Experimental Evidence of Differences in Life History Characteristics and Interactions Between Cryptic Species of *Diplostomum* (Digenea)

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ABSTRACT

Experimental Evidence of Differences in Life History Characteristics and Interactions Between Cryptic Species of *Diplostomum* (Digenea)

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Concordia University, 2018

Recent DNA studies have uncovered diversity in sympatric species of *Diplostomum* (Digenea), a cosmopolitan parasite with a three host life-cycle infecting a piscivorous bird, snail and fish at different stages during its life cycle. The goal of this dissertation was to elucidate the basic life history characteristics and interactions of molecularly delineated sympatric cryptic species within fish and bird hosts. Using nomenclature from previous molecular studies, the species were designated as *Diplostomum* sp. 1, *Diplostomum* sp. 4 and *Diplostomum baeri*. The species of *Diplostomum* studied in each chapter depended on the experimental maintenance of the life cycle in the laboratory over consecutive years. Chapter 1 tested fish host specificity and both *Diplostomum* sp. 1 and *Diplostomum* sp. 4 successfully established in phylogenetically diverse fishes classifying them as generalists. Chapter 2 tested temporal heterogeneity on establishment success in rainbow trout (*Oncorhynchus mykiss*). The data revealed no effect for *Diplostomum* sp. 4, but a decline in establishment success for *Diplostomum* sp. 1 in challenge infections. This is indicative of the importance of priority establishment for this species. Chapter 3 examined intestinal spatial distribution and range and fecundity in the bird (*Larus delawarensis*) host in single and mixed infections. There was a statistically significant shift in intestinal distribution in mixed-species infections for only for *D. baeri* when *Diplostomum* sp. 1 was also present. Further, *Diplostomum* sp. 4 experienced a decline in fecundity in mixed infections with in the presence of *Diplostomum* sp. 1 whereas fecundity of *Diplostomum* sp. 1 increased in mixed infections with *D. baeri*, indicative of interspecific interaction. Chapter 4 then examined life history characteristics and found interspecific differences in egg size and number, where *Diplostomum* sp. 4 had larger and more numerous eggs than *Diplostomum* sp. 1. However, there was no intraspecific relationship between egg size and number, nor egg and adult fluke size. Lastly, Chapter 5 revealed faster egg development and greater hatching success for *Diplostomum* sp.

4 compared to *Diplostomum* sp. 1. Overall, this dissertation provides novel experimental evidence of host specifity, interactions in both the fish and bird hosts and life-history differences, helpful in species identification, between cryptic species.

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"But as for you, be strong and do not give up, for your work will be rewarded."

2 Chronicles 15:7

DEDICATION

To Granny & Grampy,

For your endless, unconditional love and support from Heaven and Earth.

To Aunt DD,

For being a strong, intelligent, independent woman, who loves fiercely, gives boundlessly, forever loyal and dependable, a woman who always speaks her mind and is always up for a ride in the woods. You have always been and always will be my role model. You have helped shaped me into the woman I am today and for this and so much more I love you forever and always.

CONTRIBUTION OF AUTHORS

This thesis has been prepared as manuscripts for submission for publication. Dr. Daniel McLaughlin and Dr. David Marcogliese contributed to the study design and manuscript preparation. Dr. Daniel McLaughlin contributed with the snail collections, gull exposures and necropsies. For Chapter 3, Dr. Marilynn Scott contributed with statistical analyses, data interpretation and manuscript preparation.

The following manuscript based on work reported in Chapter 4 has been published:

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The following manuscript based on work reported in Chapter 2 is in preparation for submission to the Parasitology Research as:

Lapierre, A.R., J.D. McLaughlin, and D.J. Marcogliese. 2019. Impact of exposure sequence on establishment among sympatric lens-infecting species of *Diplostomum* (Digenea) in single and challenge infections in rainbow trout (*Oncorhynchus mykiss*).

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GENERAL INTRODUCTION

In nature, a single host may be infected by multiple species of parasites, the populations of which comprise an infracommunity of parasites species within an individual host (Bush et al., 2001). In addition, a species of parasite with a complex life cycle requiring a succession of host species for completion may be part of different communities at different stages of its life cycle. Probability of recruitment, interactions and sequence of establishment are some factors which can influence the structure of the infracommunity (Poulin, 2007). The outcomes of the recruitment success and interactions are largely dependent on the life history characteristics of each species of parasite.

Many more potential host-parasite relationships are possible than are actually observed in nature due to the presence of either an ecological or physiological incompatibility between parasite and host that prevents the association (Combes, 2001). Ecological factors may prevent a host from ever being exposed, and if it is exposed, physiological factors may prevent successful establishment of the particular species of parasite within the host (Combes, 2001). The number of hosts a parasite can successfully infect at one stage of its life cycle, host specificity, varies widely between and within species of parasites (Poulin, 2007). At one stage, a parasite may be restricted to a single host species (high specificity: specialist) yet infect a number of different species at another life cycle stage (low specificity: generalist) (Poulin, 2007). Characterization of species as host generalists or specialists is useful for understanding community dynamics and organization (Goater et al., 2014). However, for many parasites this information is lacking or misleading due to unequal sampling of hosts and taxonomic uncertainty in parasite identification (Poulin, 2007).

Generally, evolutionary factors favor the addition of hosts that a parasite can colonize as it adds an alternative pathway to increase transmission success (Poulin, 2007). These processes have led to the occurrence of multiple species of parasites within a single host, where the constituent species may or may not interact (Petney and Andrews, 1998; Goater et al., 2014; Rynkiewicz et al., 2015). Interactions may be direct through competition for space or resources, or indirect through host immune responses. Further, interactions can be either positive (synergistic) or negative (antagonistic) and may vary depending on the order of species recruitment (Poulin, 2007).

Previous research investigating interactions between species of parasites have focussed on the infracommunity of adult (sexually mature) parasites in vertebrate hosts, primarily those of helminth

communities found in the digestive tract (Poulin, 2001). Studies of parasite communities in fish have an added level of complexity because they consist of two components: adult parasites and larval stages that will continue to develop in host species further up in the food chain. In general, adult stage parasites are large, readily identified to species and may be interactive for example through competition or host immune responses. For the most part, the view of the larval community has been one of limited species diversity, a low level of host specificity and, because most larval stages are encysted, one with little potential for interaction (Janovy et al., 1992; Poulin and Valtonen, 2001). To complicate matters further, they are difficult to identify beyond the generic level using traditional morphology-based methods (Criscione et al., 2005; Nolan and Cribb, 2005). The poor level of taxonomic resolution available has made it difficult to study the larval community critically. Accordingly, studies on parasite communities in fish have focused on the adult component (e.g. Dezfuli et al., 2001; Kennedy and Hartvigsen, 2000), while the larval component has received relatively little attention (Désilets et al., 2013), even though larval stages are often present in greater numbers than adults and frequently form a larger proportion of the parasite community in an individual host.

If interaction does occur between two or more parasite species simultaneously infecting a single host, there are different possible outcomes. Positive interactions might occur due to facilitation, where one species actually modifies the habitat toward increased suitability for another species through mechanisms such as host immune suppression (Lotz and Font, 1991). Synergistic interactions between concurrent helminth infections in mammals have been shown to increase establishment, increase growth and fecundity, delay elimination from the host and reduce host immune response (Christensen et al., 1987; Poulin, 2007). For example, Presidente et al. (1973) found *Haemonchus contortus* (Nematoda) had an increase in fecundity and delayed evacuation in sheep (*Ovis* sp.) when concurrently infected with *Fasciola hepatica* (Trematoda). Negative interactions may be due to direct competition or cross-reactive host immunity and cause a reduction in establishment success, reduction in fecundity, a shift in the distribution of another parasite species, or earlier elimination from the host (Christensen et al., 1987; Poulin, 2007). For example, *Hymenolepis microstoma* (Cestoda) was expelled from mice (*Mus musculus*) when concurrently infected with *F. hepatica* (Lang, 1967). In addition to interactions, there are also the effects of sequence of exposure. For example, where the establishment success of *Ribeiroia ondatrae* (Digenea) in the Pacific chorus frog (*Pseudacris regilla*) decreased substantially only when the host had been previously exposed to *Echinostoma trivolvis* (Digenea) (Hoverman et al., 2013). It is also possible for no interactions to occur between parasites sharing a host. If the parasite populations are small enough,

they may not affect the host or experience competition for shared resources (e.g. food or habitat) within the host. Seemingly shared niches may also not actually overlap due to small, but consistent, differences in the use of food or habitat resources within hosts (Poulin, 2007).

Interactions, whether via direct or indirect mechanisms, are strong selective forces on individual parasites and are relevant to the evolution of parasite life histories (Goater et al., 2014). Life history strategies are combinations of biological traits such as body size, life span, age at maturity, fecundity and offspring size favored by selection due to an increase in fitness (Poulin, 2007). As not all life history traits determining reproductive success can be simultaneously maximized, any investment into one trait will be at the expense of another (Stearns, 1992). If co-occurrences are common due to shared hosts, then interspecific associations and sequence of recruitment may contribute to infracommunity structure. Co-occurrence may also drive selective forces to increase transmission success by either limiting differences between species to increase facilitation or differentiate life cycle characteristics to reduce negative interactions.

This project will focus on species of *Diplostomum* (Digenea), a cosmopolitan digenetic trematode with a three-host life cycle. The life cycle of *Diplostomum* is complex and involves a series of stages successively infecting lymnaeid snails, fish and piscivorous birds, particularly gulls (Laridae). Details of the general life cycle and brief descriptions of the stages involved and their biology are provided in Figure 1.

Species of *Diplostomum* have been well studied due to their potential consequences on the health and fitness of fish, especially those of commercial importance. Cercariae penetrate the skin or gills of the fish and migrate to their final site of establishment, the humor, retina or lens of the eye or the brain (Chappell et al., 1994). Penetration and migration to the final site have been shown to stimulate various innate immune responses (Whyte et al., 1989). Furthermore, the relatively short migration time of the cercariae have nonetheless been shown to initiate mounting an adaptive immune response should the host be exposed yet again at a later date (Whyte et al., 1989). Once cercariae have reached their final site in the fish host, they undergo development into metacercariae (Figure 1). The metacercariae are protected from the immune system of the host as the final sites of establishment are in immunologically inaccessible sites (Chappell et al., 1994). The metacercariae can cause cataracts in the lens, impairing host vision and are a significant problem in aquaculture (Chappell et al., 1994; Karvonen, 2012) and likely in nature. The metacercariae of *Diplostomum* spp. differ from those of most other genera in several aspects.

Unlike most digeneans the metacercariae lack a protective cyst and remain active and mobile in spatially restricted sites where contact with other metacercariae can occur.

Until recently, species level identification of *Diplostomum* spp. was based primarily on adult morphology. Depending on the source, there are between five and 15 nominal species reported from North American hosts (McDonald, 1969; Dubois, 1970, Yamaguti, 1975; Margolis and Arthur, 1979; McDonald and Margolis, 1995; Gibson, 1996; Hoffman, 1999). Despite this apparent diversity, virtually all of the metacercariae from the lens-infecting specimens throughout North America have by default been identified as *D. spathaceum* or a subspecies of *D. spathaceum,* a commonly reported species in Europe (McDonald and Margolis, 1995; Gibson 1996). The same is true of North American adult stages of lens-infecting specimens, where the majority have also been identified as *D. spathaceum* (Threlfall, 1968a; 1968b; Vermeer, 1969); or a subspecies of *D. spathaceum* (Dick and Rosen, 1981) based on the measurements provided in either Yamaguti (1958) or Dubois (1970). At present however, there is no DNA evidence to confirm the occurrence of this species from either larval or adult specimens in North America (Galazzo et al., 2002; Locke et al., 2010a; Désilets et al., 2013). Accordingly, most records dealing with metacercariae from the lenses of North American fish need to be viewed with caution (Gibson, 1996).

The application of DNA techniques to identify or at least distinguish larval specimens has opened up a completely new area of study in the parasitology of fishes. Recent molecular studies have shown that multiple species of *Diplostomum* are present in naturally-infected fishes (Galazzo et al., 2002; Niewiadomska and Laskowski, 2002; Locke et al., 2010a, 2010b; Désilets et al., 2013; Georgieva et al., 2013). Locke et al. (2010a, 2010b) performed extensive studies on parasites infecting fishes in the St. Lawrence River. In the first study, 12 species of *Diplostomum* were molecularly delineated with the barcode region of cytochrome c oxidase 1 of mitochondrial DNA (Locke et al., 2010a), a greater amount of cryptic diversity than ever imagined. Subsequently, Locke et al. (2010b) demonstrated a low level of host specificity among species of *Diplostomum* infecting the lens versus high host specificity among those infecting the humor. Most recently, molecular studies have also demonstrated negative interactions in terms of metacercarial abundance between three lens-infecting species in a survey of 20 species of fish from the St. Lawrence River (Désilets et al., 2013). The revelation of cryptic diversity and the lack of taxonomic resolution within the lens and humor-infecting community is a significant impediment to our understanding of the biology of species of *Diplostomum*. It has obscured important biological, life cycle

and ecological differences between species that significantly limit our understanding of the biology and coexistence of species complexes of parasitic organisms.

DNA approaches provide a definitive and repeatable method of distinguishing species of *Diplostomum* at each stage of their life cycle. Using these molecular tools for taxonomic resolution, given the recent findings of sympatric cryptic species, the objectives of this thesis are to 1) determine if there are differences in life history characteristics between the species and 2) determine if there are interactions in the fish and bird host. This will allow for the study and comparison of biological properties of different species of *Diplostomum* at various stages of their life cycle and test for effects and consequences of interspecific interactions in mixed infections in fish and bird hosts. Together this will give greater insight to how different species using similar transmission patterns and the same pool of host species coexist.

