakes, individual specimens of *S. elodes* were collected by hand or by using dip nets and then stored in Ziploc bags with lake water prior to returning to the lab. Approximately 100 snails were collected at each lake during each collection day. Larger snails, greater than 5mm in shell length, were targeted for collection rather than juveniles. This was due to the assumption that larger snails had been present in the environment for a longer time period, and thus had a greater risk of infection. Starting on September 7th, collections were made on two to three-week intervals until weather deteriorated in mid-November. Water temperatures were recorded at the collection site on every collection day using a YSI 85 Oxygen, Conductivity, Salinity and Temperature probe (Xylem Inc.).

Snails were brought back to the lab as soon as possible after field collections were completed. In the lab, snails were placed into small plastic cups (200mL) with approximately 30 mL of commercially purchased Big 8 Spring Water. Larger snails were isolated in a cup by themselves, while smaller snails were placed two per cup. Each cup had a Petri dish lid placed over the top to prevent the snails from escaping. The cups were then placed on trays and put under a timed full spectrum, fluorescent light that mimicked natural day length.

After approximately 24 hours, cups were examined under a dissecting microscope for cercariae. If snails demonstrated evidence of trematode infection, as determined by the presence of cercarial larval stages in the water or cysts on the sides of the cup, the cup was labelled according to the month, day, and year the snail was collected (i.e. MMDDYY - #). If there was shedding in a cup with two snails, they were separated to determine which snail, or if both were shedding, and checked 24 hours later. At 48 hours after collection, a second check was undertaken to confirm the presence of cercariae or cysts and to determine if there was new shedding in any other cups. Morphotype identifications (A-L), approximating family-level groupings, were made based on past categorizations of larvae using larval morphology and behaviour (Lawrence, 2011). The morphotype of the cercariae was also labelled on the cup. All snails positive for cercarial shedding were transferred to new cups with fresh Spring Water, while snails that did not release cercariae over the 48 hour time period were returned to the collection site. The infection prevalence, calculated as the number of snails infected divided by the total number collected at that site on the collection day, was recorded for each collection as both the total prevalence, and the prevalence by morphotype. A maximum of ten infected

snails were retained for each morphotype from each study lake during a collection period. These snails provided larvae for molecular work and morphological analysis.

To retain material for molecular analyses, live cercariae were pipetted from each cup into a small Petri dish half filled with 95% ethanol. From this dish, a minimum of fifty cercariae from a single snail were then selectively pipetted into a labelled Eppendorf tube (1.5 mL) that was half-filled (750 µL) with 95% ethanol. This was performed by looking into the cup using a dissecting microscope to ensure that only cercariae were transferred to the Eppendorf tubes, and any protists, snail feces, or other contaminants present in the cup and Petri dish were avoided.

When snails were shedding large numbers of cercariae, additional cercariae were preserved in 5% formalin for morphology and as reference specimens for future collections. Twenty or more cercariae were pipetted from the plastic cup into an empty Eppendorf tube (1.5 mL) until the tube was half-filled with Spring Water. The tube was then filled the rest of the way with 10% formalin. When possible, photos were also taken of live cercariae under a compound scope 100 – 400x magnification. To do this, live cercariae were mounted on glass slides under a cover slip and photographed using an ocular mounted DinoEye and DinoCapture software (AnMo Electronics). All infected snails from which cercarial samples were collected were then preserved in ethanol for reference and confirmation of identity through morphology and molecular analysis.

Molecular Work

DNA Extractions

Samples were spun in a centrifuge for 1 minute at 14 revolutions per minute. The ethanol was drawn off using a 1000 µL pipette. Remaining ethanol was then removed through air-drying or through the use of a speed vacuum. Trematode DNA was extracted using QIAGEN's DNeasy Blood & Tissue Kit following directions provided by the manufacturer. In the final elution step, DNA was eluted using 50 µL of AE buffer to make the DNA stock solution. From the stock solution, 15 µL was pipetted into labelled Eppendorf tubes (1.5 mL) to make up a working aliquot of each DNA sample to use for subsequent Polymerase Chain Reactions (PCR). Both the stocks and aliquots were stored at -20°C. Archived DNA samples, preserved in ethanol, from past collection periods were used in addition to the samples collected in 2017, following the same procedures.

PCR Amplification of Target COI Region

The Cytochrome Oxidase Subunit 1 (COI) gene is a fast-evolving mitochondrial gene that is used in DNA barcoding because it provides the potential for species-level discrimination. To differentiate between trematode species associated with *S. elodes*, I targeted a 770 base pair region (including primers) of the COI barcoding gene. Amplification of the traditional barcoding gene region is often challenging for trematode species (Van Steenkiste et al., 2015), so a section of the COI gene was amplified that was offset from the traditional barcoding region by 500 base pairs, with a 250 base pair overlap. Specific primers, PSCOIF (5' – GGTGTTGGTTGGACTTTTTA TCC-3') and PSCOIR (5'- AGAACATAATGAAAATGAGC-3'), were used to amplify the target region. Because of difficulties with the amplification of samples, a few Lake Ainslie samples were

amplified using the same forward primer, but with the reverse primer, DICE 14R (5'-TAATACGACTCACTATA-3') (Van Steenkiste et al., 2015). The targeted region of the COI gene was amplified using PCR and the target size of the amplification was 730 base pairs without primers for PSCOIF/R and 472 base pairs without primers for PSCOIF/DICE14R. The PCR reactions were 25 µL in volume, and consisted of of 2 µL of DNA solution, 12.5 µL GoTaq (consisting of buffer, 1.5 mM $MgCl_2$, dNTPs, and GoTaq DNA polymerase), 0.5 µL of both primers at 10 µM, and 9.5 µL of distilled water. PCRs were performed using BIO-RAD's MJ mini personal thermal cycler, using the following cycling conditions: 3 minutes at 94°C, 38 cycles of 1 minute at 94°C, 30 seconds at 48°C and 1 minute at 72°C, followed by 10 minutes at 72°C, and then a hold at 4°C until samples were retrieved.

After PCR was completed, gel electrophoresis was performed on a 1% agarose TBE gel with 4 µL of SYBR Safe Gel Stain. For each sample, 5 µL of the PCR products and 2 µL of 6x loading dye were mixed together and then loaded into a well. Each gel included at least one lane of 100 base pair ladder (5 µL) as a size standard for the PCR bands. Gels were imaged using AlphaImager (by Alpha Innotech), and strong samples with the correct band size were noted as positive PCR reactions.

PCR Clean-Up and Sequencing

Samples with strong PCR bands were purified using QIAGEN's QIAquick PCR purification kit, following directions provided by the manufacturer. DNA was eluted using 30 µL of buffer EB. The concentrations of the yields were measured in ng/µL using a Nanodrop 8000 spectrophotometer (Thermo Scientific Inc.). Sequencing reactions were then set up according to specifications from the Centre for Applied Genomics at the Sick Kids Hospital in Toronto and

then sent there for sequencing. Separate reactions were set up for the forward and reverse primers. The samples contained either a forward or reverse primer (1 µL of 5µM), between 30-40 ng of DNA, and distilled water so that the volume of the DNA combined with the volume of distilled water was 7 µL. Final sequences were based on forward and reverse sequences of good quality, i.e. sequences with few or no ambiguities, that were edited and assembled into a consensus sequence using Geneious v. II (Biomatters). The primers were then removed from the consensus sequences. Additionally, DNA sequences of trematodes obtained by past students were included if associated with *S. elodes*.

Analysis of Species Richness

Once all trematode sequences had been compiled for *S. elodes*, they were aligned in Geneious using default Geneious alignment parameters and examined visually for any insertions or deletions that would cause a misalignment. An outgroup sequence was added to the dataset using sequence from a trematode morphotype not present in *S. elodes* and known to be distantly related to the ingroup sequences based on analyses of a larger dataset. The chosen outgroup was an F morphotype associated with the snail genus *Helisoma*. I then explored the relationships amongst my trematode sequences from *S. elodes* using the Neighbour-Joining distance based method in Geneious following the Jukes Cantor model. This dataset incorporated both new samples and archived samples. The resulting distance-based phylogeny was used to identify genetically discrete lineages – those lineages clearly separated by substantial genetic distances that likely represented different species groupings. To examine the level of sequence divergence across these lineages, I chose one representative sequence from each of the 17 discrete lineages (=assumed species) and constructed a pairwise distance

matrix using uncorrected p-distances in Geneious. Given that the smallest level of sequence divergence across lineages was 4.5%, I selected a percent difference of 3% as the level of divergence indicative of species-level differences. This value was used as a cut-off to decide between conspecific and congeneric matches in BLAST searches below.

To determine if newly acquired sequences corresponded to known parasites, I undertook Basic Local Alignment Search Tool (BLAST) searches on NCBI (National Centre for Biotechnology Information; https://blast.ncbi.nlm.nih.gov/Blast.cgi). One representative sequence from each of the 17 distinct lineages was chosen to perform a BLAST search in GenBank to determine the closest match and species identity, where possible. The percent similarity value was used for determining the closest match to the query sequence on GenBank instead of the *E*-score because some fragments only overlapped with GenBank sequences by 300-400 base pairs, and the percent similarity values also gave more reasonable matches to the query sequences. Percent matches ≥97% to taxa on GenBank were assumed to be species-level matches.

Following tentative identifications of trematode species, I searched the literature for studies that provided information on the life cycles of these species. This involved recording the second intermediate hosts and definitive hosts of the species, where known. This information was important for providing the links between the presence of trematode species and other organisms acting as hosts in these local environments. To compare the species richness of trematodes in Blacketts Lake and Lake Ainslie to other comparable aquatic environments, I also undertook a search for similar studies in North America. This included doing a search for North

American studies that had examined *S. elodes* or any of its conspecifics (i.e. *Lymnaea emarginata*) extensively in relation to its trematode assemblage.