This dissertation is presented as five chapters involving experimental laboratory studies, all depending heavily on DNA-based identifications. Chapter 1 examines host specificity by experimentally testing metacercarial establishment success of different lens-infecting species of *Diplostomum* in phylogenetically different species of fish. Chapter 2 investigates the effect of an existing infection in rainbow trout (*Oncorhynchus mykiss*) of one lens-infecting species of *Diplostomum* on the establishment success of a challenge exposure with either the conspecific or a congener. Chapter 3 examines intestinal distribution, range and fecundity of species of *Diplostomum* in single and mixed infections in the ring-billed gulls (*Larus delawarensis*) host. Chapter 4 examines life history parameters of the eggs by collecting adult flukes from gull feces and counting eggs *in utero* to test for species differences in egg size. Further, adult size will be measured to determine if there is an intraspecific relationship between fluke size and egg size and number. Lastly, Chapter 5 explores potential interspecific differences between two lens infecting species of *Diplostomum* in the developmental parameters of prepatent period, embryonation rates, embryonation success and hatching success.

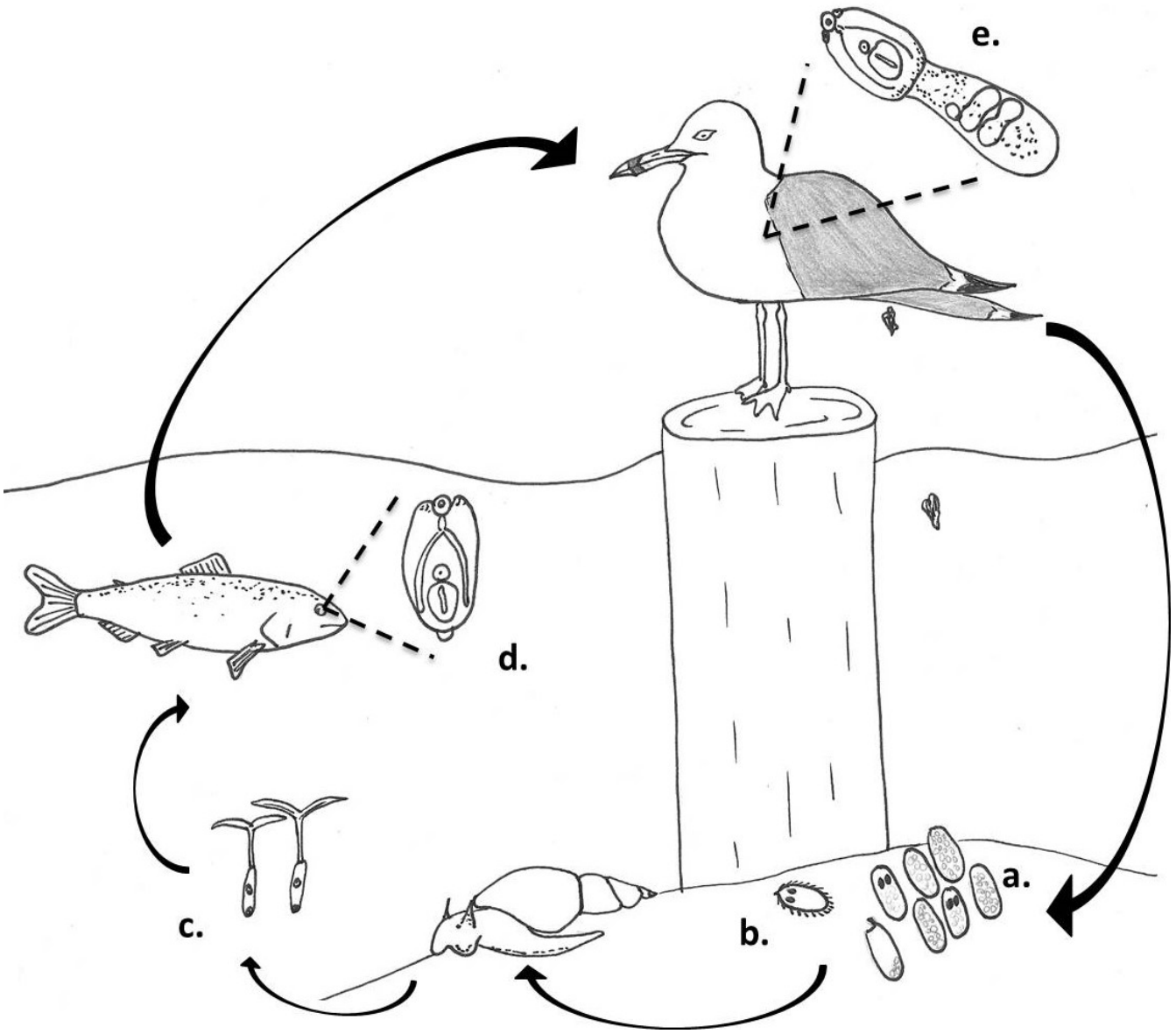
The uncertainty in the taxonomy of *Diplostomum* spp. has left us virtually in the dark on the biological and ecological aspects of these parasites' life history. The ability to distinguish species of *Diplostomum* using DNA at different stages of the life cycle provides a unique opportunity to compare life history parameters and to examine species interactions. Collectively these experimental observations allow comparisons of interactions of molecularly-identified sympatric parasite species in both an intermediate and the definitive host and contribute greater insight into factors affecting parasite community structure.

Figure 1. Generalized life cycle of *Diplostomum* (Digenea).

The typical life cycle of species of *Diplostomum* includes three different hosts. Adult parasites occur in the intestinal tract of a fish-eating bird and larval stages in snails and then fish or, less commonly, amphibians. Chappell et al. (1994) reviewed and described the general life cycle of *Diplostomum spathaceum*. Embryonated eggs (a) are passed in the feces of a gull (Laridae) or other piscivorous birds, which after a period of development in water under favorable conditions will release miracidia (b). The short-lived (24 - 48 hours), non-feeding miracidium actively searches for and penetrates the first intermediate host, a lymnaeid snail. Once in the gonads of the snail, the miracidium develops into a mother sporocyst which asexually produces and releases daughter sporocysts. The daughter sporocysts migrate to the digestive gland and asexually produce cercariae (c) between four to ten weeks post-infection. The short lived cercariae will exit the snail and actively search for and penetrate and the second intermediate host, typically a fish. Once inside the fish, cercariae migrate to a site-specific location of infection within approximately 24 hours. *Diplostomum spathaceum* infects the lens, but other species have been reported from the humor, retina and brain. Once in the final infection site, they develop into metacercariae (d). These are infective after about eight weeks, and remain un-encysted and active within the host for over a year, and possibly many years. When the fish is eaten by the definitive host, a gull or other piscivorous bird, the metacercariae develop into adults (e) and reproduce sexually in the intestine of the bird, releasing embryonated eggs in the host feces after about three days post-infection.

The figure has been adapted from:

Karvonen, A. 2012. *Diplostomum spathaceum* and related species. Pages 260- 269 *in* Woo, P. T. K., and K. Buchmann. (Eds). Fish parasites: Pathology and protection. CAB International, Oxfordshire, U.K.


e.
d.
c.
b.
a.

CHAPTER 1. *Diplostomum* spp., a jack of all trades: Establishment of two lens-infecting species in phylogenetically different fish

ABSTRACT: Metacercariae of species belonging to the genus *Diplostomum* (Digenea) are common parasites of fish. Most species establish in the lens with others infecting the vitreous humor and fewer the brain. Species of *Diplostomum* are cosmopolitan but little is known regarding the diversity of species infecting fish due to the difficulty of identifying the metacercariae to species level based on morphology alone. In this study we examined establishment of two molecularly delineated lens-infecting species, *Diplostomum* sp. 1 and *Diplostomum* sp. 4, in five species of fish. This included Central American convict cichlids (*Amatitlania nigrofasciata*, Cichlidae, Perciformes), northern redbelly dace (*Chrosomus eos*, Cyprinidae, Cypriniformes), guppies (*Poecilia* sp., Poeciliidae, Cyprinodontiformes), rainbow trout (*Oncorhynchus mykiss*, Salmonidae, Salmoniformes) and walleye (*Sander vitreus*; Percidae, Perciformes). *Diplostomum* sp. 4 successfully established in dace, guppies and rainbow trout and had a significantly higher number of metacercariae, whereas *Diplostomum* sp. 1 successfully established in all species of fish except walleye. Both *Diplostomum* sp. 1 and *Diplostomum* sp. 4 had a high index of host specificity, indicating they are generalists, capable of establishing in a wide variety of fish hosts. These are the first experimental results to test host specificity among a range of phylogenetically diverse fish hosts for two lens-infecting species of *Diplostomum*. Results support field studies reporting lens-infecting species as generalists.

INTRODUCTION

Host specificity among parasites varies (Poulin, 2007). It may be restricted to a single host species at one stage in its life cycle (high specificity: specialist) yet infect a number of different species at another stage (low specificity: generalist) (Poulin, 2007). There are benefits and costs for either strategy. Specialists, occurring in a single or few host species, are capable of exploiting the host to its fullest potential thereby maximizing their fitness, yet limit their survival based on the survival of their host (Poulin and Keeney, 2008). Generalists, in contrast, can avoid extinction due to their ability to exploit alternative hosts (Koh et al., 2004); however, relaxing host specificity may render the parasite less capable of dealing with competition from other species or the host's immune response (Keeney et al., 2015), resulting in differing intensities of infection in different host species. Therefore, host specificity is multifaceted. Basic host specificity takes into consideration only the number of hosts used by a parasite whereas structural

specifity includes differing intensities of infections in different host species and phylogenetic host specifity includes host relatedness (Poulin et al., 2011). Recent studies have revealed an over or under estimation of host specifity based solely on field surveys (Poulin and Keeney, 2008).

Species of *Diplostomum* (Digenea) are common parasites with complex life cycles infecting piscivorous birds, primarily gulls, as their definitive hosts, and lymnaeid snails and aquatic vertebrates, primarily fish, as first and second intermediate hosts respectively (Figure 1). Many field studies have reported metacercariae of *Diplostomum* spp. infecting the lens in numerous fish species (Chappell et al., 1994). Penetration and migration of cercariae to the final site within the fish host have been shown to stimulate various innate immune responses (Whyte et al., 1989) as well as initiate an adaptive immune response should the host be exposed yet again at a later date (Whyte et al., 1989). However, once cercariae have reached their final site in the fish host, species of lens-infecting *Diplostomum* are located within a protective site where they are shielded from the host-immune response (Shariff et al., 1980; Whyte et al., 1990). This may allow them to have broader host specificity than the non-lens-infecting species (Sitjà-Bobadilla, 2008; Locke et al., 2010b; Locke et al., 2015).

Three previous studies have experimentally studied host specificity in the lens-infecting *Diplostomum spathaceum*. Sweeting (1974), in the first and only combined field and experimental study on the host specificity of *D. spathaceum*, found natural infections of varying intensities in 13 species of Cyprinidae, three species of Salmonidae, two species of Percidae and one species each of the Esocidae, Cottidae, Cobitidae, Gasterosteidae and Thymallidae. Sweeting (1974) also successfully experimentally infected seven species of Cyprinidae, two species of Percidae and one species each of Salmonidae, Anguillidae and Pipidae. The same is true in a study by Betterton (1974) who found establishment of varying intensities in two species of Salmonidae, brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*). Lastly, Speed and Pauley (1984) found fish survival times to be negatively associated with number of cercariae they were exposed to for four species of Salmonidae, rainbow trout, cutthroat trout (*Oncorhynchus clarkia*), brook trout (*Salvelinus fontinalis*) and coho salmon (*Oncorhynchus kisutch*).

While the results of the three previous studies revealed *D. spathaceum* in both natural and experimental settings to have low host specificity at this stage in its life cycle, there are two important issues to consider. First, in the natural infections, all the metacercariae were assumed to be *D. spathaceum* due to the infection site; and second, it was assumed that all the naturally infected snails used in the experimental infections were shedding the same species of *Diplostomum*. As recent molecular studies

have revealed the presence of cryptic species of *Diplostomum* (Locke et al., 2010a; 2010b; Georgeiva et al., 2013), the previous results may be an amalgamation of the host specificity of several lens-infecting species of *Diplostomum*. This leads to uncertainty in the knowledge of specifity at the level of the fish host which can have implications in the diversity of parasites in the intermediate and definitive hosts and our understanding of what species are involved in the transmission process.

This chapter experimentally explores host specificity of two sympatric lens-infecting species of *Diplostomum*. The two species of *Diplostomum* examined herein have been reported from the same localities from a large variety of species of fish (Locke et al., 2010a, 2010b; Désilets et al., 2013). The goal is to assess host specificity across a range of phylogenetically different native and exotic species of fish hosts. Based on the field data of Locke et al. (2010a; 2010b), which found lower specificity in lens-infecting species of *Diplostomum* in comparison to humor infecting species, we should see no difference between the two lens-infecting species examined here. Each should be capable of infecting the different fish hosts, at potentially different intensities, irrespective of host phylogeny.

MATERIALS AND METHODS

Snail collection and molecular identification of cercariae

Snails (*Lymnaea* sp.) were haphazardly collected manually off the rocky littoral zone of the Saint-Lawrence River, Pointe-aux-Cascades, Quebec, Canada (45°10'08.3"N 66°59'56.2"W) in late August and early September 2015. The snails were isolated for cercarial shedding in individual cups containing 250 ml of dechlorinated water. Snails were fed *ad libitum* with fresh lettuce daily. Two snails shedding furcocercariae were isolated and a sample of cercariae was collected from each snail and killed by freezing. Their DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen)^{TM} following the manufacturer's protocol. Amplification and sequencing of the partial internal transcribed spacer region of ribosomal DNA (ITS-rDNA) was performed using the primers D1 (F) (5' – AGG AAT TCC TGG TAA GTG CAA G - 3') and D2 (R) (5' CGT TAC TGA GGG AAT CCT GGT – 3') and protocols described by Galazzo et al. (2002) and Locke et al. (2010a). The successful amplicons were sequenced in both directions using the forward and reverse PCR primers at the Genome Quebec Innovation Centre, McGill University in Montreal, Quebec. The successful chromatograms were manually edited and contiguous sequences were assembled in Geneious R8 version 8.0.4 (Kearse et al., 2012) and submitted to GenBank (Accession #s: MH108584 - MH108585).