Results:

Field Collection and Analysis

In Blacketts Lake, *S. elodes* specimens were found in the muddy sediment and on submerged rocks and boulders. Access to the rocks was limited during windy days when the water was rough. There was a strong seasonal trend in the decline of water temperature across collection periods. The highest water temperature recorded during collections was 20.8°C (September 6th) and the lowest was 8.8°C (November 18th) (Figure 3; Table 3). Over the six sampling periods, 550 snails were collected, of which 97 exhibited trematode infections for a prevalence of 17.6%. Trematode family-level groupings A, D, G, and L were identified based on cercarial morphology and behaviour. Morphotype C was not found during these collections but has been found previously in this lake (Lawrence, 2011). Morphotype G was found during every collection, but this was not the case for the other morphotypes (Figure 3). Morphotype A was only found during the October collections, morphotype D was found during one September collection and one October collection, and morphotype L was found in late October and mid-November (Figure 3).

In Lake Ainslie, *S. elodes* specimens were visible on the sediment and rocks when the water was calm and could be collected by hand. On windy days, however, snails had to be collected using dipnets (Rawlings, personal communication). Lake Ainslie also exhibited a strong seasonal decline of water temperature at both sites. The highest water temperature recorded during collections was 21.6°C (June 29th – Site B) and the lowest was 5.8°C (November 18th – Site A) (Figure 4a,b). At Lake Ainslie Site A, 451 snails were collected, of which 62 had trematode infections, resulting in a prevalence of 13.7%. At Lake Ainslie Site B, 147 snails were collected

with 32 exhibiting trematode infections, resulting in a prevalence of 21.8%. Family-level groupings A, C, and G were found at Lake Ainslie collection Site A and family-level groupings C, D, and G were found at Lake Ainslie collection Site B (Figure 4; Table 3). No representatives of family-level grouping L were found at Lake Ainslie over the study period, even though cercariae from this family (Schistosomatidae) were found by Farley (1967b). At Lake Ainslie Site A, morphotype G was found at every collection, morphotype A was found during the June collection, the early September collection, and the early October collection (Figure 4). Morphotype C was found during the June collection, the first October collection, and during the November collection (Figure 4). At Lake Ainslie collection Site B, morphotype G was found at during all three collections, morphotype C was found in October and morphotype D was found in September (Figure 4). Collections at Site B ceased after October 7th due to time constraints.

Infection prevalence, calculated as the number of snails infected divided by the total number collected at that site on the collection day, differed from collection to collection and also varied between collection sites (Figures 3;4). No seasonal trends were apparent. In Blacketts Lake: the highest prevalence of infection was on October 28th (23.6%), and the lowest prevalence was seen on October 7th (13.6%), the collection preceding the one that exhibited the highest prevalence of infection. In contrast, at Lake Ainslie collection Site A, the highest prevalence of infection was from the June 29th collection (21.9%), and the lowest was from the October 28th collection (7.7%). At Lake Ainslie collection Site B, the highest infection prevalence was seen on October 7th (22.6%) and the collection on June 29th yielded the lowest prevalence of infection (17.7%). Family-level grouping G remained the most prevalent morphotype across all sites and collections with a prevalence of 15.8% in Blacketts Lake, 11.1% at Lake Ainslie

collection Site A, and 20.4% at Lake Ainslie collection Site B. Of the 97 infected snails from Blacketts Lake, 89.7% were infected with morphotype G. At Lake Ainslie Site A, 80.6% of the 62 infected snails were infected with G cercariae, and 93.8% of the 32 infected snails from Lake Ainslie Site B exhibited morphotype G infections.

Identification of Cercariae

Five different cercarial morphotypes were identified based on collections from Blacketts Lake and Lake Ainslie, including morphotypes A, C, D, G, and L (Table 4). Cercariae belonging to morphotype A (Families: Diplostomidae & Strigeidae) exhibited a forked tail, and swam in the water column as opposed to aggregating in the bottom of the cup. These cercariae were sometimes difficult to collect with the pipette for preservation. Those cercariae belonging to morphotype C (Family: Notocotylidae), were very different from the others because they encysted on the snail's shell or on the sides of the cup, with their detached tails remaining at the bottom of the cup. Morphotype D (Family: Echinostomatidae) cercariae were relatively large compared to the other morphotypes and were found crawling on the bottom of the cup. These cercariae moved around by elongating the anterior end of their body and then contracted to pull the posterior end forward. As previously mentioned, the most commonly found morphotype was morphotype G (Family: Plagiorchiidae). These cercariae could be found on the bottom of the cup, aggregated on the sides of the cup, or in the water column. When they were found on the bottom of the cup, they moved much like the morphotype D cercariae. However, they could be differentiated because the Gs were much smaller. When they were present in the water column or on the sides of the cup, the cercariae would roll up and thrash around. These cercariae also had a stylet at their anterior end that was visible under high

magnification with a dissecting scope and when photographing the cercariae using a compound microscope. Finally, morphotype L (Family: Schistosomatidae) also had a forked tail, but it was shorter and appeared wrinkled. These cercariae also had visible eyespots. The one L infection that was present on the last collection day, November 18th, exhibited cercariae that were separating the heads from the tails and then remaining at the top of the water column.

Molecular Analysis

DNA was extracted from samples preserved from collections made in 2017, as well as a limited number of samples from previous years that had not been examined. DNA extractions focused on samples from the family-level grouping G because it was the most abundant across collections and sites. From the Blacketts Lake samples, DNA was extracted from 36 samples of preserved Gs, each from separate snails, of which 34 were amplified successfully using PCR. DNA was also extracted from 3 D samples, 1 A sample, and 2 L samples. From the Lake Ainslie samples, DNA was extracted from 16 Gs, of which 12 were amplified successfully using PCR. Some Lake Ainslie samples did not amplify using PSCOIF/R primers, so these were amplified using a PSCOIF/DICE14R primer pair. Samples with strong PCR bands were chosen for sequencing. From Blacketts Lake, 32 G samples were sequenced successfully, and 12 G samples from Lake Ainslie were sequenced successfully. Other morphotypes resulted in messy sequences, with lots of background noise or conflicting signal, for either the forward or reverse sequence, and sometimes both. Morphotype C was difficult to amplify, although useable sequence from one reaction was obtained. Morphotype A was also challenging, but for one sample both a forward and reverse sequence were obtained. No useable sequences were acquired for morphotype L samples from 2017 due to difficulties in obtaining cercariae and

during sequencing. Morphotype D did not result in any positive PCR bands with either 1 µL or 2 µL of DNA used in the PCR reactions. However, both morphotypes D and L have undergone successful sequencing in the past, so with further attempts, and different primer pairs, it could be possible to obtain sequences for these specimens.

A distance-based phylogeny of Blacketts Lake and Lake Ainslie sequences was generated using Geneious. This tree identified 17 discrete lineages, 4 of which were exclusively from Lake Ainslie, and the remaining 13 were from Blacketts Lake with one of the clades showing a Lake Ainslie sequence nested within Blacketts Lake sequences (Figure 5). This tree was based on 71 sequences, with the primer sequences edited out, using an F morphotype from a *Helisoma* sp. snail as the outgroup. All three species groupings from Lake Ainslie were from the family level grouping G. The 13 distinct lineages from Blacketts Lake consisted of new samples from the 2017 sampling period and archived samples from past collections (2011-2016) and were represented by family-level groupings G (2 lineages), A (6 lineages), C (1 lineages), D (2 lineages) and L (2 lineages) (Figure 5).

Within the phylogeny, Lineages 1 and 2, made up of D morphotypes, were collected in May and June during three separate years. Although based on a small dataset, this morphotype has been present in collections of *S. elodes* from May to October (Rawlings lab, unpublished data). Lineage 3 is based on a partial sequence for a C morphotype. These cercariae were not present during the 2017 collections in Blacketts Lake, and have been difficult to amplify (Rawlings, personal communication). However, morphotype C was present in Lake Ainslie, but has not yet been sampled for DNA. Lineages 4-12 were from morphotype A cercariae. This was the most species rich group of the cercariae collected in Blacketts Lake. These cercariae were

present in collections from May to October. Morphotype G represented lineages 13-17. Lineages 14, 15, and 16 were unique to Lake Ainslie, and lineage 17 had one Lake Ainslie sequence within it. Morphotype G cercariae could be found during collections from June to November. Even within well sampled lineages (i.e. lineage 17), collection dates spanned several months, indicating little seasonality of cercarial emergence.

The distinct lineages represented on the tree were each assumed to represent one species based on levels of genetic divergence. After performing a pairwise matrix for sequence divergence, using one sequence from each of the 17 distinct lineages, the smallest sequence divergence was found to be between two A lineages (species groups 11 and 12) at 4.5% (Table 5). Therefore, 3% sequence divergence was used as the cut off value for classifying a query sequence as conspecific with a GenBank sequence.

To assess the species identities of each genetic lineage, one sample from each species grouping was blasted in GenBank to find the closest match for that clade. Of 17 species groups, 10 matched within 3% of the percent similarity of a sequence on GenBank, suggesting they were conspecifics. These 10 groups included 1 G, 1 C, 2 D, and 6 A lineages (Table 6). The G lineage was identified as a member of the family Plagiorchiidae, the C lineage as the family Notocotylidae, both D lineages as members of the family Echinostomatidae. Two A lineages were identified as a members of the family Strigeidae and the remaining 4 A lineages matched with members of the family Diplostomidae (Table 6). These corresponded well with prior family-level morphotype groupings. Of the remaining 7 groups, even though high percent matches (≥ 97%) were not found, these were classified as members of the families

Schistosomatidae (2 lineages), Diplostomidae (1 lineage), and Plagiorchiidae (4 lineages) (Table 6).