The two newly generated ITS-rDNA sequences were aligned, trimmed and were compared with 28 published ITS-rDNA sequences of *Diplostomum* spp. from Canada and Europe (Galazzo et al., 2002; Locke et al., 2010a; Georgieva et al., 2013; Blasco-Costa et al., 2014; Pérez-Del-Olmo et al., 2014; Locke et al., 2015; Selbach et al., 2015) in Geneious R8 version 8.0.4 (Kearse et al., 2012). The trimmed alignment was then used to construct a number-of-difference based neighbor-joining tree with 1000 bootstrap replicates and pairwise deletion of gaps using a published sequence from GenBank for *Tylodelphus scheuringi* (Mosczynska et al., 2009) for the outgroup (Blasco-Costa et al., 2014; Selbach et al., 2015) in MEGA version 6 (Tamura et al., 2013). The NJ analysis of the ITS-rDNA dataset (based on a final dataset with gaps removed of 808 base pairs) provided robust evidence where one isolate clustered with *Diplostomum* sp. 1 (GQ292522.1, Locke et al., 2010a; KT186794.1, Locke et al., 2015) and one isolate clustered with *Diplostomum* sp. 4 (GQ292520.1, Locke et al., 2010a) (Appendix 1). While it is rare for snails to be concurrently infected with more than one species of parasite (Soldánová et al., 2012), the trimmed alignments (Appendix 2) were analyzed for diagnostic sites (Appendix 3) and the peaks of the original chromatograms (Appendix 4) were re-examined at the diagnostic sites to validate species identification. No ambiguities were observed, confirming that each individual snail was infected with a single species of *Diplostomum*. The cercariae were hence molecularly delineated to species as two lens-infecting species, *Diplostomum* sp. 1 and *Diplostomum* sp. 4, using provisional nomenclature based on Locke et al. (2010a).

Experimental fish and exposure protocol

All the animals used in this experiment were maintained and handled in accordance with the guidelines of the Canadian Council of Animal Care. The experiments were officially approved by the Concordia University Animal Research Ethics Committee (AREC, certificate #30000269).

The experimental procedure consisted of two groups. The first group was exposed to cercariae of *Diplostomum* sp. 1 and the second group exposed to cercariae of *Diplostomum* sp. 4. The different species of naïve fish chosen for this experiment were based on availability at the time of the experiment and phylogenetic differences: rainbow trout (Salmoniformes, Salmonidae: *Oncorhynchus mykiss*) obtained from a commercial farmer in Sainte-Eldwidge, Quebec, Canada; northern redbelly dace (Cypriniformes, Cyprinidae: *Chrosomus eos*) obtained from a privately owned pond in Dunvegan, Ontario, Canada; Central American convict cichlids (Perciformes, Cichlidae: *Amatitlania nigrofasciata*) obtained from a lab-reared population in the Dr. James Grant laboratory Concordia University, Montreal; guppies (Cyprinodontiformes, Poeciliidae: *Poecilia* sp.) obtained from a commercial pet store in Montreal; and

walleye (Perciformes, Percidae: *Sander vitreus*) obtained from a commercial farmer in Wotton, Quebec. Depending on availability of the fish, the sample sizes varied from 12 to 66 fish in each of the experimental groups. The experiments were performed over two months contingent on the availability of space, cercariae and fish.

The evening prior to fish-exposure, each infected snail was placed in 250 ml of fresh water to stimulate cercarial shedding in ambient artificial light. Exposure beakers were filled with 250 ml of dechlorinated water at a temperature of 17 - 18°C and aerated overnight to fully saturate the water with oxygen. The amount of water in the beakers varied with the fork length of the fish (≤ 50 mm: 250 ml (guppies, dace, rainbow trout, cichlids); 51 – 100 mm (dace, rainbow trout, cichlids): 500ml; > 100 mm: 750 ml (walleye)) to ensure adequate coverage and oxygen for the fish for the duration of the experiment. The cercariae of each species of *Diplostomum* were collected the following morning, approximately 12 hours later. The morning of the experiment, the aeration of the exposure beakers was stopped and a dose of 20 haphazardly chosen cercariae was added to each beaker by pipette. In order to get accurate indications representative of natural infections, a small exposure dose was chosen over a larger dose of either 50 or 100 cercariae (Poulin, 2010). Cercarial density was calculated as the number of cercariae per litre of water (Höglund, 1995; Karvonen et al., 2003).

For each treatment, once dosed, the fish were placed individually in the beakers and left undisturbed for one hour. The beakers were not aerated during the one-hour exposure to avoid possible effects on cercarial swimming behaviour. Upon completion of exposure, fish were removed from the beakers with the use of a net and the fish was rinsed with water between transfers to reduce any carry over of cercariae and placed in aerated aquariums, separated by host and parasite species. The fish were maintained and fed commercial fish food daily for two days.

Fish examination and necropsy

The movement of cercariae from the penetration site to the lens of the eye is usually completed within 24 hours (Chappell et al., 1994). All fish for each of the treatments were euthanized 48 hours post exposure (PE) with an overdose a 0.1% concentration of clove oil and frozen at -20°C until dissection. The total fork length (tip of the snout to the end of the middle caudal fin rays) of the fish was measured (± 1.0 mm). The lenses of the fish were removed, the left and right lenses dissected separately and the number of metacercariae counted for each lens using a stereomicroscope. Establishment is defined herein as the number of metacercariae recovered from the lens of the host.

Data analysis

Descriptive statistics for the fish of mean fork length (mm) and range were calculated. Descriptive statistics for the metacercariae of prevalence (percentage of fish infected), mean (± standard deviation, SD), intensity (mean number of parasites of a given taxon per infected fish) (± SD) and range values for each species of *Diplostomum* for each species of fish were calculated. Outliers were assessed by converting raw data into standardize scores, where cases with a *z*-score ± 3.00 were considered potential outliers (Tabachnick and Fidell, 2006). Normality was tested with skew and kurtosis ($p < 0.01$) and homoscedasticity was tested with Levene's Test ($p < 0.05$: Tabachnick and Fidell, 2006).

The fork length for the fish data had a single outlier in the dace sample exposed to *Diplostomum* sp. 1 and it was maintained in the analyses. The data were normal, but heteroscedastic. Transformations on fork length were unsuccessful in converting the data from heteroscedastic to homoscedastic and the appropriate parametric test was selected. A Welch's Test (as the data were heteroscedastic) was calculated to determine if there was a difference among treatments in the mean fork length of the fish. Eta squared effect size was calculated to measure the proportion of variation in mean fork length attributed to the variation between treatments. Tamhane's T2 (adjusting for heteroscedasticity) post-hoc tests were calculated to measure differences among relevant pairwise comparisons. Hedges' *g* effect sizes were calculated to measure how much each treatment differed from one another; where $g \leq 0.2$ has little effect, $g \leq 0.5$ has a medium effect and $g \geq 0.8$ has a large effect (Ellis, 2010). Subsequently fish fork length was entered as a covariate in further analyses. Sex of the fish was not taken into consideration as sex has been shown to not a significant determinant of infection success of metacercariae for *Diplostomum* spp. (Marcogliese et al., 2001; Désilets et al., 2013).

The data for the number of metacercariae had several outliers (one outlier for dace and guppies and two outliers for rainbow trout which were exposed to *Diplostomum* sp. 1, and one outlier for guppies exposed to *Diplostomum* sp. 4), all which were retained within the analyses. The data were all negatively skewed and heteroscedastic and transformations were unsuccessful. There were no significant differences between the numbers of metacercariae established in the left and right eye for each species of fish (non-parametric related samples sign test; $0.146 \leq p \leq 1.00$) and the total number of metacercariae for both eyes was combined.

Count data generally follow a Poisson distribution; however, over-dispersion can invalidate the Poisson assumption that variance equals mean (Zuur et al., 2009). To accommodate for over-dispersion in the data, a negative binomial regression model with an estimated dispersion parameter was constructed with all the effects. The model treated number of metacercariae as the dependent variable, species of fish and *Diplostomum* as independent variables and fish fork length and cercarial density as covariates because the fish varied in size and were exposed to different cercarial densities depending on their size. The fit of the data to the negative binomial regression model was confirmed using the Likelihood ratio chi-square goodness-of-fit test. Probabilities of ≤ 0.05 were considered significant and Bonferroni corrected when needed to adjust for multiple comparisons. Statistical analyses were conducted using the SPSS® 24.0 software package.

There are various indexes to estimate host specificity. Historically, the number of host species that a parasite was known to infect was used to assess host specificity (Poulin and Keeney, 2008). However, the number of host species does not include structural or phylogenetic components of host specificity. Common diversity indexes such as the Simpson or Shannon index takes into consideration the prevalence / abundance data, but are sensitive to sample size and ignore host phylogenetic data. The index of host specificity (S_{TD}*) combines structural and phylogenetic specificity into a single index. Further, the S_{TD}* does not require a phylogenetic tree of hosts and is not sensitive to phylogenetic construction tree methods in comparison to other indexes which do (Cadotte et al., 2010). Therefore, the index of host specificity (S_{TD}* = $\sum\sum_{i<j} w_{ij}(p_i p_j) / \sum\sum_{i<j} (p_i p_j)$) was calculated to determine the level of host specificity for both species of *Diplostomum*, where *w* is the taxonomic distances between host species *i* and *j* and p_i and p_j are the prevalence's of the parasite in host species *i* and *j* respectively (Poulin and Mouillot, 2005). The S_{td}* is on a scale of one to five, and the higher the specificity the lower the scale value and it takes into consideration the phylogenetic difference of the host species and the prevalence of the parasite in each host species. The phylogenetic distance of host species is calculated as the number of steps in a Linnaean hierarchy to a common taxon based on the classifications of species, genus, family, order, class (Poulin and Mouillot, 2005). Therefore, apart from cichlids and walleye which were three units from one another, all other species pairs were four units apart.

RESULTS

A total of 470 fish representing five species of varying sample sizes for each species of fish were exposed to cercariae of *Diplostomum* (Table 1). All of which fish survived. The mean (± the SD) fork length

(mm) of the fish in each treatment ranged from 12 – 138 mm, with guppies being the smallest and walleyes being the largest (Table 1). There was a significant difference in mean fish fork length between host species (Welch's Test, $F_{9, 111.658} = 2409.730$, $p < 0.001$) with an eta squared effect size of 0.92. Post-hoc comparisons indicated no significant difference in mean fork length within experimental groups (i.e. between the same species of fish exposed to either *Diplostomum* sp. 1 or *Diplostomum* sp. 4; $p = 1.000$). Pairwise comparisons between species of fish showed no significant difference in mean fish length between dace and rainbow trout ($p = 1.000$, no effect for Hedges' $g = 0.05$ for *Diplostomum* sp. 1, no effect for Hedges $g = 0.11$ for *Diplostomum* sp. 4), between dace and cichlids exposed to *Diplostomum* sp. 1 ($p = 0.015$, large effect for Hedges' $g = 2.41$) nor between rainbow trout and cichlids exposed to *Diplostomum* sp. 1 ($p = 0.015$, large effect for Hedges' $g = 1.27$). However, there were significant differences in mean fish length between all other species pairs ($p = 0.001$, large effect for Hedges' $g \geq 1.46$). For each of the treatments, there were several fish with no metacercariae recovered and the prevalence of infection ranges from 0.0 to 96.4 % (Table 1).

The negative binomial regression model was a good fit to the data (Likelihood ratio $X^2_{12} = 300.424$, $p < 0.001$, Table 1, Figure 2). The main factors of species of host (negative binomial regression model, Wald $X^2_3 = 120.358$, $p < 0.001$, $B = 3.170$) and species of *Diplostomum* (negative binomial regression model, Wald $X^2_1 = 3.822$, $p = 0.05$, $B = 1.791$) were statistically significant in predicting the number of established metacercariae. There was a significant interaction between species of *Diplostomum* and host species (negative binomial regression model, Wald $X^2_2 = 50.021$, $p < 0.001$, $B = 0.768$) where the species of *Diplostomum* did not establish equally in each host, but no interaction between species of *Diplostomum* and fish size (negative binomial regression model, Wald $X^2_1 = 1.812$, $p > 0.05$, $B = 0.015$), such that smaller parasitized fish were not associated with a particular species of *Diplostomum*. For host species, rainbow trout had the greatest prevalence followed by cichlids, guppies, dace and then walleye. For species of *Diplostomum*, *Diplostomum* sp. 4 had a larger mean establishment in rainbow trout than *Diplostomum* sp. 1. There was a significant effect for the covariate of fish length (negative binomial regression model, Wald $X^2_1 = 6.411$, $p = 0.011$, $B = -0.035$), where smaller fish had a greater number of metacercariae, but not cercarial density (negative binomial regression model, Wald $X^2_1 = 1.282$, $p > 0.05$, $B = -0.011$).

Diplostomum sp. 4 was successful at establishing (though at varying intensities) in the dace (0.10 ± 0.30), guppies (0.28 ± 0.62) and rainbow trout (2.52 ± 0.53) and *Diplostomum* sp. 1 was successful at establishing in all species of fish except walleye, and also at varying intensities: cichlid (1.17 ± 1.53); dace

(0.13 ± 0.38); guppies (0.54 ± 1.17); rainbow trout (0.58 ± 0.97: Table 1, Figure 2). The species of fish used in the experiment differed at the order or family (cichlids and walleye) taxonomic level. Both *Diplostomum* sp. 1 and *Diplostomum* sp. 4 had an S_{TD}* of 4.00, indicative of these species being generalists, capable of establishing in a wide variety of fish hosts.