Thirteen trematode species associated with *S. elodes* were found in Blacketts Lake. This level of diversity falls within that reported from other studies, with Blacketts Lake in the middle of this range. These other studies include two from Douglas Lake Michigan (Cort et al., 1937; Keas & Blankespoor, 1997), one from Lake Ainslie (Farley, 1967b), and a study from Alberta that examined six lakes (Gordy et al., 2016). In Douglas Lake in 1937, 16 trematode species were present (Cort et al., 1937). More than 50 years later, only 8 species remained (Keas & Blankespoor, 1997; Figure 6). Farley (1967b) found 10 species in Lake Ainslie (Figure 6). And Gordy and colleagues (2016) found a range of 8-15 species (Figure 6). Such studies incorporate morphological and, in some cases, molecular tools in species identifications, which can lead to varying estimates of species diversity, as can differences in sampling intensities. For instance, in Lake Ainslie, only 5 trematode lineages were found, along with 2 morphotypes from which DNA was not extracted, meaning that there are at least 7 species associated with *S. elodes*. Based on this, Lake Ainslie has a low species richness compared to the studies above, but sampling is ongoing for this lake. Also, obtaining sequences from challenging samples may also result in an increase of species diversity.

Discussion:

Lake Comparisons

The two study sites, Blacketts Lake and Lake Ainslie, are visibly different habitats in terms of general location and size. Blacketts Lake is deeper, on average, and much smaller than Lake Ainslie (Table 2). Blacketts Lake is also more developed with houses built along the shoreline and a relatively busy road along one edge. Lake Ainslie is a large, rural lake, with less human activity. In terms of similarities, these lakes are only approximately 110 kilometers apart and share the same Cape Breton climate. These lakes have similar shoreline development index values (Table 2), which reflects the potential for development of plant communities along the edge of the lake, and associated biological productivity. Additionally, *S. elodes* is present at both sites, suggesting that the parameters of both lakes fall within the range of conditions that are tolerable to this snail.

Over the late summer/fall sampling period, infection prevalence ranged from 13.7% (Lake Ainslie Site A) to 21.8% (Lake Ainslie Site B), with Blacketts Lake intermediate between the two Lake Ainslie sites at 17.6%. Not only did the prevalence vary between lakes, but it also varied across collection dates at each site. Despite variation in prevalence, there was an obvious overlap of parasite families infecting *S. elodes* at both lakes, if not identical species. Cercariae from morphotypes A, D, G, and L emerged from *S. elodes* in Blacketts Lake. All four of these have been found during previous sampling at this lake. Morphotype C was not present in any of the 2017 collections in Blacketts Lake but has been found to infect *S. elodes* in previous years (Rawlings lab, unpublished data). In Lake Ainslie, morphotypes A, C, D, and G were present during the collection period, but morphotype L was not (Table 6). Morphotype L

belongs to the family Schistosomatidae, of which Farley (1967a; 1967b) found a representative (*Trichobilharzia stagnicolae*) during collections in June and July in Lake Ainslie.

Both lakes have been subjected to the introduction of non-native fish species (smallmouth bass and chain pickerel) within the past 18 years (LeBlanc, 2010; Madden et al., 2010). The first occurrence of the smallmouth bass in Cape Breton occurred in Lake Ainslie in 2000, and it was then documented to have appeared in Blacketts Lake in 2009 along with the introduction of the chain pickerel (LeBlanc, 2010; MacLeod, 2012). Both of these fish species are known to have detrimental effects on native fish species (Madden et al., 2010). They are able to quickly grow in numbers and take over niches previously occupied by trout or salmon (Madden et al., 2010). The smallmouth bass is recognized as a top predator and preys upon salmonoids (Department of Fisheries and Oceans Canada, 2016). Chain pickerel have been known to force out native trout populations (Mitchell et al., 2013). The loss of these fish species from their native habitats could affect trematode species and their life cycles if these native fish acted as second intermediate hosts or definitive hosts in their life cycles (Huspeni et al., 2005).

It is possible that both the smallmouth bass and the chain pickerel could act as second intermediate hosts for the trematode species in Blacketts Lake and Lake Ainslie. The smallmouth bass is known to be host to 15 families of trematodes in North America (Gibson, 1996; Hoffman, 1999), including the families Diplostomidae, found in both Cape Breton lakes studied, and Strigeidae, found only in Blacketts Lake (Table 7). The chain pickerel is host to 9 trematode species in North America (Gibson, 1996; Hoffman, 1999) including the family Diplostomidae. Because some of the families parasitizing these introduced species are found in these two Cape Breton lakes, these fish species could fill the gaps created in the trematode life

cycles caused by the loss of native fish species. This was observed by Ondračková et al. (2009) in Europe. Of the 9 trematode species found to infect the introduced Bighead goby, only 4 were known to infect this fish in its native habitat, suggesting that the Bighead goby was incorporated into the life cycles of trematodes present in the new habitat (Ondračková et al., 2009).

Trematode species richness and assemblage data from Blacketts Lake before the introduction of smallmouth bass and chain pickerel are not available, but Lake Ainslie was sampled in 1967, 33 years before the introduction of the smallmouth bass. In Lake Ainslie during collections in 1967, 10 trematode species infected *Lymnaea emarginata* (Farley, 1967b). In 2017, approximately 18 years after the introduction of the smallmouth bass, at least 7 trematode species were associated with *S. elodes*. This includes 2 morphotypes (C, D) from which DNA was not extracted, but are assumed to represent a minimum of 2 different species. We are considering *L. emarginata* and *S. elodes* to be conspecifics as this is the only large pulmonate snail present in Lake Ainslie and *Stagnicola* is often used interchangeably with *Lymnaea*. While there is a correlation between the introduction of a new species to the lake and a decrease in trematode species, this does not mean causation. This was the first year that we collected *S. elodes* at Lake Ainslie, so there is no archival data similar to that compiled for Blacketts Lake. This lake was sampled at two different sites, A and B, both of which presented a different infection prevalence, while Farley (1967b) collected snails at six sites. Since Lake Ainslie is so large, it would not be unreasonable to expect to see differences in the species collected at each site reflecting the presence of different definitive hosts. Ideally, one should be able to sample in just one site as a representative of the whole lake. However, it would be

difficult to find one area of the study environment that accurately represented the entire habitat (Lafferty, 1997). Also, we only sampled this lake one time in the summer and then every two weeks in the fall of one year. To have a more comprehensive understanding of the lake, it would require returning to the same sites year after year. Perhaps further sampling in Lake Ainslie will reveal a greater species richness.

Comparison to Previous Studies

Thirteen trematode species in Blacketts Lake and 7 in Lake Ainslie were found to exploit *S. elodes*. In comparison to the species richness values found in other studies in North America, Blacketts Lake falls within the middle of this range, and Lake Ainslie has the second lowest value (Figure 6). The trematodes found in these two Cape Breton lakes came from the families Diplostomidae, Strigeidae, Notocotylidae, Echinostomatidae, Plagiorchiidae, and Schistosomatidae, all of which have been found in previous studies of *S. elodes* in North America (Cort et al., 1937; Farley, 1967b; Keas & Blankespoor, 1997; Gordy et al., 2016; Table 1).

In a perfect environment, how many trematodes would we expect to be associated with a freshwater snail intermediate host, such as *S. elodes*? Cort et al. (1937) seems to have studied a relatively pristine environment compared to the other studies. In 1937, Cort et al. examined *S. emarginata*, a conspecific of *S. elodes* (Correa et al., 2010), from four areas in Douglas Lake, Michigan. These four sites were all beach areas, and only one site had waterfront properties (Cort et al., 1937). As such, the level of species diversity found during this study could be the baseline expectation for a healthy environment. During collections at Douglas Lake (Cort et al., 1937) cercariae from 16 species of trematodes emerged from this snail (Cort et al., 1937). Of

these 16 species, 5 were from the family Strigeidae, 6 from the family Plagiorchiidae, 2 were from the family Schistosomatidae, and one each was from the families Echinostomatidae, Diplostomidae, and Notocotylidae (Cort et al., 1937). All 5 of these families were found across Blacketts Lake and Lake Ainslie.

If parasites are common in a given habitat, it might be reasonable to expect snail intermediate hosts to be infected by more than one species of trematode. Multiple infections of the same snail with different trematode species were not observed in the snails collected from Blacketts Lake or Lake Ainslie. However, Cort et al. (1937) found that 12.3% of their infected snails exhibited multiple infections. Multiple infections observed included double, triple and quadruple infections (Cort et al., 1937). The greatest number of multiple infections occurred in snails that were infected with trematodes from the families Strigeidae and Schistosomatidae (39% of the total multiple infections). Of the 529 multiple infections discovered, 96.6% were double infections and the remaining 3.4% were triple and quadruple infections (Cort et al., 1937). Only one quadruple infection was noted, with 2 trematodes from the family Strigeidae, 1 schistosome, and 1 diplostome (Cort et al., 1937). These multiple infections could be attributed to the fact that prevalence ranged from 27.1 – 95.9% at different sampling sites (Cort et al., 1937). With prevalence values that high, it would have been difficult to infect a snail that had not previously been infected.