DISCUSSION

Host specificity is a key aspect of understanding parasite community structure and evolutionary history (Poulin, 2007). Species of *Diplostomum* have a three-host life cycle and the level of fish host specificity will play a role in the transmission dynamics to the definitive host. Evidence of low specificity was suggested by Locke et al. (2010a; 2010b) for molecularly-delineated lens-infecting species of *Diplostomum* in naturally infected fish communities. The data presented here are the first experimental results to test host specificity among a range of phylogenetically diverse fish hosts for two sympatric lens-infecting species of *Diplostomum*. As expected, both species of *Diplostomum* studied herein had a high S_{TD}*, indicative of being generalists, capable of establishing in a wide variety of fish hosts. Both species of host and parasite were significant predictors of infection success. There was successful establishment of *Diplostomum* sp. 1 in cichlids, dace, guppies and rainbow trout, representative of four different taxonomic orders, but not walleye. *Diplostomum* sp. 4 successfully established in significantly greater number in the rainbow trout and but overall infected fish representatives from only three orders, being unsuccessful in cichlids and walleye, both Perciformes. There was a significant negative association between fork length and mean establishment, but no association for either cercarial density or fork length with species of *Diplostomum*. Similar to our experimental design where cercarial density varied, *D. spathaceum* had greater mean establishment in smaller rainbow trout (Höglund, 1995). Likewise, Karvonen et al. (2003) also found the number of metacercariae per fish to increase with cercarial density. While previous results (Höglund, 1995; Karvonen et al., 2003) have shown cercarial density to play a role in establishment, here, cercarial density did not have a significant association with mean establishment.

Various components of evolution, behavior, ecology, physical, physiology and immune function will all help determine host specificity (Secombes and Chappell, 1996). There are benefits and costs of being either a specialist or generalist. The benefit of specialization towards the physiology and immune response of one host would lead to an increase in performance in few hosts, whereas generalists can utilize greater hosts but with potentially unequal efficiency (Poulin, 2007). Here, the lens-infecting species of *Diplostomum* show a generalist pattern, capable of establishing in fish hosts from varying taxonomic

groups with different levels of metacercarial establishment. This is in agreement with previous experimental work on *D. spathaceum*, also a lens-infecting species (Betterton, 1974; Sweeting, 1974; Speed and Pauley, 1984). However, while the S_{TD}* is equal and high for both species suggesting they are generalists, there are subtle differences for both structural and phylogenetic specificity between *Diplostomum* sp. 1 and *Diplostomum* sp. 4. *Diplostomum* sp. 4 had greater structural specificity with greater mean establishment in rainbow trout and *Diplostomum* sp. 1 had lower phylogenetic specificity with establishment in cichlids. Even though the S_{TD}* takes into consideration both prevalence and host phylogenetic differences, it did not differ between the two species as the taxonomic distances of the fish hosts were similar and in general low prevalence. The patterns of host specifity have been suggested to be governed by physiological characteristics rather than ecological for host-parasite relationships within the Diplostomoidea as the patterns observed were similar in distinct fish communities and the eye is an immune privileged site (Locke et al., 2010b).

Reasons for variable establishment remain unclear; however, speculatively, exploring physiological characteristics which may been involved, anatomical differences, fish size and innate immune components will be explored. Two physiological differences standout for the walleye, the only fish uninfected by either species of *Diplostomum.* First, the *tapetrum lucidum* covering the eyes of walleye could potentially inhibit cercariae from crossing the eye epithelium (Ali and Anctil, 2011). Second, the walleye were the largest fish in our experiment and overall, smaller fish had greater mean establishment for either species of *Diplostomum*. Larger hosts represent more resources which should favor increased colonisation (Poulin, 2007). However, larger fish, depending on the site of penetration, may present a longer migration route and greater energy reserves would be required to reach the lens, decreasing mean establishment. Désilets et al. (2013) found a negative association between fish length and intensity with *Diplostomum* sp. 1, but no significance with *Diplostomum* sp. 4 from field collected data. Herein however, there was no significant interaction between fork length and species of *Diplostomum* on metacercarial establishment. Further, walleye have been reported with natural infections in the lens with a range of 1 to 116 metacercariae (Marcogliese et al., 2001) which in light of molecular data are presumed to be *Diplostomum* sp. 1 and/or *Diplostomum* sp. 4 as observed by Locke et al. (2010a).

Hypothetically, there could be differences among host species in one or more aspects of the innate response. Species of *Diplostomum* have been shown to elicit both an innate and adaptive immune response in rainbow trout (Chappell et al., 1994) and the differences between the species may be due to

differences in immune responses among them. The innate immune system of fish to respond to a novel infection consists of physical barriers and cellular components (Magnadóttir, 2006) and only innate immune system components would have potentially played a role here as all the fish were naïve, exposed once and subsequently euthanized 48 hours PE, before the adaptive immune response would have been initiated (Chappell et al., 1994).

Beginning with external attachment through to establishment, size and/or phylogenetic differences may have played a role in the varying mean establishment. For example, increased epithelial mucous interfering with cercarial infiltration (Jones, 2001) or differences in cercarial penetration stimulus (Haas et al., 2007). Once penetrated, cercariae cause tissue damage stimulating an inflammatory response (Smyth, 1962); however, as the innate immune system is non-specific, there should be little to no difference between macrophages engulfment (Whyte et al., 1989) and stimulation of reactive oxygen species in the phagocytosis of the cercariae (Chappell et al., 1994). Finally, upon reaching the lens, they are protected from further innate or adaptive immune responses as the eye is protected against inflammation and the lens acts as an additional defensive barrier (Shariff et al., 1980; Stein-Streilein and Streilein, 2002; Sitjà-Bobadilla, 2008). The evasion from the fish immune system once inside the lens would not select for specialization.

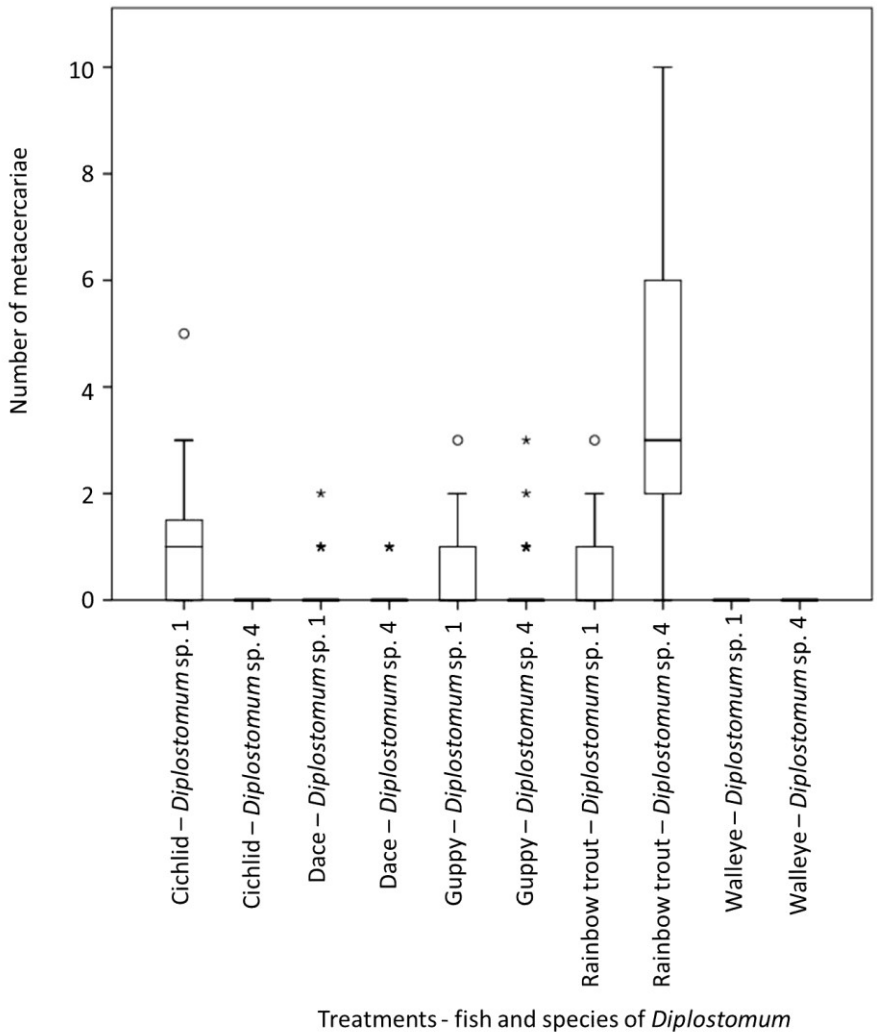
In summary, experimental results confirm lens-infecting *Diplostomum* sp. 1 and *Diplostomum* sp. 4 to be generalists. These species of *Diplostomum* are capable of infecting phylogenetically different species of fish, at varying intensities, increasing their probability of reaching the final gull host. There was a greater mean establishment in smaller fish, which may suggest that fish are more prone to infection at an earlier point in their life; however, cercarial density was not shown to be of significant association. Future studies examining host specificity data are needed to improve our understanding of parasite flow infection rates and if the opportunistic behavior of the cercariae leads to an increase in transmission rates.

Table 1. Summary statistics describing the distributions of the fish (sample size (n), mean fork length and range) and metacercariae (prevalence, mean abundance, mean intensity and range) for each experimental infection with *Diplostomum* sp. 1 or *Diplostomum* sp. 4. The species of fish were: Central American convict cichlids (*Amatitlania nigrofasciata*); northern redbelly dace (*Chrosomus eos*); guppies (*Poecilia* sp.); rainbow trout (*Oncorhynchus mykiss*); and walleye (*Sander vitreus*). There was a significant difference in mean fish fork length between host species (Welch's Test, $F_{9,\ 111.658} = 2409.730$, $p < 0.001$) with an eta squared effect size of 0.92. Post-hoc comparisons indicated no significant difference in mean fork length within experimental groups nor between dace and rainbow trout, dace and cichlids or rainbow trout and cichlids exposed to Diplostomum sp. 1 with significant differences between all other species pairs ($p = 0.001$).

	Fish				**Metacercariae number**		
	Sample size (n)	**Mean fork length (mm) ± SD**	**Range (mm)**	**Prevalence**	**Mean abundance ± SD**	**Mean intensity ± SD**	**Range**
	Cichlid						
Diplostomum sp. 1	12	65.00 ± 15.42	42 - 96	58.33%	1.17 ± 1.53	2.00 ± 1.53	0 - 5
Diplostomum sp. 4	12	70.00 ± 12.69	55 - 91	0.00%	0	0	0
	Dace						
Diplostomum sp. 1	63	43.17 ± 7.40	27 - 54	12.70%	0.13 ± 0.38	1.14 ± 0.38	0 - 2
Diplostomum sp. 4	59	44.37 ± 6.33	27 - 64	10.17%	0.10 ± 0.30	1.00 ± 0.00	0 - 1
	Guppy						
Diplostomum sp. 1	48	19.58 ± 4.13	12 - 27	27.08%	0.54 ± 1.17	2.00 ± 1.47	0 - 5
Diplostomum sp. 4	46	19.24 ± 2.92	14 - 26	21.74%	0.28 ± 0.62	1.3 ± 0.67	0 - 3
	Rainbow Trout						
Diplostomum sp. 1	45	42.49 ± 18.32	24 - 84	40.00%	0.58 ± 0.97	1.44 ± 1.04	0 - 5
Diplostomum sp. 4	55	45.84 ± 17.56	23 - 77	96.36%	2.52 ± 0.53	4.09 ± 2.44	0 - 10

	Walleye						
Diplostomum sp. 1	66	119.91 ± 6.50	105 - 133	0.00%	0	0	0
Diplostomum sp. 4	64	117.86 ± 6.14	104 - 138	0.00%	0	0	0

Figure 2. Box and whisker plots of total number of metacercariae for *Diplostomum* sp. 1 and *Diplostomum* sp. 4 in experimental infections in the fish intermediate hosts: Central American convict cichlids (*Amatitlania nigrofasciata*); northern redbelly dace (*Chrosomus eos*); guppies (*Poecilia* sp.); rainbow trout (*Oncorhynchus mykiss*); and walleye (*Sander vitreus*). The box represents the interquartile range and the horizontal line within the box represents the median. The whiskers end at the largest and smallest value excluding any outliers, and the circles and asterisks are non-statistically significant outliers. A negative binomial regression model was calculated and found the main factors of species of host (Wald X^2_3 = 120.358, $p < 0.001$) and species of *Diplostomum* (Wald X^2_1 = 3.822, $p = 0.05$) were statistically significant in predicting the number of established metacercariae. There was a significant interaction between species of *Diplostomum* and host species (Wald X^2_2 = 50.021, $p < 0.001$) where the species of *Diplostomum* did not establish equally in each host.



CHAPTER 2. Impact of exposure sequence on establishment among sympatric lens-infecting species of *Diplostomum* (Digenea) in single and challenge infections in rainbow trout (*Oncorhynchus mykiss*)

ABSTRACT: Negative associations between metacercariae of lens-infecting species of *Diplostomum* have been demonstrated in natural communities. However, it remains to be tested experimentally whether temporal heterogeneity in the colonization process is associated with infracommunity composition. Metacercarial establishment success was evaluated and compared in experimental single and challenge infections in rainbow trout (*Oncorhynchus mykiss*) of two lens-infecting species of *Diplostomum* (Digenea), previously designated by their DNA sequences as *Diplostomum* sp. 1 and *Diplostomum* sp. 4. In challenge infections, fish were exposed to one species and then exposed five days later to either the same or the second species. In single species infections, there was no significant difference in establishment success between *Diplostomum* sp. 1 and *Diplostomum* sp. 4. In challenge infections, there was a significant decline in establishment success of *Diplostomum* sp. 1 in fish that had been previously exposed to either *Diplostomum* sp. 1 or *Diplostomum* sp. 4. There was no difference in mean establishment success for *Diplostomum* sp. 4 in either challenge exposure. These results demonstrate the importance of establishment priority for the infection success of *Diplostomum* sp. 1.