The methods undertaken by Cort et al. (1937) for determining whether or not a snail was infected were very similar to ours. The snails were placed in half-pint milk bottles with water, after which the water was examined using a microscope to determine whether or not cercariae were present (Cort et al., 1937). Cort et al. (1937) discussed that often the multiple

infections were noted at this stage through morphological and behavioural differences, but were sometimes found during the autopsy of the snail. It is possible then, that multiple infections were occurring in the snails collected in Blacketts Lake and Lake Ainslie and were not identified because we did not dissect the snails. This was also the case for Keas and Blankespoor (1997) who did not find snails infected by more than one species of trematode. Farley (1967b) crushed the snails that were collected to determine infection status and did not find evidence of multiple infections. Lower prevalence values and degraded habitats, relative to Douglas Lake in the mid-1930s, are possible explanations for the lack of multiple infections observed in these more recent studies. The study done by Gordy et al. (2016) was the only other study mentioned that found evidence of multiple infections. A total of 8 snails were found to exhibit infections from two different trematode genera. Seven of 8 multiple infections included a member from the family Plagiorchiidae, along with another family.

Cort et al. (1937) laid the foundation for a long-term survey of the trematode community present in Douglas Lake, Michigan. Keas and Blankespoor (1997) returned to Douglas Lake Michigan 50 years after Cort et al. (1937) and only found 8 trematode species associated with *S. emarginata*. The suggested explanation for the large decline in trematode species richness in Douglas Lake was an increase in shoreline development, which deterred definitive host species (i.e. birds) from going near the water (Keas & Blankespoor, 1997). Furthermore, there was an increase in recreational activities in and around the water, such as boating, which could have increased the noise and pollution around the lake (Keas & Blankespoor, 1997). Of the 8 trematode species present, 3 belonged to the family Strigeidae, and one belonged to each of the following families: Schistosomatidae, Diplostomidae,

Echinostomatidae, Plagiorchiidae, and Notocotylidae (Keas & Blankespoor, 1997), all of which were found during the present study. Two trematode species present during this study, *Diplostomum flexicaudum* and *Cercaria emarginatae* both infect gulls as a definitive host (Keas & Blankespoor, 1997). At one collection site, Phragmites Flats, the prevalence of *D. flexicaudum* declined, while that of *C. emarginatae* increased. A change in the number of definitive host species likely was not the reason for the infection prevalence changes, because it would be expected that both trematodes would exhibit the same response. Keas and Blankespoor (1997) posited that this disparity was due to a decrease in the number of second intermediate host fish species of *D. flexicaudum*. Keas and Blankespoor (1997) do not propose a reason for the decrease in the second intermediate host fish species. However, it is mentioned that there was an increase of boating activities on the lake, so perhaps recreational fishing or pollution created conditions that were not tolerable to this species and caused the decline in numbers.

In Lake Ainslie, Farley (1967b) collected *L. emarginata* monthly from May to October of 1966. During these collections, cercariae from 10 trematode species emerged from this snail (Farley, 1967b). Farley (1967b) expressed disappointment that compared to the study by Cort et al. (1937), Lake Ainslie had relatively low species richness. In 2017, we only found 7 species of trematodes associated with *S. elodes* (=*L. emarginata*). There are several factors that could be playing a role in the differences seen between sampling years and lakes. One is that the sampling done in 1967 was largely in the summer, while the 2017 collections only had one sampling date in the summer. Farley (1967b) found that at four of six collection sites, the percent infection peaked in June and then decreased rapidly. If these patterns are still in effect today, the collections in 2017 could have missed species whose peak infectivity of *S. elodes* was

in June. Furthermore, the sizes of the lakes are vastly different, with Lake Ainslie being larger and shallower than Blacketts Lake. A larger, more rural lake would suggest a more suitable environment for definitive host species to spend time near the water, as this type of habitat would have a larger area for migratory birds to aggregate, and more shoreline habitat available for other vertebrate intermediate hosts. However, this does not seem to be reflected in the species richness values found from both lakes.

Gordy et al. (2016) studied 6 lakes in central Alberta in which *S. elodes*, along with other freshwater snails, were present. These lakes included Pigeon Lake, Wabamun Lake, Lac la Nonne, Buffalo Lake, Isle Lake and Gull Lake (Gordy et al., 2016). The number of species found to be exploiting *S. elodes* in these lakes ranged from 0-15 species (Figure 6), with 26 species in total found to infect *S. elodes*. These species spanned the six families mentioned earlier, along with 3 trematode families (Gorgoderidae, Haematoloechidae, and Telorchiidae). Pigeon Lake (9670 ha) had no Lymnaeid snails (*L. stagnalis* and *S. elodes*) that were infected with trematodes, however, only 49 were collected. The only snails that were infected from this site were Physids (*Physella gyrina*), which had a 3.49% infection prevalence with only 3 snails showing signs of infection. This lake is shallow with an average depth of 6.5 m (Gordy et al., 2016) and was sampled less frequently than the others, along with Lac la Nonne (Gordy et al., 2016). Wabamun Lake (8180 ha), Lac la Nonne (1180 ha), and Buffalo Lake (9350 ha) contained 8, 10, and 12 species, respectively, that infected *S. elodes* (Gordy et al., 2016). These three lakes fall within the middle of the range of species richness values. At the high end of the range were Isle Lake (2300 ha) with 14 species, and Gull Lake (8060 ha) with 15 species (Gordy et al., 2016). These differences could be due to differences in the sampling intensity across the lakes, which

is the case in Pigeon Lake and Lac la Nonne (Gordy et al., 2016), or to differences in the lake parameters. All of the lakes were classified as eutrophic except Isle Lake and Lac la Nonne, which were classified as hypereutrophic (Gordy et al., 2016). Learning more about the histories of these lakes could help us to understand more about the assemblages of trematode species, i.e. through host fauna in the area and differences in lake parameters. Furthermore, this study could serve as a model system to explore the use of trematodes as a monitor for habitat health. These lakes experience the same climate, and all have parameters that are tolerable for *S. elodes*. Additionally, there has been extensive, detailed sampling within these lakes, and a combination of morphological and molecular techniques have been used to identify the trematode species collected from these lakes. If the healthiest lake of the six, based on independent measures such as habitat integrity, and known human impacts, also demonstrated the highest species richness of trematodes, this would help advocate the use of trematodes as bioindicators.

When comparing the above studies to the present study, no trematode species were shared across all studies. For this study, many tentative species identifications could not yet be determined due to lack of close matches on GenBank, so this species list may become more complete in the future. Four species were common to three of five studies, including *Schistosomatium douthitti* (Family Schistosomatidae; (Cort et al., 1937; Keas & Blankespoor, 1997; Gordy et al., 2016), *Diplostomum flexicaudum* (Family Diplostomidae; Cort et al., 1937; Farley, 1967b; Keas & Blankespoor, 1997), *Plagiorchis muris* (Family Plagiorchiidae; Cort et al., 1937; Farley, 1967b; Keas & Blankespoor, 1997), and *Plagiorchis miracanthos* (Family Plagiorchiidae; Cort et al., 1937; Farley, 1967b; Keas & Blankespoor, 1997). Of these, 2 are from

the family Plagiorchiidae, which was the most common family sampled from both Blacketts Lake and Lake Ainslie in 2017. Family Plagiorchiidae was also the only family of the six associated with *S. elodes* that was common to all of the above studies (Table 7). It appears that the family Plagiorchiidae along with the families Schistosomatidae, Diplostomidae, Strigeidae, Echinostomatidae, and Notocotylidae are most commonly associated with *S. elodes* (Table 7). Since there are 25 trematode families (Cribb et al., 2003), it would be interesting to explore any reasons why these 6 are the most prevalent.

It is a common misconception that an ecosystem that is unhealthy will exhibit a larger diversity of parasite species (Marcogliese, 2005). Instead, parasites are an expected component of healthy ecosystems and habitat degradation can lead to a decrease in the species richness of trematode species (Huspeni & Lafferty, 2004; Lafferty & Kuris, 1999, 2005). A study performed in California, using the snail *Cerithidea californica*, aimed to demonstrate that trematodes were good candidates to assess habitat quality through the restoration of a degraded saltmarsh habitat (Huspeni & Lafferty, 2004). Before the restoration occurred, the mean trematode species richness was 4.5 species (Huspeni & Lafferty, 2004). The restoration began in August of 1997, and the goal was to restore this saltmarsh to what it had resembled in the early to mid-1900s. During the first year of the restoration, the mean species richness decreased (Huspeni & Lafferty, 2004). After year one, the mean species richness increased every year, except between years 4 and 5 when it appeared to remain the same (Huspeni & Lafferty, 2004). At 6 years after restoration, the mean species richness had surpassed the value taken from the control site to 9 species (Huspeni & Lafferty, 2004). The authors proposed that the reasons for the increase in trematode species richness were because birds had returned to the area and that more snails

had moved into this restored habitat (Huspeni & Lafferty, 2004). There is clearly a correlation between habitat restoration and trematode species richness in this study, as the species richness doubled within six years of the restoration. This study provides more evidence that trematodes are good candidates to monitor changes within their habitat.