INTRODUCTION

It is rare in nature for vertebrate hosts to be infected by a single species of parasite (Poulin, 2007). Generally, an individual host is infected concurrently by two or more species (Petney and Andrews, 1998; Poulin, 2007), the populations of which constitute the parasite infracommunity within that individual host (Bush et al., 2001). Within an individual host, the infracommunity of parasites may be interactive through direct or indirect means. Direct interactions through competition for a shared resource, such as food or space (Behnke et al., 2001), would have a negative impact on the subordinate species (Poulin, 2007). Indirect interactions through host immune system responses could have negative effects due to cross immunity or positive effects from immunosuppression (Behnke et al., 2001). Whether the interspecific interactions are positive or negative, the tangible result could be observed as a numerical effect (e.g. asymmetrical differences in parasite numbers, sizes, or fecundity) or a functional effect (e.g. adjustments to infection site) (Poulin, 2007). Evidence of both positive and negative interactions has been reported

from parasite infracommunities. Positive indirect interactions have resulted in an increase in parasite intensities (e.g. Behnke et al., 1978; Presidente et al., 1973; Cattadori et al., 2008). Negative interactions, both direct and indirect, have been reported as a reduction in numbers (e.g. Lang, 1967; Behnke et al., 1977; Dash, 1981; Holland, 1984), fecundity (Silver et al., 1980; Holland, 1984) or a shift in spatial distribution (Holmes, 1961; 1962; Stock and Holmes, 1988; Bush and Holmes, 1986a; 1986b; Patrick, 1991; Haukisalmi and Henttonen, 1993; Ellis et al., 1999). The majority of studies of parasite communities examine adult parasites (sexually mature) which are large and readily identified to species. Fewer studies have examined larval stages that mature in host species higher in the food chain which are typically small, normally encysted and thought to consume few resources (Poulin and Valtonen, 2001).

Furthermore, the relative timing of parasite exposure and infection can have different effects on parasite infracommunities. Concurrent as well as challenge exposures have been shown to affect community dynamics of co-infecting species of parasites (Rynkiewicz et al. 2015). For example, there was a significant decrease in infection success for *Acanthoparyphium* sp. (Digenea) in both simultaneous and challenge infections with *Curtuteria australis* (Digenea) in the bivalve cockle (*Austrovenus stutchburyi*) host (Leung and Poulin, 2011). Further, establishment success of *Ribeiroia ondatrae* (Digenea) in the Pacific chorus frog (*Pseudacris regilla*) decreased by 14% when the host had been previously exposed to *Echinostoma trivolvis* (Digenea) (Hoverman et al., 2013).

Species of *Diplostomum* have a three-host life cycle involving a piscivorous bird as a definitive host, and lymnaeid snails and aquatic vertebrates, primarily fish, as first and second intermediate hosts respectively (Figure 1). The metacercariae are not encysted and those of most species infect the lens, while a few species infect other parts of the eye (vitreous humor, retina) and fewer still, the brain. Species of *Diplostomum* have received much attention due to the fish disease diplostomiasis, most notable with lens-infecting species of *Diplostomum* that cause cataracts, which can be detrimental to the fish host (Karvonen, 2012). Recent studies have shown that multiple species of lens-infecting *Diplostomum* are present in naturally-infected fishes (Galazzo et al., 2002; Niewiadomska and Laskowski, 2002; Locke et al., 2010a; 2010b; Désilets et al., 2013; Georgieva et al., 2013). Furthermore, previous exposure to lens-infecting species of *Diplostomum* has been shown to reduce metacercarial establishment in subsequent exposures (Höglund and Thuvander, 1990; Whyte et al., 1990; Karvonen et al., 2004; Scharsack and Kalbe, 2014; Klemme et al., 2015). Molecular studies have also revealed negative interactions in terms of metacercarial abundance between three lens-infecting species in a survey of 20 species of fish from the

St. Lawrence River, Quebec, Canada (Désilets et al., 2013). Infections are acquired over time (Chappell et al., 1994) and it is unlikely that multiple species are acquired simultaneously (Marcogliese et al., 2001). Therefore, direct and/or indirect interactions along with sequence of establishment could all play a role in infracommunity variation within individual hosts and consequently shape the parasite community of a host population.

Previous experimental studies on interactions between metacercariae of *Diplostomum* spp. have been limited to those occupying different habitats within the host due to difficulties in species level identifications. Despite not sharing the same habitat, establishment/infection success of lens- and humor-infecting *Diplostomum* spp. were negatively affected by both simultaneous and challenge infections (Karvonen et al., 2009; Seppälä et al., 2009; Seppälä et al., 2012). As the lens species must pass through the humor to reach their final destination there may be a brief period of direct interaction between lens and humor species; however, longer term direct interactions could occur between coinciding species (e.g. between two or more lens species). Indirect interactions may also be acting in the short term. Even though metacercariae of species of *Diplostomum* escape the host immune system 24 hours post-cercarial penetration, they have been shown to induce both innate and adaptive immune responses (Chappell et al., 1994). Penetration and migration of cercariae to the final site within the fish host have been shown to stimulate various innate immune responses (Whyte et al., 1989) as well as initiate an adaptive immune response should the host be exposed yet again at a later date (Whyte et al., 1989). This leaves questions of temporal heterogeneity in the colonization process and patterns on infracommunity composition of sympatric lens-infecting species experimentally unverified.

Here, rainbow trout (*Oncorhynchus* mykiss) were exposed to either single or challenge infections with two molecularly-delineated lens-infecting species of *Diplostomum*. The single exposures will allow comparisons of susceptibility of rainbow trout to each species of *Diplostomum* and provide a basis for comparison of average establishment success. The challenge exposures will subsequently expose fish to either the conspecific or a second lens-infecting congener. Challenge exposures will be performed before the onset of the adaptive immune responses (Chappell et al., 1994) to test for interactions and the role of exposure sequence in the establishment of sympatric and taxonomically related parasites. Discerning the presence or absence of interaction and any effect of exposure sequence will provide a greater understanding of variation within the infracommunities of an individual host, the impacts on the overall parasite distribution in the host population and the transmission process to the definitive hosts.

MATERIALS AND METHODS

Cercarial identification

Snails, *Lymnaea* sp., were collected manually from the rocky littoral zone of the Saint-Lawrence River at Pointe-aux-Cascades, Quebec, Canada (45°10'08.3"N 66°59'56.2"W) in late August and early September 2015. Each snail was placed in an individual cup containing 250 ml of dechlorinated water and fed fresh lettuce daily. Two snails shed furcocercariae and a sample of cercariae were collected from each and killed by freezing. The snails described here were the same snails used in the experiments for chapter 1. DNA was extracted from the snails using a DNeasy Blood & Tissue Kit (Qiagen)™ following the manufacturer's protocol. Amplification and sequencing of the partial internal transcribed spacer region of ribosomal DNA (ITS-rDNA) was performed using the primers D1 (F) (5' – AGG AAT TCC TGG TAA GTG CAA G - 3') and D2 (R) (5' CGT TAC TGA GGG AAT CCT GGT – 3') and protocols described by Galazzo et al. (2002) and Locke et al. (2010a). The successful amplicons were sequenced in both directions using the forward and reverse PCR primers at the Genome Quebec Innovation Centre, McGill University in Montreal, Quebec. The successful chromatograms were manually edited and contiguous sequences were assembled in Geneious R8 version 8.0.4 (Kearse et al., 2012) and submitted to GenBank (Accession #s: MH108584 - MH108585).

The two newly generated ITS-rDNA sequences were aligned, trimmed and compared with 28 published ITS-rDNA sequences of *Diplostomum* spp. from Canada and Europe (Galazzo et al., 2002; Locke et al., 2010a; Georgieva et al., 2013; Blasco-Costa et al., 2014; Pérez-Del-Olmo et al., 2014; Locke et al., 2015; Selbach et al., 2015) with Geneious R8 version 8.0.4 (Kearse et al., 2012). The trimmed alignment was used to construct a number-of-difference based neighbor-joining tree with 1000 bootstrap replicates and pairwise deletion of gaps using a published sequence from GenBank for *Tylodelphus scheuringi* (Moszczynska et al., 2009) for the outgroup (Blasco-Costa et al., 2014; Selbach et al., 2015) in MEGA version 6 (Tamura et al., 2013). The NJ analysis of the ITS-rDNA dataset (based on a final dataset with gaps removed of 808 base pairs) provided robust evidence where one isolate (C290) clustered with *Diplostomum* sp. 1 (GQ292522.1, Locke et al., 2010a; KT186794.1, Locke et al., 2015) and one isolate (C305) clustered with *Diplostomum* sp. 4 (GQ292520.1, Locke et al., 2010a) (Appendix 1). While it is rare for snails to be concurrently infected with more than one species of parasite (Soldánová et al., 2012), the trimmed alignments (Appendix 2) were analyzed for diagnostic sites (Appendix 3) and the peaks of the original chromatograms (Appendix 4) were re-examined at the diagnostic sites to validate species

identification. No ambiguities were observed, confirming that each individual snail was infected with a single species of *Diplostomum*. Based on these results, the cercariae were molecularly delineated to species as two lens-infecting species, *Diplostomum* sp. 1 and *Diplostomum* sp. 4, using provisional nomenclature based on Locke et al. (2010a).

Exposure protocol

All the animals used in this experiment were maintained and handled in accordance with the guidelines of the Canadian Council of Animal Care. The experiments were officially approved by the Concordia University Animal Research Ethics Committee (AREC, certificate #30000269).

Naïve rainbow trout, between 22 – 83 mm, were obtained from a commercial farmer in Sainte-Eldwidge, Quebec. The fish were maintained in aerated aquariums and fed commercial fish food daily. The experiment involved seven treatments for a total of 34 fish replicates per treatment. The treatments consisted of two single exposure groups (Single 1 and Single 4), and four challenge experiments where the pair symbolize 1st exposure followed by 2nd exposure: *Diplostomum* sp. 1 X *Diplostomum* sp. 1 (1 X 1); *Diplostomum* sp. 1 X *Diplostomum* sp. 4 (1 X 4); *Diplostomum* sp. 4 X *Diplostomum* sp. 1 (4 X 1); *Diplostomum* sp. 4 X *Diplostomum* sp. 4 (4 X 4). The two single exposure groups were exposed only once to either *Diplostomum* sp. 1 (Single 1) or *Diplostomum* sp. 4 (Single 4) to determine mean metacercarial number and length seven days post-exposure (PE). The four experimental groups were initially exposed to one species and then challenged five days later by subsequent exposure to either the conspecific or the congener. The control group underwent the full experimental procedure but was not exposed to any cercariae to ascertain the protocol did not cause fish mortality.

The evening prior to fish exposure, each infected snail was placed in 250 ml of fresh water to stimulate cercarial shedding in ambient artificial light. Exposure beakers were filled with 250 ml of dechlorinated water at a temperature of 17 - 18°C and aerated overnight to fully saturate the water with oxygen. The cercariae of each species of *Diplostomum* were collected the following morning, approximately 12 hours later. The aeration of the exposure beakers was stopped and a dose of 20 haphazardly chosen cercariae was added to each beaker by pipette. In order to get accurate indications representative of natural infections, a small exposure dose was chosen over a larger dose of either 50 or 100 cercariae (Poulin, 2010).

Once the beakers were dosed for each treatment, a single fish was placed in an individual beaker and left undisturbed for one hour. The beakers were not aerated during the exposure to avoid possible effects on cercarial swimming behaviour. Upon completion of exposure, fish were removed from the beakers with the use of a net and the fish was rinsed with water between transfers to reduce any carry over of cercariae and placed in an aerated aquarium separated by treatment to which they were exposed.

After five days, fish in the four experimental groups were challenged with either the same species as the original exposure (1 X 1 and 4 X 4) or to the congener (1 X 4 and 4 X 1). The protocol for the challenge treatments was identical to the first exposure protocol. Upon completion of the second exposure, the fish were returned to their separate aquariums identified by treatment. The experiment was replicated on over a period of one month (dependent on space and cercarial availability). The movement of cercariae from the penetration site to the lens of the eye is usually completed within 24 hours (Chappell et al., 1994). The five-day delay between initial and challenge exposure was to allow for growth of the metacercariae in the initial infection, thus permitting differentiation by size from those in the second exposure.

All fish for each treatment were euthanized seven days post initial exposure, with an overdose a 0.1% concentration of clove oil and frozen at -20°C until dissection. The total fork length (tip of the snout to the end of the middle caudal ray fin) of the fish was measured (± 1 mm). The lenses of the fish were removed and dissected separately and the number of metacercariae determined for each lens using a stereomicroscope.

Metacercariae 48 hours PE were smaller than those seven days PE (Klemme et al., 2015; Sweeting, 1974). They were sorted by size using a stereomicroscope to separate them into first and second exposure cohorts. If all the metacercariae in the challenge exposures were of the same size, they were compared to conspecific metacercariae from the single exposure group to confirm the exposure cohort. A haphazard sample of 10 metacercariae per treatment from 10 different fish per exposure with varying infection levels was selected to measure their lengths (μm) using a Leica Microsystem stereomicroscope with LAS X version 3.8.

Data analysis

Descriptive statistics of prevalence (percentage of fish infected), mean (± standard deviation, SD) along with median and range values for each species of *Diplostomum* for each treatment for the following variables were calculated: fish size (mm), metacercarial length (μm) and number. Outliers were assessed by converting raw data into standardize scores, where cases with a *z*-score ± 3.00 were considered potential outliers (Tabachnick and Fidell, 2006). Normality was tested with skew and kurtosis ($p < 0.01$) and homoscedasticity was tested with Levene's Test ($p < 0.05$: Tabachnick and Fidell, 2006).

Fish fork length data were normal and homoscedastic and had no outliers. A one-way analysis of variance (ANOVA) was used to determine if there was a difference in fish length among treatments. Pearson correlations were calculated to determine if fish length was correlated with the establishment success for each species of *Diplostomum* in the single exposures.