Challenges

Using trematode diversity as a bioindicator is best used to monitor changes in one habitat over time (Sures, 2004). It is more difficult to use it as a means of comparing two lakes due to confounding differences in habitat, size, and lake chemistry, as well as sampling inconsistencies. There are still many challenges to overcome in order to validate the use of this system as an indicator of ecosystem health (Lafferty, 1997). For instance, it is difficult to define specific parameters that make an ecosystem healthy or unhealthy as this is not something that can be directly quantified (Marcogliese, 2005). Furthermore, there are many factors that can influence variations in trematode communities that would make it difficult to narrow the cause down to a single element (Lafferty, 1997). These factors could include a loss of host species, changes in lake chemistry (i.e. pH), pollutants, nutrient levels, sewage input or climate change, along with habitat loss and shoreline development (Keas & Blankespoor, 1997; Lafferty, 1997; Marcogliese, 2005). So, it is possible to say that trematodes can indicate changes to the environment, but not the specific cause of the changes (Keas & Blankespoor, 1997). However, the present study is an interesting first step in studying creating a benchmark of the trematode community in Blacketts Lake for use in studying changes across years. Using the snail-trematode relationship to assess habitat integrity in this way causes little disturbance to the environment, and can reveal the presence of other organisms that live in this community

through the elucidation of life cycles (Huspeni et al., 2005; Marcogliese, 2005). This is possible because life cycles can provide information on the presence of other organisms, i.e. the second intermediate hosts and the definitive hosts (Table 8).

The extent to which cercarial emergence is linked to other intermediate and definitive hosts in a local environment will be dependent to some extent in whether definitive hosts are migratory, or seasonal, as compared to non-migratory. Non-migratory birds, for instance, could be infected by trematodes in their environment as the definitive host, and then shed eggs in their feces, restarting the cycle (Lafferty & Kuris, 1999). This causes an increase in the prevalence of the infective trematodes, because they are remaining in the environment (Lafferty & Kuris, 2005). When birds are not present in the area due to such reasons as habitat degradation, there is an observed decrease in species richness (Lafferty & Kuris, 1999). Migratory birds, or hosts visiting lakes seasonally, on the other hand, can release trematode eggs into the water, causing the snail intermediate host to become infected. This can occur even if the second intermediate and definitive hosts are not present in the environment. This could result in the observation of a cercarial infection in the snail, but ultimately the life cycle may not be completed due to a lack of proper hosts. Therefore, when elucidating life cycles from cercarial infection alone, it is necessary to consider the means by which the trematode species was introduced to the environment.

Additionally, only 10 samples of the same morphotype were preserved from each collection date due to time limitations. It is possible, then, that trematode infections that were not preserved for later molecular work could have been representatives of rare species. Furthermore, since the snails were returned to the collection site, it is possible that snails were

collected more than once. If any of these snails were resistant to infection, this would alter the prevalence of infection from that lake site. Finally, it can be observed in Table 6 that a number of the samples from the present study have low percent query covers with their closest match on GenBank. This could be due to the fact that many researchers amplify the traditional barcoding area of the COI gene, and this study used an area of the traditional barcoding area that was offset form this region by 500 base pairs and only overlapped with 250 base pairs. Accordingly, the percent matches found on GenBank could have been inflated because there was a smaller area to match on the query sample.

Future Studies

Increasing the sampling in Lake Ainslie should help to better understand the trematode community present and provide a comparison of the community over 50 years since Farley (1967b) first sampled this lake at six different sites. Creating an archive of trematode samples for Lake Ainslie could prove useful for studying this lake across a long period time to monitor environmental changes. Furthermore, molecular techniques have advanced since the late 1960s, allowing for more accurate species identifications. Also, comparing the assemblage and life cycles of parasites present in the lakes sampled in Cape Breton, Michigan, and Alberta could help us to understand why certain trematode species are present in Blacketts Lake, while others are not. This could be studied through the presence or absence of intermediate and definitive host species in the habitat, along with visiting migratory species.

Additionally, a threatened species of freshwater bivalve, *Lampsilis cariosa*, has only a few known populations in Canada, one of which is in Blacketts Lake (Davis et al., 2004). Bivalves are possible first or second intermediate host for trematodes (Cribb et al., 2003). To further

understand the trematode community in Blacketts Lake, it would be interesting to determine, for conservation efforts, whether or not these bivalves are being exploited by trematode species, and if so, which species. It is also a possibility that trematode larval stages are acting as a food source for these bivalves and other organisms in the lake, resulting in fewer infective stages present in the water column (Preston et al., 2013). Furthermore, the maintenance of habitat integrity is important for species that are threatened, so using trematodes as an indicator of habitat health could inform us about possible threats to this mussel population.

At this stage, there appears to be a reasonable diversity of species in Blacketts Lake related to other lakes sampled for *S. elodes*. However, this is only stage one of a longer study. For the purposes of this study, the distance-based phylogeny suited our needs, but perhaps another phylogeny model could indicate more differences between the distinct lineages found during this study.

In the future, this study could benefit from direct communication with the Department of Fisheries and Oceans and the Department of Natural Resources. In addition to collections, these resources could provide us with more information on these lake environments and the organisms present within them, as well as the historical records of the lakes including species introductions, nutrient levels, and the addition of copper sulfate for algal control.

Tables:

Table 1. Trematode parasites that have been documented in the snail *Stagnicola elodes* in North America, according to the NHM Host-Parasite Database[1], Farley, 1967[2], Gordy et al., 2016 Gordy et al., 2017, and a Google Scholar search.

Parasites found in *S. elodes* in North America	Morpho-type[3]	References
Family: Cyclocoelidae		
Cyclocoelum mutabile	?	McKindsey & McLaughlin, 1995a; McKindsey & McLaughlin, 1995b
Family: Diplostomidae		
Diplostomum flexicaudum	A	Cort et al; 1937; Pratt & Barton, 1941; Farley, 1967b; Keas & Blankespoor, 1997
Diplostomum baeri	A	Gordy et al., 2016
Diplostomum huronense	A	Gordy et al., 2016
Diplostomum indistinctum	A	Gordy et al., 2016
Diplostomum sp.	A	Poulin et al., 1999; Sangster et al., 2004; Gordy et al., 2016
Neodiplostomum americanum	A	Gordy et al., unpublished data
Family: Plagiorchiidae		
Plagiorchis elegans	G	Lowenberger & Rau, 1993; Lowenberger at al., 1994; Lowenberger & Rau, 1994; Zakikhani & Rau, 1998; Zakikhani & Rau, 1999; Poulin, 2006
Plagiorchis muris	G	Cort et al., 1937; McMullen, 1937; Pratt & Barton, 1941; Farley, 1967b; Keas & Blankespoor, 1997
Plagiorchis miracanthos	G	Cort et al., 1937; McMullen, 1937; Farley, 1967b; Keas & Blankespoor, 1997
Plagiorchis proximus	G	Cort et al., 1937; McMullen, 1937; Farley, 1967b; Keas & Blankespoor, 1997
Plagiorchis noblei	G	Webber et al., 1986; Webber et al., 1989
Plagiorchis sp.	G	Gordy et al., 2016
Eustomos chelydrae	G	Cort et al., 1937; Keas & Blankespoor, 1997
Family: Echinostomatidae		
Echinostoma revolutum	D	Cort et al., 1937; Keas & Blankespoor, 1997; Sorenson & Minchella, 1998; Sandland & Minchella, 2003
Echinostoma caprioni	D	Gordy et al., 2016
Echinostomatide gen. sp.	D	Gordy et al., unpublished data
Echinostoma trivolvis	D	Gordy et al., 2016
Family: Haematolechidae *Haematoloechus* sp.	J	Gordy et al., 2016

Continued

Table 1. Continued

Parasites found in *S. elodes* North America	Morpho-type[3]	References
Family: Strigeidae		
Strigeidae gen. sp.	A	Gordy et al*.,* 2016
Apatemon sp.	A	Gordy et al., 2016
Ichthyocotylurus sp.	A	Gordy et al*.,* 2016
Cotylurus gallinulae	A	Gordy et al*.,* 2016
Cotylurus communis	A	Cort et al., 1937; Farley, 1967b
Cotylurus cornutus	A	Barragán-Sáenz et al., 2009
Cotylurus flabelliformis	A	Cort et al., 1937; Campbell, 1997; Keas & Blankespoor, 1997
Australapatemon burti	A	Gordy et al*.,* 2016; Gordy *et al.,* 2017
Australapatemon mclaughlini	A	Gordy et al*.,* 2017
Australapatemon sp.		
Cercaria yogena	A	Gordy et al*.,* 2017
Cercaria laruei	A	Cort et al., 1937; Pratt & Barton, 1941; Keas & Blankespoor, 1997
Cercaria emarginatae	A	Cort et al., 1937; Pratt & Barton, 1941; Keas & Blankespoor, 1997
Cercaria dohema	A	Keas & Blankespoor, 1997
	A	Cort et al., 1937; Keas & Blankespoor, 1997
Family: Schistosomatidae		
Schistosomatidae gen.	L	Gordy et al*.,* 2016
Schistosomatium douthitti	L	Cort et al., 1937; Keas & Blankespoor, 1997; Schwanz, 2006; Brant & Loker, 2009b; Gordy et al*.,* 2016
Trichobilharzia sp.	E	Farley, 1967a, b
Trichobilharzia stagnicolae	E	Loker, 1979; Keas & Blankespoor, 1997; Leighton et al., 2000; Brant & Loker, 2009a; Gordy et al*.,* 2016
Trichobilharzia ocellata	E	Loken et al., 1995; Graham, 2003
Cercaria stagnicolae	E	Cort et al., 1937
Family: Notocotylidae		
Notocotylus urbanensis	C	Cort et al., 1937; Keas & Blankespoor, 1997
Notocotylus sp.	C	Gordy et al., 2016
Family: Gorgoderidae		
Gorgoderina sp.	?	Gordy et al*.,* 2016

[1] From a search of the Host-Parasite Database using the terms: "*S. elodes*", "*S. catascopium*","*S. emarginata*", "*S. elrodi*" and "*S. bonnevillensis*" based on molecular data from Remigio (2002) and Correa et al. (2010). The data compiled in the database contains records with publication dates up until 2003.

[2] Farley studied *Lymnaea emarginata*, which we believe to be the same species as *S. elodes*.

[3] As defined in Lawrence (2011), Novorolsky (2017)

Table 2. Physical parameters of the two Cape Breton study sites, taken from surveys done by the Department of Fisheries and Oceans[1] over several sampling periods and from Alexander et al. (1986)[2].

Lake Parameters	Blacketts Lake	Lake Ainslie
Surface area	172 ha	5736 ha
Average Depth	9.5 m	5.7 m
Maximum Depth	30 m	18 m
pH	5.9 – 7.5	6.6 – 8.0
Lake Volume	16 400 000 m^3	329 486 637 m^3
Shoreline Development[3]	2.4	2.1
Ortho Phosphorus	<0.01 mg/L	0.001 mg/L
Total Phosphorus	No data	0.004 mg/L
Total Nitrogen	No data	0.12 mg/L
$CaCO_3$	35.7 mg/L	28.40 mg/L

[1] Sampling by the Department of Fisheries and Oceans (https://data.novascotia.ca/Environment-and-Energy/Nova-Scotia-Lake-Chemistry-Data/vn55-yjyi) occurred at 3 separate times in Blacketts Lake: July 1975, May 1989, and September 2000. Lake Ainslie was sampled on seven separate occasions: August 1977, August 1978, three times in the summer of 1982, May 1989, and August 2001.

[2] Sampling by Alexander et al. (1986) occurred in 781 Nova Scotian lakes, including Blacketts Lake and Lake Ainslie, from 1964-1981.

[3] Calculated by dividing the length of the shoreline (in meters) by the surface area (m^2). A value greater than 1 reflects the potential for development of plant communities along the edge of the lake, and associated biological productivity.

Table 3. A summary of the morphotypes present at Blacketts Lake and Lake Ainslie, from June – November 2017 based on the number of snails collected during each sampling period.

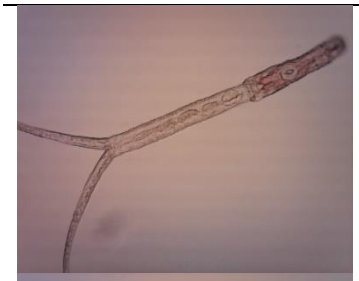
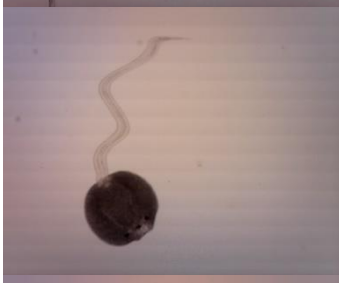
Site	G morphotype	D morphotype	A morphotype	C morphotype	L morphotype	Total # snails collected
Date: June 29th, 2017						
Lake Ainslie – Site A	**4**	0	**2**	**3**	0	41
Lake Ainslie – Site B	**3**	0	0	0	0	17
Date: September 6th, 2017						
Blacketts Lake	**15**	**2**	0	0	0	102
Date: September 7th, 2017						
Lake Ainslie – Site A	**5**	0	**1**	0	0	70
Lake Ainslie – Site B	**4**	**1**	0	0	0	24
Date: September 23rd, 2017						
Blacketts Lake	**15**	0	0	0	0	109
Date: October 7th, 2017						
Lake Ainslie – Site A	**22**	0	**1**	**1**	0	113
Lake Ainslie – Site B	**23**	0	0	**1**	0	106
Blacketts Lake	**12**	**1**	**2**	0	0	110
Date: October 28th, 2017						
Lake Ainslie – Site A	**8**	0	0	0	0	104
Blacketts Lake	**26**	0	**2**	0	**2**	127

Continued

Table 3. Continued

Site	G morphotype	D morphotype	A morphotype	C morphotype	L morphotype	Total # snails collected
Date: November 18th, 2017						
Lake Ainslie – Site A	**11**	0	0	**4**	0	123
Blacketts Lake	**19**	0	0	0	**1**	102
Total	**167**	**4**	**8**	**9**	**3**	**1148**

Table 4. Light microscope images of cercariae collected in Blacketts Lake and Lake Ainslie, 2017, along with their morphotype and family. All cercariae are less than 1 mm in length.

Photo	Morphotype	Family	Blacketts Lake	Lake Ainslie
	A	Diplostomidae & Strigeidae	X	X
	C	Notocotylidae	★	X
	D	Echinostomatidae	X	X
	G	Plagiorchiidae	X	X
	L	Schistosomatidae	X	★

★ - indicates that this morphotype was not present during collections in 2017, but is present in historical data from these sites. Morphotype C was found during collections performed by the Rawlings lab in Blacketts Lake, and morphotype L was found by Farley (1967a; b) in Lake Ainslie.

Table 5. A pairwise distance matrix of uncorrected p values, of percent sequence difference, using one representative sequence from each of the 17 lineages shown in Figure 5. The highlighted cell shows the two lineages that were most closely-related with only a 4.5% sequence divergence between them (lineages 11A/12A).

Clade	1D	2D	3C	4L	5L	6A	7A	8A	9A	10A	11A	12A	13G	14G	15G	16G
1D																
2D	18.6															
3C	34.8	27.8														
4L	40.5	29.8	30.5													
5L	39.1	29.0	31.0	7.4												
6A	33.6	26.4	27.8	31.0	30.1											
7A	31.9	26.8	30.2	29.2	30.3	12.7										
8A	32.8	27.1	30.7	28.7	29.4	12.2	7.8									
9A	35.3	27.1	29.0	30.2	30.1	12.7	13.3	12.7								
10A	30.3	28.0	29.5	30.0	29.7	16.8	16.7	16.2	15.5							
11A	31.1	26.5	28.5	31.8	32.2	17.1	16.2	16.0	17.1	12.6						
12A	30.7	25.9	27.8	30.9	31.8	15.9	15.2	14.8	16.0	11.7	4.5					
13G	28.6	22.7	22.4	27.6	28.4	26.0	25.3	27.3	26.6	26.7	26.5	25.6				
14G	28.2	25.4	24.1	28.7	29.4	26.2	26.8	26.1	26.3	26.3	27.1	26.1	15.9			
15G	31.1	21.9	22.1	28.1	28.2	25.6	25.9	24.9	25.8	24.9	25.1	23.3	14.1	16.3		
16G	26.5	22.2	20.9	31.0	29.6	25.0	25.0	23.6	24.1	24.8	23.2	22.5	13.8	18.1	12.6	
17G	29.0	23.6	24.1	28.9	29.2	26.6	27.0	26.2	26.4	25.7	26.5	26.6	14.8	16.0	12.9	12.4

Table 6. Identification of genetically distinct lineages shown in Figure 5. One sample from each of the 17 distinct lineages was queried in GenBank to find the closest match available, and to assign species identification, where possible. A GenBank percent match ≥97% was considered to be a conspecific.

Species Group	Morphotype & Snail Genus	Lake	DNA Code	Tentative ID	Family	GB % Match	Query Cover (%)	Query Length (#of bps)	Match Length with GB Sequence	Accession Number
1	D-Stagnicola	BL	070413-1	*Hypoderaeum sp.*	Echinostomatidae	99*	100	582	582	KT831350
2	D-Stagnicola	BL	070915-5	*Echinostoma trivolvis*	Echinostomatidae	99*	53	1074	579	KM538091
3	C-Stagnicola	BL	061413-5	*Notocotylus* sp.	Notocotylidae	99*	52	406	215	KM538104
4	L-Stagnicola	BL	072115-7	*Schistosomatium douthitti*	Schistosomatidae	92	99	717	716	AY157193
5	L-Stagnicola	BL	082513-7	*Schistosomatium douthitti*	Schistosomatidae	96	98	460	460	AY157193
6	A-Stagnicola	LkA	121717-7	*Diplostomum* sp.	Diplostomidae	100*	37	730	277	KR271383
7	A-Stagnicola	BL	112016-6	*Diplostomum baeri*	Diplostomidae	100*	32	730	240	GQ292501
8	A-Stagnicola	BL	061413-2	*Diplostomum* sp.	Diplostomidae	100*	28	730	211	KR271394
9	A-Stagnicola	BL	112016-5	*Diplostomum* sp.	Diplostomidae	100*	37	730	277	KR271234
10	A-Stagnicola	BL	121717-5	*Icthycotylurus* aff.	Strigeidae	99*	33	708	235	KT831371
11	A-Stagnicola	BL	061713-3	*Neodiplostomum americanum*	Diplostomidae	85	78	730	575	KY851304
12	A-Stagnicola	BL	073015-6	*Cotylurus* aff. *gallinulae*	Strigeidae	99*	30	730	220	KT831347
13	G-Stagnicola	BL	081212-2	*Plagiorchis koreanus*	Plagiorchiidae	90	55	730	404	KJ533418
14	G-Stagnicola	LkA	121717-12	*Plagiorchis elegans*	Plagiorchiidae	85	52	706	368	KJ533416
15	G-Stagnicola	LkA	012618-3	*Plagiorchis* sp.	Plagiorchiidae	90	55	730	404	KY513259
16	G-Stagnicola	LkA	012518-6	*Plagiorchis* sp.	Plagiorchiidae	100*	48	435	210	KT831380
17	G-Stagnicola	BL & LkA	121417-5	*Plagiorchis elegans*	Plagiorchiidae	90	55	730	404	KJ533413

*Considered conspecific

Table 7. A comparison of the six trematode families in Blacketts Lake to trematode families associated with *Stagnicola elodes* in other lakes including Lake Ainslie (Cape Breton, Nova Scotia), Douglas Lake (Michigan) and across six lakes in central Alberta.