Metacercarial length data were normal but heteroscedastic and had no outliers. Transformations on metacercarial length were unsuccessful in transforming the data from heteroscedastic to homoscedastic. A Welch's Test (as the data were heteroscedastic) was calculated to determine if there was a difference in metacercarial size between first and second exposures for each challenge treatment with Tamhane's T2 (adjusting for heteroscedasticity) post-hoc tests calculated to measure the difference among relevant pairwise comparisons of the exposures.

Data for the number of metacercariae had two outliers which were retained within the analyses. Data were skewed and heteroscedastic and transformations were unsuccessful. Non-parametric related samples sign tests were used to determine if there were significant differences between the numbers of metacercariae established in the left and right eye for each exposure for each treatment. There were no significant differences between left and right lenses in number of metacercariae established for any of the exposures (p between 0.312 and 1.000) and the total number of metacercariae for the left and right eyes were combined for each fish for subsequent analyses. A non-parametric Kruskal-Wallis test was calculated to determine if there was a significant difference in metacercarial numbers between treatments and exposures followed by Mann Whitney U post-hoc tests to measure the difference among relevant pairwise comparisons. Sex of the rainbow trout was not taken into consideration as the fish used in the experiment were not sexually mature. Probabilities were Bonferroni corrected to adjust for multiple

comparisons and values of ≤ 0.005 were considered significant. Statistical analyses were conducted using the SPSS® 24.0 software package.

RESULTS

Overall fish infections

No fish died prematurely during the experiment. Some fish failed to become infected in each cercarial-exposure treatment and prevalence ranged from 70.59 – 97.06 % (Table 2). There was no statistically significant difference in fish length between any of the treatments (ANOVA, $F_5 = 0.054$, $p = 0.998$: Table 2). There was a significant negative association between fish length and the number of metacercariae established for both species where smaller fish had a greater number of metacercariae (*Diplostomum* sp. 1: Pearson correlation: $r_{34} = -0.796$, $p < 0.001$; *Diplostomum* sp. 4: Pearson correlation: $r_{34} = -0.543$, $p = 0.001$).

Metacercarial length

There was a significant difference in mean metacercarial length between exposures and species (Welch's Test, $F_{9,\ 36.514} = 2025.987$, $p < 0.001$). Post-hoc comparisons indicated the mean length of metacercariae of *Diplostomum* sp. 1 (175.11 ± 2.82) was significantly larger than those of *Diplostomum* sp. 4 (163.51 ± 3.12: $p < 0.001$) from single and first exposures (Table 3). The mean length of metacercariae from the second exposures for each species was significantly smaller than that of the conspecifics or congeners from the first exposures ($p < 0.001$). The difference in metacercarial size between first and second exposures allowed for visual separation into cohorts (Table 3).

Number of metacercariae

The mean (± SD) number of metacercariae for the single exposure treatments was 4.88 (± 4.21) and 3.62 (± 3.80) for *Diplostomum* sp. 1 and *Diplostomum* sp. 4 respectively (Table 4). There was a significant difference in the number of metacercariae among treatment groups (Kruskal-Wallis, $H_9 = 74.899$, $p = 0.001$). Post-hoc Mann Whitney U comparisons indicated no significant difference in mean number of metacercariae between the species in the single exposure groups ($p = 0.261$), rainbow trout were equally susceptible to either species of *Diplostomum* (Table 4, Figure 3). Further, there was no difference between initial exposures compared to the baseline single exposure establishment of the same

species (*Diplostomum* sp. 1 first exposure in 1 X 1: $p = 0.605$; *Diplostomum* sp. 1 first exposure in 1 X 4: $p = 0.196$; *Diplostomum* sp. 4 first exposure in 4 X 1: $p = 0.508$; *Diplostomum* sp. 4 first exposure in 4 X 4: $p = 0.528$: Table 4, Figure 3). There was a significant decrease in the number of metacercariae of *Diplostomum* sp. 1 in challenge exposures compared to initial exposure of either the conspecific or congener ($p < 0.001$: Table 4, Figure 3). There was no significant difference in number of metacercariae of *Diplostomum* sp. 4 in challenge exposures in fish initially exposed to either the conspecific ($p = 0.733$) or the congener ($p = 0.305$: Table 4, Figure 3).

DISCUSSION

Effects of temporal heterogeneity in establishment success in the fish host for sympatric lens infecting species of *Diplostomum* had been previously untested. In single species infections, *Diplostomum* sp. 1 and *Diplostomum* sp. 4 had equal intensity in establishment success in rainbow trout. *Diplostomum* sp. 4 showed no difference in establishment success between single exposure, conspecific or congener challenge exposures. However, there was a significant decline in establishment of *Diplostomum* sp. 1 in challenge infections with both the conspecific and congener, as compared to the baseline single exposure establishment. Our results revealed evidence of a 10% (intraspecific) to 18.6% (interspecific) reduction in mean number of established metacercariae in challenge exposures of *Diplostomum* sp. 1, suggesting importance the of primary establishment for *Diplostomum* sp. 1.

Naïve rainbow trout were equally susceptible to *Diplostomum* sp. 1 and *Diplostomum* sp. 4 in single species exposures. This suggests little difference between these two species of *Diplostomum* in their ability to successfully attach, penetrate and migrate to the lens of previously unexposed rainbow trout under the conditions described here. These results differ from previous unpublished experimental observations (Chapter 1), which showed *Diplostomum* sp. 4 to have a greater infectivity than *Diplostomum* sp. 1. The samples sizes used herein, may not have been large enough to detect a difference between infectivity in single exposures. Another explanation may be that the age of infection of the snail is unknown and the quality of the furcocercariae released from the snail may change over time reducing their infectivity.

Smaller fish had a greater number of metacercariae for both species of *Diplostomum*. A negative association with fish size and metacercarial establishment has previously been reported for *D. spathaceum* in rainbow trout due to differences in cercarial density (Höglund, 1995). Here, however,

cercarial density was kept constant. And while larger hosts potentially represent more resources to favor an increase in colonisation (Poulin, 2007), the longer migration route to the final destination may require greater energy reserves and decrease establishment success.

Earlier studies have reported a decrease in establishment success of *Diplostomum* spp. in previously exposed rainbow trout (Höglund and Thuvander, 1990; Whyte et al., 1990; Karvonen et al., 2004; Scharsack and Kalbe, 2014; Klemme et al., 2015). However, there was no difference in establishment success for *Diplostomum* sp. 4 in challenge infections following initial exposure to its conspecific or its congener. Yet, the exact opposite was observed for challenge infections of *Diplostomum* sp. 1. If the reduction in challenge *Diplostomum* sp. 1 seen here is a direct mode of action, the benefit of excluding both conspecifics and congeners would be due to a restriction of space or resources (Poulin, 2007). However, even though the size of the lens must be taken into consideration, hundreds of metacercariae have been reported per lens (Karvonen, 2012). Further, an increase in metacercarial establishment would not only increase transmission success (Karvonen, 2012), but also increase the genetic diversity and mating possibilities within the definitive host (Poulin, 2007), making direct interaction a less likely cause.

While this study was not designed to test mechanisms, conceivably indirect interactions through host immune system responses may be negatively impacting challenge *Diplostomum* sp. 1 establishment. Here, the immune response would be reflecting a generalized innate immune response to infection as it is mounted whether the fish is first exposed to either *Diplostomum* sp. 1 or *Diplostomum* sp. 4, but the mounted response is only effective at reducing subsequent challenges of *Diplostomum* sp. 1. Additionally, as the challenge exposure was only five days PE and acquired immune responses take several weeks to be initiated (Chappell et al., 1994), speculatively, only innate immune responses would have been activated.

Nonspecific cellular responses with an increase in neutrophils and monocytes within two weeks of exposure have been associated with *D. spathaceum* exposures in rainbow trout (Höglund and Thuvander, 1990). Further, increases in innate and not adaptive responses were observed in three-spined stickleback (*Gasterosteus aculeatus*) to repeated exposures of *Diplostomum pseudospathaceum* with an overall decrease in infection success (Scharsack and Kalbe, 2014). As Kurtz (2005) argues, if certain innate immune functions remain enhanced for a period of time following the initial exposure, there may be a reduction of infection upon subsequent exposure and there is no need for such immunological priming to be specific.

However, variations in genotypes of *D. pseudospathaceum* in sticklebacks (*Gasterosteus aculeatus*) were shown to have varying levels of infection success (Rauch, Kalbe and Reusch, 2006) and were later shown to produce different innate immune responses (e.g. increased transcription of genes in phagocytosis, cell migration, antigen presentation or complement activation), which had different levels of success on different genotypes (Haase et al., 2016). Asymmetrical interactions between infection success were observed with a reduction in ranavirus infection success when larval grey treefrogs (*Hyla versicolor*) were previously infected with the trematode *Echinoparyphium* sp. (Wuerthner, Hua and Hoverman, 2017). Similar results were reported in the Pacific chorus frog where *R. ondatrae* had reduced establishment success in challenge infections two days PE with conspecifics or with *E. trivolvia* (Hoverman et al., 2013). However, Hoverman et al. (2013) attributed the decreased establishment to adaptive immune responses even though the challenge infection was administered before a naïve host would have been capable of mounting a specific defense mechanism. Rare innate immune cells such as group 2 innate lymphoid cells have been shown to be long lived, have memory and are activated through various pathways, sharing characteristics with adaptive cells (Webb and Wonjo, 2017). Therefore, it is conceivable that different species of *Diplostomum* trigger different innate immune responses and the mechanism would have an effect on subsequent *Diplostomum* sp. 1 cercariae only, implying potential specificity and at least short-term memory of innate response.

Diplostomum sp. 1 and *Diplostomum* sp. 4 have been reported from the same localities (Locke et al., 2010a, 2010b; Désilets et al., 2013). Further, these two lens infecting species have been reported to be negatively associated with *Diplostomum* sp. 1 infecting smaller fish (Désilets et al., 2013). The results here tentatively corroborate the field data in that *Diplostomum* sp. 1 would not be as successful at infecting a fish previously infected with *Diplostomum* sp. 4.

In conclusion, there is evidence of the importance of priority of establishment for infection success in challenge exposures of *Diplostomum* sp. 1 but not for *Diplostomum* sp. 4. Further, as smaller, hence younger, fish had a greater number of metacercariae, there is an effect of host age on establishment success that requires further inquiry. A short term innate immune response may be negatively impacting the establishment of *Diplostomum* sp. 1, while leaving *Diplostomum* sp. 4 unaffected. As hosts are exposed to parasites throughout their lifetime, future studies investigating priority effects and multiple repeated exposures will increase our understanding of factors affecting

parasite community assemblages and dynamics and are essential for understanding host–parasite interactions.

Table 2. Summary statistics describing the overall prevalence of infection and size of rainbow trout (*Oncorhynchus mykiss*) for each experimental treatment involving either single or challenge infections of two molecularly-delineated species: *Diplostomum* sp. 1 and *Diplostomum* sp. 4. Treatments for single exposures are designated as Single 1 (fish were exposed to *Diplostomum* sp. 1) or Single 4 (fish were exposed to *Diplostomum* sp. 4). The experimental group pairs symbolize 1st exposure X 2nd exposure: *Diplostomum* sp. 1 X *Diplostomum* sp. 1 (1 X 1); *Diplostomum* sp. 1 X *Diplostomum* sp. 4 (1 X 4); *Diplostomum* sp. 4 X *Diplostomum* sp. 1 (4 X 1); *Diplostomum* sp. 4 X *Diplostomum* sp. 4 (4 X 4). There was no statistically significant difference in fish length between any of the treatments (ANOVA, $F_5 = 0.054$, $p = 0.998$).

Treatment	Sample size (n)	Prevalence	Mean length (mm) ± SD	Range (mm)
Single 1	34	73.53%	47.85 ± 16.20	46.00 - 72.00
1 X 1	34	97.06%	42.82 ± 15.86	47.00 - 71.00
1 X 4	34	97.06%	46.56 ± 16.00	25.00 - 71.00
Single 4	34	70.59%	47.91 ± 16.03	26.00 - 82.00
4 X 1	34	82.35%	46.62 ± 15.45	23.00 - 72.00
4 X 4	34	91.18%	46.76 ± 13.39	26.00 - 70.00

Table 3. Summary statistics describing the length of metacercariae recovered for each experimental treatment involving either single or challenge infections of two molecularly-delineated species: *Diplostomum* sp. 1 and *Diplostomum* sp. 4. Treatments for single exposures are designated as Single 1 (fish were exposed to *Diplostomum* sp. 1) or Single 4 (fish were exposed to *Diplostomum* sp. 4). The experimental group pairs symbolize 1st exposure X 2nd exposure: *Diplostomum* sp. 1 X *Diplostomum* sp. 1 (1 X 1); *Diplostomum* sp. 1 X *Diplostomum* sp. 4 (1 X 4); *Diplostomum* sp. 4 X *Diplostomum* sp. 1 (4 X 1); *Diplostomum* sp. 4 X *Diplostomum* sp. 4 (4 X 4). There was a significant difference in mean metacercarial length between exposures and species (Welch's Test, $F_{9,\ 36.514} = 2025.987$, $p < 0.001$). Post-hoc comparisons indicated the mean length of metacercariae of *Diplostomum* sp. 1 was significantly larger than those of *Diplostomum* sp. 4 ($p < 0.001$) from single and first exposures. The mean length of metacercariae from the second exposures for each species was significantly smaller than that of the conspecifics or congeners from the first exposures ($p < 0.001$). Significant differences are indicated in bold.