Trematode Family	**Blacketts Lake 2011-2017**	**Douglas Lake 1937 (Michigan)**	**Douglas Lake 1997 (Michigan)**	**Central Alberta (6 lakes) 2016**	**Lake Ainslie (1967)**	**Lake Ainslie (2017)**
Strigeidae	X	X	X	X	X	
Diplostomidae	X	X	X	X	X	X
Notocotylidae	X	X	X	X		X
Echinostomatidae	X	X	X	X		X
Plagiorchiidae	X	X	X	X	X	X
Schistosomatidae	X	X	X	X	X	

Table 8. Second intermediate and definitive hosts (where known) for trematode species assigned tentative identifications based on GenBank matches ≥ 97%. The grey spaces indicate that the 2nd intermediate hosts species could not be found in the literature.

Tentative Species ID	2nd Intermediate Host	Definitive Host	References
Hypoderaeum sp.	2nd species of freshwater snails: *Physella acuta* or *Gyraulus chinensis*	Aquatic bird species	Muñoz-Antoli et al., 2000
Echinostoma trivolvis	Wood frog larvae (*Rana* sp.)	Aquatic bird species, Muskrat	Belden, 2006; Koprivnikar et al., 2006; Griggs & Belden, 2008; Kanev, 1994
Notocotylus sp.		Avian species, Voles, Muskrat	Herber, 1942; Beckett and Gallicchio, 1967; Haukisalmi, 1986
Diplostomum baeri	Stickleback, eyes of Yellow Perch	Wild mallard, Herring gull; Ring-billed gull	Hoffman & Hundley, 1957; Ching, 1985; Shostack et al., 1987; Locke et al., 2010
Diplostomum sp.	Eyes of fish species	Fish-eating birds, particularly gulls	Pietrock et al., 2002; Voutilainen et al., 2009
Icthyocotylurus aff.	Round goby fish		Pronin et al., 1997
Cotylurus aff. *gallinulae*		Aquatic bird species	Graham et al., 1937
Plagiorchis sp.	Insect larvae (of chironomids, dragonflies, mayflies, and mosquitoes)	Muskrats, red-winged blackbirds, meadow voles, bats	Macy, 1931; McMullen, 1937; Blankespoor, 1974; Webber et al., 1987; Zakikhani & Rau, 1999

Figures:

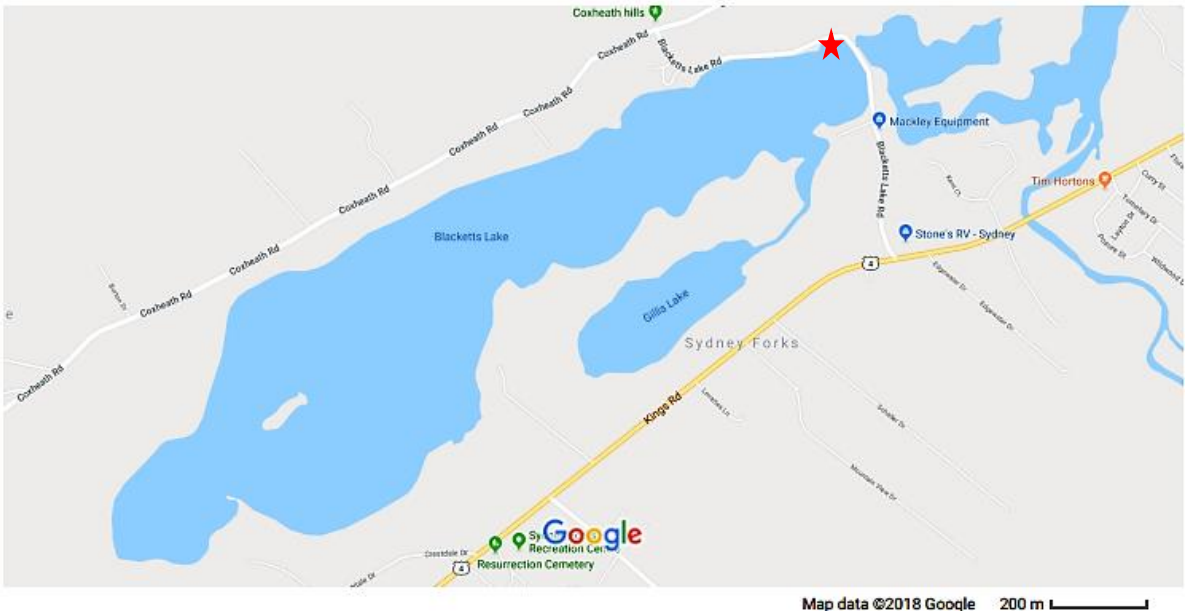


Figure 1a. Map of Blacketts Lake (Google Maps) in Sydney Forks, Nova Scotia. The red star indicates the site where *S. elodes* specimens were collected. The total lake area is 172 hectares.

Figure 1b. An image of Blacketts Lake, Nova Scotia taken on November 13th, 2017 from the site where *S. elodes* specimens were collected.

Figure 2a. Map of Lake Ainslie (Google Maps) in Inverness County, Nova Scotia. The red circles indicate the location of collection sites A and B where *S. elodes* specimens were collected. The total lake area is 5735.8 hectares.

Figure 2b. An image of Lake Ainslie, Nova Scotia, taken on October 7th, 2017 from collection site A.

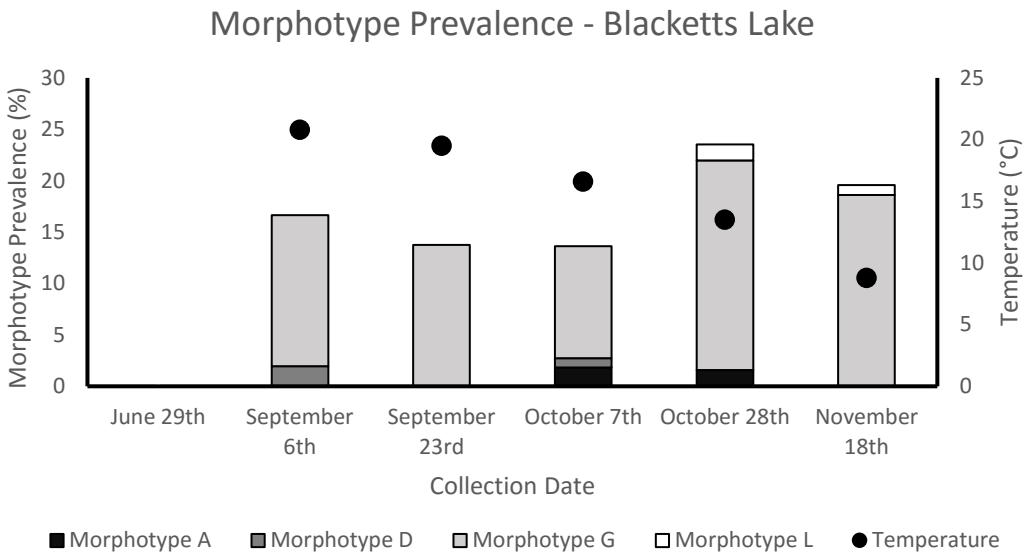


Figure 3. Prevalence of the cercarial morphotypes associated with *S. elodes* found in Blacketts Lake across 5 sampling periods, and in association with water temperature.

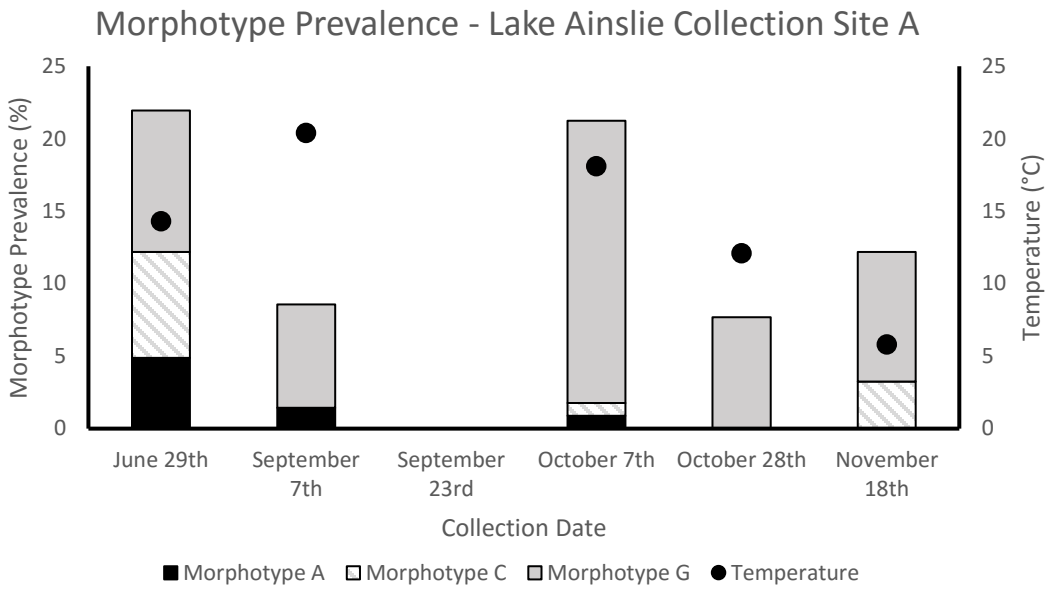


Figure 4a. Prevalence of the cercarial morphotypes found at Lake Ainslie collection Site A during 5 collections from June – November 2017 in association with water temperature.

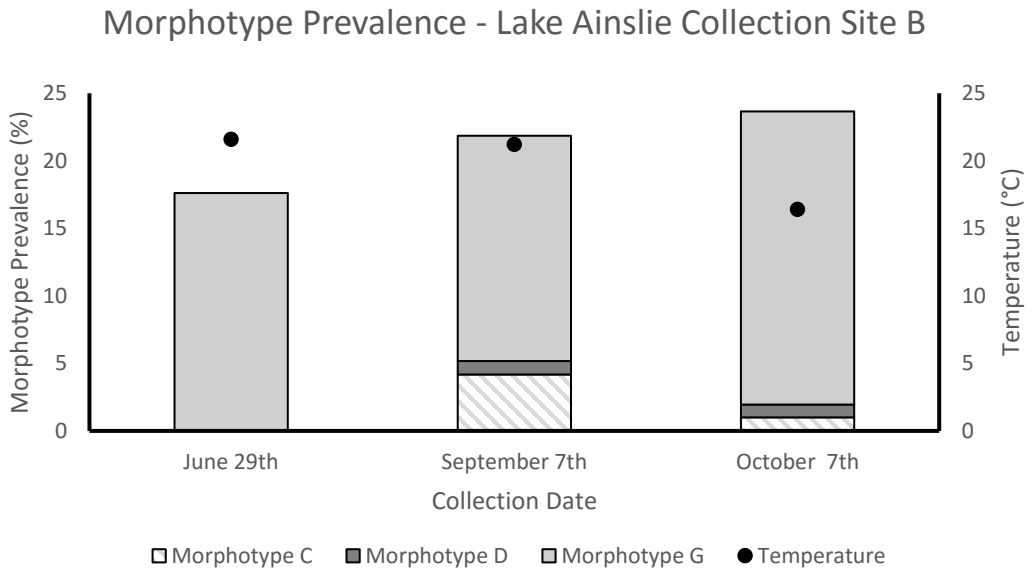


Figure 4b. Prevalence of the cercarial morphotypes found at Lake Ainslie collection Site B during 3 collections from June – October 2017 in association with water temperature.

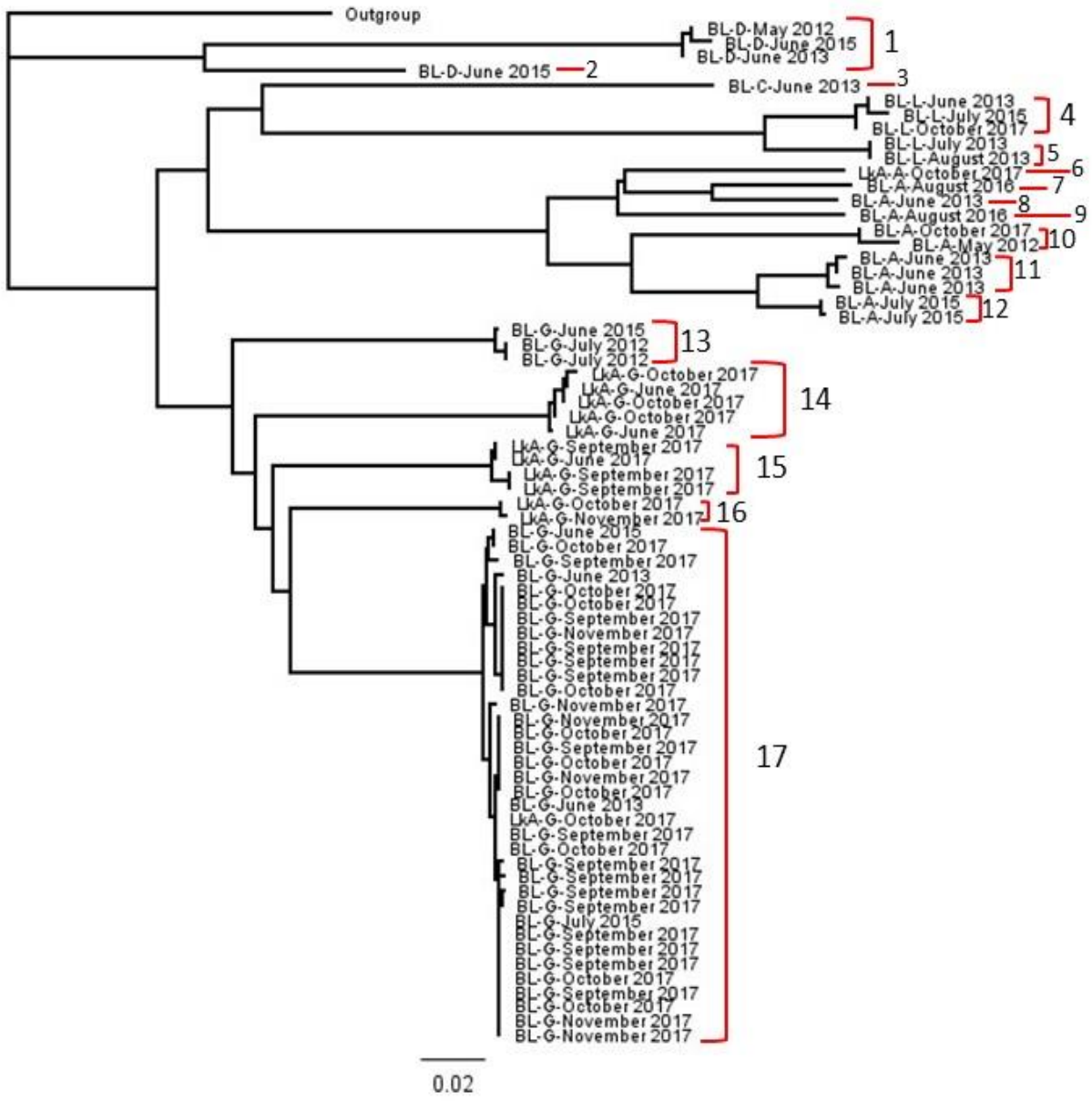


Figure 5. A distance-based phylogeny of trematodes associated with *Stagnicola elodes* in Blacketts Lake and Lake Ainslie. Labels refer to individual sequences and include lake (BL=Blacketts Lake, LA=Lake Ainslie), morphotype (A-L), and month and year that the samples were collected in the field. Each numbered lineage is assumed to represent a distinct species based on genetic distance. Of the 17 distinct lineages, 4 are exclusively from Lake Ainslie. The remaining 13 include 12 that are exclusive to Blacketts Lake, and one lineage that includes one Lake Ainslie sample among Blacketts Lake samples (lineage 17).

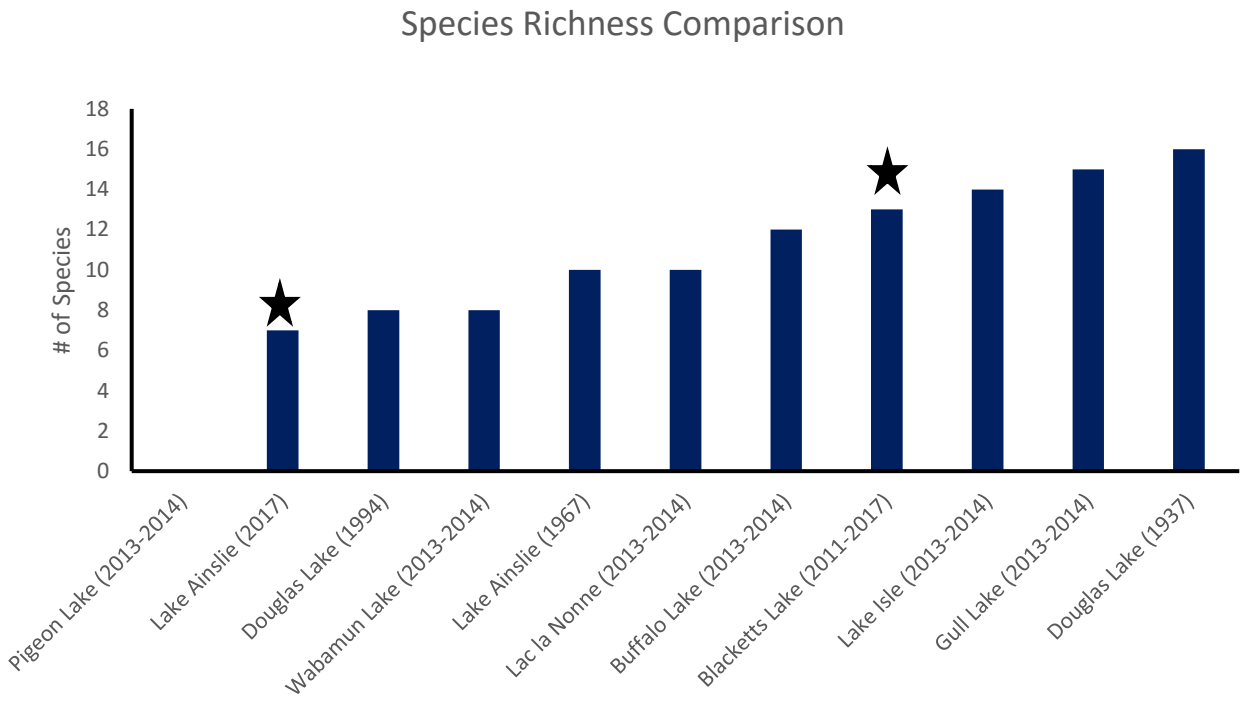


Figure 6. A comparison of the diversity of trematode species known to exploit *S. elodes* in 9 different lakes in North America. All lakes with data from 2013-2014 are from Gordy et al. (2016). The lowest species richness value was from Pigeon Lake, Alberta, where no trematodes infected *S. elodes* even though these snails were present. The highest was from Douglas Lake, Michigan (Cort et al. 1937). The star symbols indicate sampling that was undertaken by the Rawlings lab.

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