Treatment	Species	Sample size (n)	Mean (µm) ± SD	Range (µm)
Single 1	***Diplostomum* sp. 1**	10	175.11 ± 2.82	171.85 - 180.4
1 X 1	1st exposure: *Diplostomum* sp. 1	10	175.65 ± 2.19	171.69 - 178.75
	Challenge: *Diplostomum* sp. 1	10	135.96 ± 2.33	132.26 - 138.84
1 X 4	1st exposure: *Diplostomum* sp. 1	10	175.29 ± 1.64	172.65 - 177.65
	Challenge: *Diplostomum* sp. 4	10	109.55 ± 1.41	107.66 - 111.99

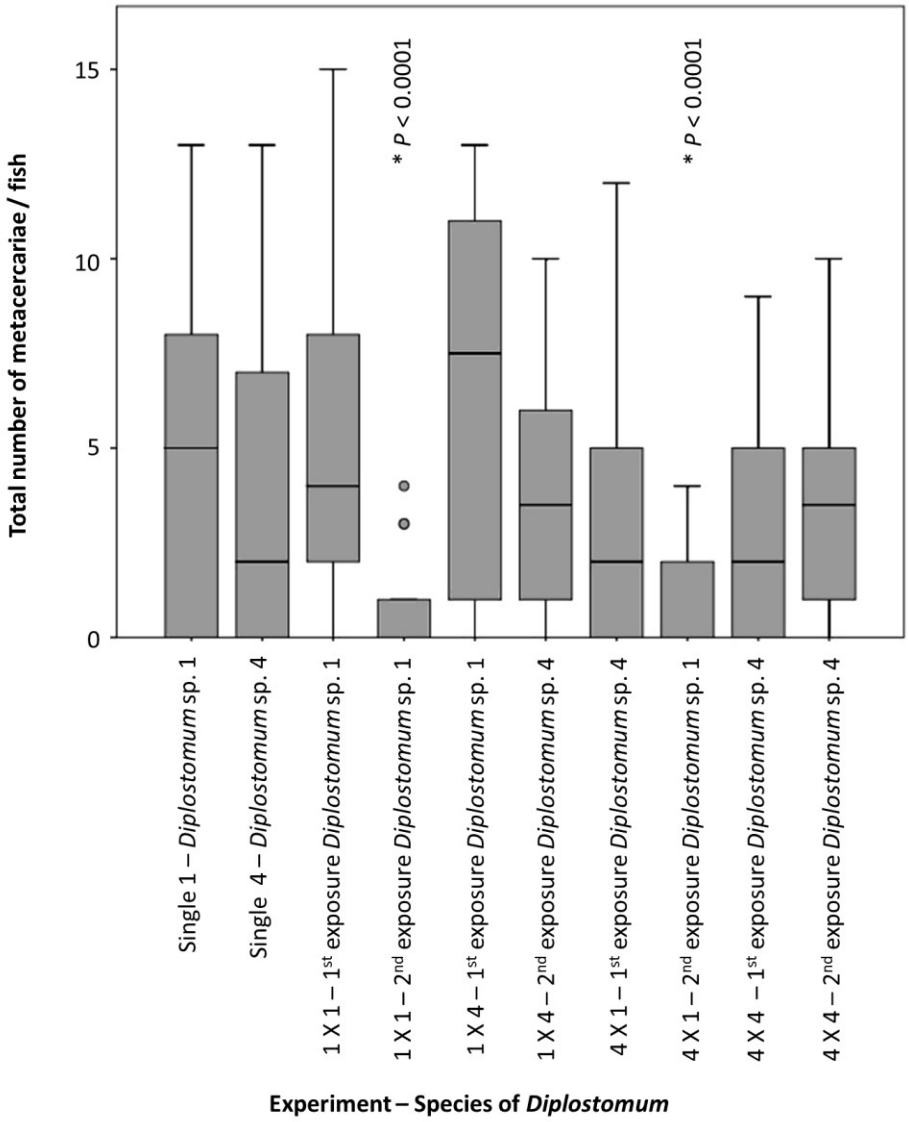
Single 4	*Diplostomum* sp. 4	10	163.51 ± 3.12	160.03 - 169.19
4 X 1	1st exposure: *Diplostomum* sp. 4	10	163.60 ± 3.07	159.99 - 168.51
	Challenge: *Diplostomum* sp. 1	10	133.99 ± 2.17	131.27 - 137.22
4 X 4	1st exposure: *Diplostomum* sp. 4	10	162.25 ± 1.33	160.05 - 164.54
	Challenge: *Diplostomum* sp. 4	10	110.39 ± 1.50	108.00 - 112.93

Table 4. Summary statistics describing the number of metacercariae recovered for each experimental treatment involving either single or challenge infections of two molecularly-delineated species: *Diplostomum* sp. 1 and *Diplostomum* sp. 4. Treatments for single exposures are designated as Single 1 (fish were exposed to *Diplostomum* sp. 1) or Single 4 (fish were exposed to *Diplostomum* sp. 4). The experimental group pairs symbolize 1st exposure X 2nd exposure: *Diplostomum* sp. 1 X *Diplostomum* sp. 1 (1 X 1); *Diplostomum* sp. 1 X *Diplostomum* sp. 4 (1 X 4); *Diplostomum* sp. 4 X *Diplostomum* sp. 1 (4 X 1); *Diplostomum* sp. 4 X *Diplostomum* sp. 4 (4 X 4). There was a significant difference in the number of metacercariae among treatment groups (Kruskal-Wallis, $H_9 = 74.899$, $p = 0.001$). Post-hoc Mann Whitney U comparisons indicated no significant difference in mean number of metacercariae between the species in the single exposure groups. Further, there was no difference between initial exposures compared to the baseline single exposure establishment of the same species. There was a significant decrease in the number of metacercariae of *Diplostomum* sp. 1 in challenge exposures compared to initial exposure of either the conspecific or congener. There was no significant difference in number of metacercariae of *Diplostomum* sp. 4 in challenge exposures in fish initially exposed to either the conspecific or the congener. Significant differences are indicated in bold.

Treatment	**Species**	**Mean ± SD**	**Median**	**Range**
Single 1	*Diplostomum* sp. 1	4.88 ± 4.21	5.00	0 - 13
1 X 1	1st exposure: *Diplostomum* sp. 1	5.21 ± 4.17	4.00	0 - 15
	Challenge: *Diplostomum* sp. 1	0.50 ± 0.99	0.00	0 - 4
1 X 4	1st exposure: *Diplostomum* sp. 1	6.21 ± 4.78	7.50	0 - 13
1 X 4	Challenge: *Diplostomum* sp. 4	3.94 ± 2.88	3.50	0 - 10

Single 4	*Diplostomum* sp. 4	3.62 ± 3.80	2.00	0 - 13
4 X 1	1st exposure: *Diplostomum* sp. 4	2.88 ± 3.15	2.00	0 - 12
	Challenge: *Diplostomum* sp. 1	0.91 ± 1.19	0.00	0 - 4
4 X 4	1st exposure: *Diplostomum* sp. 4	2.82 ± 2.78	2.00	0 - 9
	Challenge: *Diplostomum* sp. 4	3.44 ± 2.75	3.50	0 - 10

Figure 3. Box and whisker plots for the total numbers of metacercariae of *Diplostomum* sp. 1 and *Diplostomum* sp. 4 in experimental single-species and challenge infections of rainbow trout (*Oncorhynchus mykiss*). The box represents the interquartile range and the horizontal line within the box represents the median. The whiskers end at the largest and smallest value excluding any outliers, and the circles are non-statistically significant outliers. There was a significant difference in the number of metacercariae among treatment groups (Kruskal-Wallis, $H_9 = 74.899$, $p = 0.001$). An asterisk and significant *p* values have been indicated on the figure for the experimental groups which differ from the conspecific single exposure treatments.



CHAPTER 3. The spatial distribution and fecundity of sympatric species of *Diplostomum* (Digenea) in single and mixed species infections in the intestine of the ring-billed gull (*Larus delawarensis*)

ABSTRACT: Interactions between parasite species may influence their distribution and abundance within communities. Experimental single species infections of *Diplostomum* spp. in the gut of the definitive host, ringed-billed gulls (*Larus delawarensis*), were compared to mixed species infections to explore interactions among parasites. Three species of *Diplostomum* (Digenea), designated as *Diplostomum* sp. 1, *Diplostomum* sp. 4 and *Diplostomum baeri* were examined for intestinal distribution and fecundity in single and mixed infections. In single species infections, most specimens of *Diplostomum* sp. 1 and *D. baeri* were recovered from the mid-region of the intestine, whereas *Diplostomum* sp. 4 were mainly present in the anterior region. Significant spatial displacement was observed only for *D. baeri* when *Diplostomum* sp. 1 was also present. Intensity was directly correlated to the number of occupied intestinal segments, and there was no significant difference in mean linear span for each species between single and mixed species infections. *Diplostomum* sp. 4 had the highest mean number of eggs per worm *in utero* in single species infections. In mixed species infections, *Diplostomum* sp. 4 experienced a dramatic decline in fecundity in the presence of *Diplostomum* sp. 1 whereas fecundity of *Diplostomum* sp. 1 increased in mixed infections with *D. baeri*. These results highlight interspecific interactions which may play a role in population dynamics of *Diplostomum* spp. and community structure.

INTRODUCTION

A parasite's niche, its physical distribution and ecological role, is dependent on many different factors such as the presence of other species of parasites and potential interactions among them. In single species infections, adult parasites normally occupy a specific and predictable site within the host where conditions are optimal for growth and reproduction (Holmes and Price, 1986). However, the factors that influence the parasite niche are difficult to quantify. For gut parasites, parasitologists often use the linear axis of the intestine as a simplified but informative measure of the spatial dimension of niche (Poulin, 2007). The fundamental niche, in terms of spatial location, is the potential distribution of the parasite in the host where it can develop, whereas the realized niche is a subset of the fundamental niche and consists of sites actually occupied by parasites in the host due to antagonistic interactions with a competing species (Poulin, 2007). In natural conditions an individual host may be infected by multiple

species of parasites, some of which may share an optimal site or resources within that host (Poulin, 2007). In cases where species of parasites are likely to co-occur in potentially large numbers and where their fundamental niches overlap, competition may exert selective pressures on the niche dimensions of the species within the infracommunity if resources are limited (Poulin, 2007). However, in low density populations or in situations where co-occurrence is rare, interspecific interactions are rarely the driving forces shaping infracommunity structure and composition (Price, 1980). Rather, optimal sites for reproductive success or resource acquisition would determine the realized niche (Poulin, 2007).

Interspecific interactions can result in a numerical effect (e.g. asymmetrical differences in parasite numbers, reduction in body mass or fecundity) or a functional effect (e.g. adjustments to infection site) where small interspecific differences in ability to exploit host resources will determine infection success (Poulin, 2007). An antagonistic numerical effect was demonstrated in the nematode *Nippostrongylus brasiliensis* which displayed a decrease in establishment success and fecundity in rats with a previously established acanthocephalan, *Moniliformis dubius* (Holland, 1984). A synergistic numerical effect was observed in sheep (*Ovis* sp.) where the nematode *Haemonchus contortus* experienced an increase in fecundity and delayed expulsion when concurrently infected with the trematode *Fasciola hepatica* (Presidente et al., 1973). Stock and Holmes (1988) observed a functional effect where the most anterior and median individuals of the cestode, *Wardium paraporale*, exhibited a posterior shift when the numbers of the co-occurring cestode, *Diorchis* sp., increased within the gut of four species of grebes (Family: Podicipedidae). Functional effects have also been demonstrated in the helminth community of flying squirrels (*Glaucomys volans*) where the nematodes, *Strongyloides robustus* and *Capillaria americana*, had overlapping fundamental niches in experimental single infections but very little overlap in their realized niches in co-infections (Patrick, 1991). These examples illustrate effects of interspecific interactions on infracommunity structure and dynamics.

Species of *Diplostomum* (Subclass: Digenea) have a three-host life cycle. A generalized life cycle of *Diplostomum* spp. includes a piscivorous bird, primarily gulls, as their definitive hosts, a lymnaeid snail as first intermediate host and an aquatic vertebrate, primarily fish, as second intermediate host. Cercariae emerging from the snail attach and penetrate a fish host. Once in the fish, the cercariae migrate to the lens, humor or retina of the eye or in rare cases to the brain, depending on the species, where they transform into un-encysted metacercariae. Infections have been reported from various species of fish hosts and are acquired over time (Chappell et al., 1994). Further, host age has been shown to have a

significant effect of the infracommunity structure of lens-infecting species of *Diplostomum*, where older fish had higher infection intensities and species richness (Désilets et al., 2013). There is a high probability of mixed species of *Diplostomum* in the gut of the definitive host in natural conditions due to the diversity of sympatric species of *Diplostomum* metacercariae in fishes (Locke et al., 2010a, 2010b; Désilets et al., 2013; Georgieva et al., 2013), as well as the occurrence of mixed species infections in individual fish (Désilets et al., 2013).

Previous experimental (Rees, 1955; Hoffman and Hundley, 1957; Dick and Rosen, 1981; Niewiadomska, 1984; Shostak et al., 1987; Field and Irwin, 1995; McKeown and Irwin, 1995; Karvonen et al., 2006) and field (Karvonen et al., 2006) studies have examined the distribution of several species of adult *Diplostomum* in the gut of various species of avian hosts (Dick and Rosen, 1981; Shostak et al., 1987; McKeown and Irwin, 1995; Field and Irwin, 1995). However, until recently, different species could not be distinguished without molecular techniques (Locke et al., 2010a, 2010b; Désilets et al., 2013; Georgieva et al., 2013). Thus, the reliability of reported spatial distributions is unclear. Two studies have examined mixed species infections (Dick and Rosen, 1981; Karvonen et al., 2006) but these were not compared to controlled single species infections. In experimental mixed infections *Diplostomum spathaceum indistinctum* was reported from the anterior 20% and *D. baeri bucculentum* the anterior 60% of the intestine of herring gulls (*Larus argentatus*) (Dick and Rosen, 1981). In both field and experimental studies, *D. pseudospathaceum* occupied a predominantly anterior position and *D. spathaceum* a posterior position in herring gulls and common gulls (*Larus canus*) (Karvonen et al., 2006). Two experimental studies used chickens (Field and Irwin, 1995; McKeown and Irwin, 1995) rather than the natural definitive hosts, which may result in differences in the optimal infection site compared to that in the natural host. For example, *D. spathaceum* was reported from the anterior third in experimentally infected chickens (Field and Irwin, 1995; McKeown and Irwin, 1995), but from the anterior and middle thirds in experimentally infected black-headed gulls (*Larus ridibundus*) (Niewiadomska, 1984), posteriorly from naturally infected herring and common gulls and both anteriorly and posteriorly in experimental infections (Karvonen et al., 2006). A single study examined fecundity in field and experimental mixed infections of *D. spathaceum* and *D. pseudospathaceum* and observed no differences in mean egg number, but comparisons to control single infections were not made (Karvonen et al., 2006).

In this study, laboratory raised ring-billed gull (*Larus delawarensis*) chicks, a natural definitive host, were experimentally exposed to single or mixed species infections of molecularly delineated species

of *Diplostomum.* The first objective was to establish the spatial distribution of each species along the length of the gut in controlled single-species infections. If two species of *Diplostomum* occupy the same site and there is spatial overlap within the gull intestine in single-species infections, there may be a spatial displacement in terms of range and average position in the mixed-species infections due to competition for space and/or nutrients. The second objective was to estimate fecundity in terms of number of eggs present *in utero* at the time of recovery in controlled, single-species infections and then test for a numerical effect by comparing it to the fecundity of the same species in the presence of a potential competitor. If the co-infecting species compete for space or resources, this may be manifested in a reduction in fecundity of the subordinate species when they co-occur (Poulin, 2007).

MATERIALS AND METHODS

Snail collection and molecular identification of cercariae

Marsh pond snails (*Lymnaea elodes*) were haphazardly collected manually from the rocky littoral zone of Wheaton Lake, Bocabec, New Brunswick, Canada (45°10'08.3"N 66°59'56.2"W) during September 2012, September and October 2013, and June 2014. Snails were transported to the laboratory in aerated coolers (45.5 L) filled with lake water. Upon arrival at the laboratory, snails were placed in individual cups with approximately 250 ml of dechlorinated water to stimulate cercarial shedding. The following morning, the water was examined for cercariae with the use of a stereomicroscope. Snails shedding furcocercariae were identified by labelling the individual cup and a sample of cercariae (approximately 50) shed by each snail was collected and killed by freezing. Snails were maintained in the laboratory at ambient temperature, exposed daily to nine hours of artificial ambient light (9:00 am – 6:00 pm) in individual cups with approximately 250 ml of dechlorinated water and fed lettuce *ad libitum*, with weekly water changes.

The DNA of the cercariae was subsequently extracted using a DNeasy Blood & Tissue Kit (Qiagen)^{TM} following the manufacturer's protocol. Amplification of the partial internal transcribed spacer region of ribosomal DNA (ITS-rDNA) was performed using the primers D1 (F) (5' – AGG AAT TCC TGG TAA GTG CAA G - 3') and D2 (R) (5' CGT TAC TGA GGG AAT CCT GGT – 3') and protocol described in Galazzo et al. (2002) and Locke et al. (2010a).

The amplicons were sequenced in both directions using the forward and reverse PCR primers at the Genome Quebec Innovation Centre, McGill University in Montreal, Quebec, Canada. The chromatograms were manually edited and contiguous sequences were assembled and submitted to

GenBank (Accession #s KY358236 – KY358240). The new ITS-rDNA sequences generated were aligned with 28 published ITS-rDNA sequences of species of *Diplostomum* from Canada and Europe (Galazzo et al., 2002; Locke et al., 2010a, 2015; Georgieva et al., 2013; Blasco-Costa et al., 2014; Pérez-Del-Olmo et al., 2014; Selbach et al., 2015). Editing, assembly and alignment were all performed in Geneious R8 versions 8.0.4 (Kearse et al., 2012). The trimmed alignments were then used to construct a number-of-difference based neighbor-joining (NJ) tree with 1000 bootstrap replicates and pairwise deletion of gaps using a published sequence from GenBank for *Tylodelphus scheuringi* by Moszczynska et al. (2009) as the outgroup (Blasco-Costa et al., 2014; Selbach et al., 2015) in MEGA version 6 (Tamura et al., 2013).

The NJ analysis of the ITS-rDNA dataset (based on a final dataset with gaps removed of 884 base pairs) provided robust evidence that 12 isolates clustered with *Diplostomum* sp. 1 (GQ292522.1, Locke et al., 2010a), four isolates clustered with *Diplostomum* sp. 4 (GQ292520.1, Locke et al., 2010a) and seven isolates clustered with *D. baeri* (JX986856.1, Georgieva et al., 2013) (Appendix 5). While it is rare for snails to be concurrently infected with more than one species of parasite (Soldánová et al., 2012), the trimmed alignments (Appendix 2) were analyzed for diagnostic sites (Appendix 3) and the peaks of the original chromatograms (Appendix 4) were re-examined at the diagnostic sites to validate species identification. No ambiguities were observed, confirming that each individual snail was infected with a single species of *Diplostomum*. The cercariae were designated as *Diplostomum* sp. 1 and *Diplostomum* sp. 4, both of which are found in the lens, using provisional nomenclature based on Locke et al. (2010a) and *D. baeri*, which infects the humor, using nomenclature based on Georgieva et al. (2013).

Laboratory establishment of the *Diplostomum* life cycle

All the animals used in this experiment were maintained and handled in accordance with the guidelines of the Canadian Council of Animal Care. Naïve rainbow trout (*Oncorhynchus mykiss*), approximately 5 cm in total length, were obtained from a commercial farmer in Sainte-Eldwidge, Quebec. Newly hatched day-old ring-billed gull chicks (*L. delawarensis*) were captured by hand from their nests from Île Deslauriers, Quebec, (45°42'45.1"N 73°26'25.0"W) in May 2013 and 2014 and maintained under authority of permits issued by the Canadian Wildlife Service (Quebec). The experiments were officially approved by the Concordia University Animal Research Ethics Committee (AREC, certificate #30000269).

Experimental infection of fish

The evening prior to fish-exposures, the water was changed for each infected snail to stimulate fresh cercarial shedding. The cercariae of each species of *Diplostomum* were collected the following morning, approximately 12 hours later. Previously uninfected rainbow trout (approximately 5 cm in total length) were exposed in six groups of 10 individuals each to approximately 250-500 cercariae of *Diplostomum* sp. 1 for one hour in an aerated tank containing approximately 5L of water at 18°C. Two similar groups of 60 fish were exposed in the same manner to cercariae of *Diplostomum* sp. 4 and *D. baeri* respectively. The fish exposed to each species of fluke were placed in separate flow-through tanks (170.3 L) and fed commercial fish food once daily for a minimum of two months prior to use.

Experimental infection of gulls

The gulls were held in the laboratory in a single enclosure (3.6 m X 1.8 m X 2.4 m), with two heat lamps during the first two weeks, exposed daily to artificial ambient light (6:00 am – 9:00 pm) at a room temperature of approximately 22°C. The gulls were fed wet or dry commercial dog or cat food and maintained for a period of at least eight weeks in the lab before initial exposure. At approximately eight weeks of age, before exposure, the gulls were identified with a labelled plastic leg band.

Chicks can potentially be infected with parasites via feeding by their parents; however, to date there has been no evidence for this to be the case for species of *Diplostomum*. To be certain, the feces of each gull chick were examined one day prior to the start of the experiment to check for a previously established infection. Each gull was isolated for one hour in a wire bottom cage over a tray of water from which feces were collected and examined for eggs. The fecal matter was washed with water through a series of mesh screens (120 and 70 µm), allowing the sediment to settle and subsequently examined for eggs in water using a stereomicroscope (Lapierre et al., 2018). No eggs were recovered from the feces of the gull chicks.

To assess infection success in the fish prior to the experiment and to estimate the mean intensity of metacercariae per fish eye, a sample of rainbow trout were necropsied and the eyes or lenses dissected. The mean ± the standard deviation (SD) infection levels were 22.5 (± 11.0) for *Diplostomum* sp. 1 (sample of 12 fish), 7.7 (± 3.2) for *Diplostomum* sp. 4 (sample of 12 fish) and 7.8 (± 2.4) for *D. baeri* (sample of 2 fish).

To confirm that metacercariae of each species were infective to gulls, three gulls approximately two months old were infected with two-month-old metacercariae of a single species of *Diplostomum*. The

morning of infection, the fish were euthanized (AREC approved protocol) and the eyes removed in a sterile physiological saline solution (0.85%). As metacercariae are unlikely to survive the passage through the stomach if they are administered unprotected, the gulls were fed intact lenses for *Diplostomum* sp. 1 and sp. 4 or whole eyes for *D. baeri* from 10 exposed fish. Each bird was isolated one-week post-infection for feces collection as described above. Eggs were recovered from the feces of all three gulls.

Experimental Design

The experiment consisted of five treatments with a minimum of five gulls per treatment. There were three single species treatments (*Diplostomum* sp. 1; *Diplostomum* sp. 4; *D. baeri*) and two mixed species treatments (*Diplostomum* sp. 1 and *Diplostomum* sp. 4; *Diplostomum* sp. 1 and *D. baeri*). The experiments were executed over successive field seasons, depending on the availability of cercariae for fish infections. During the first year of experiments *Diplostomum* sp. 1 and *Diplostomum* sp. 4 were successfully maintained in the lab, whereas *Diplostomum* sp. 1 and *D. baeri* were successfully maintained during the second year. The differences in the availability of the species of *Diplostomum* in the snails from one year to the next may have been due to sampling effects or seasonal/annual infection differences in the definitive host. Metacercariae approximately three months old were used to infect the gulls. Each gull in a single species treatment received the lenses/eyes from 10 fish. Gulls in mixed species treatments received the lenses/eyes from 20 fish, 10 fish from each exposure to a single species of *Diplostomum*. Due to time constraints, the exposures were performed over a two-day period where half of the gulls for the single and mixed species treatments were exposed on the first day and the other half the second day. The mixed species infections were administered simultaneously in order to prevent priority effects due to earlier establishment of one species (Poulin, 2007).

Gull necropsy and specimen examination

The birds were euthanized (AREC approved protocol) three weeks post-infection and immediately necropsied. The intestine from anterior duodenum to anus was removed, positioned along a measuring stick and the length recorded. The intestine was then partitioned into 5-cm sections, tying off each partition anteriorly with a thread, and each section was numbered, starting with section 1 being the most anterior portion of duodenum. The last section may not have measured the full 5-cm, but was numbered as a section nonetheless. Each section was opened longitudinally, placed in a dish of saline solution, and washed repeatedly with the saline solution using a pipette to dislodge any flukes. The mucosa and

washings were examined with a stereomicroscope. Flukes from each section were collected, counted, killed with hot (≈100°C) saline solution (0.85%) and preserved in 95% ethanol. The infrapopulation is defined as the number of flukes of each species of *Diplostomum* in a single gull host (Bush et al., 1997).

Mixed species identifications

In the mixed infections of *Diplostomum* sp. 1 and *D. baeri* in gulls, the adult flukes were morphologically distinguishable, based on differences in the length of the hindbody (*Diplostomum* sp. 1, 1207.5 – 1610.0 µm; *D. baeri*, 241.5 – 725.5 µm) and distance between the anterior testes and the junction of the fore- and hindbody (*Diplostomum* sp. 1: 402.5 – 885.5 µm; *D. baeri*: 48.3 – 112.7 µm) which did not overlap between the species. The specimens were visually separated during dissection and then stained in acetocarmine to confirm their identifications.

For expediency due to the large number of specimens, the forebodies of specimens in mixed species infections where the two species were not distinguishable morphologically (*Diplostomum* sp. 1 and *Diplostomum* sp. 4) were sent to the Canadian Center for DNA Barcoding (CCDB) in Guelph, Ontario for DNA extraction, amplification, sequencing and editing of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) barcode region for species identification. Amplification of *cox*1 was performed using the primers Plat-diploCOX1F (5' – CGT TTR AAT TAT ACG GAT CC - 3') and Plat-diploCOX1R (5' AGC ATA GTA ATM GCA GCA GC – 3') and protocol described in Mosczynska et al. (2009). All of the samples of the mixed flukes were successfully sequenced by the CCDB.

The new *cox*1 sequences generated were aligned with 26 published sequences of species of *Diplostomum* from Canada and Europe (Locke et al., 2010a, 2010b, 2015) in Geneious R8 versions 8.0.4 (Kearse et al., 2012). The trimmed alignment was then used to construct a number-of-difference based NJ tree with 1000 bootstrap replicates and pairwise deletion of gaps using a published sequence from GenBank for *Tylodelphus scheuringi* (Locke et al., 2010b) for the outgroup (Blasco-Costa et al., 2014; Selbach et al., 2015) in MEGA version 6 (Tamura et al., 2013). The NJ analysis of the *cox*1 dataset (based on a final dataset with gaps removed of 460 base pairs) provided robust evidence for all but three flukes in the anterior region to be *Diplostomum* sp. 4 and all but one fluke in the posterior region to be *Diplostomum* sp. 1 (Appendix 6). In addition, the trimmed alignments (Appendix 7 and 8) were analyzed for diagnostic sites and the peaks of the original chromatograms were re-examined at the diagnostic sites

to validate species identification. As a single adult develops asexually from a single cercariae, no ambiguities were observed, there were no hybrid adults of *Diplostomum* spp.

Estimation of fecundity

A sample of 20 adult specimens haphazardly selected from different gulls from each treatment were hydrated, stained in 1% acetocarmine, dehydrated through absolute ethanol, cleared in xylene, mounted in Permount™ and examined microscopically for eggs. If there were less than or equal to 20 flukes in a treatment, then all of the adult specimens were stained as described above. Fecundity was estimated as the number of eggs present in the uterus of each stained adult specimen (Poulin and Hamilton, 2000).

Data analysis

Descriptive statistics of mean ± SD and range values for the number of flukes per infected gull, intestinal distribution (spatial position along the length of the intestine), intestinal length, linear span (number of intestinal sections occupied) and fecundity (number of eggs *in utero* per fluke) were calculated for each treatment. There was no significant difference in the mean intensity data for the single treatment of *Diplostomum* sp. 1 between both years and the data were combined (Kruskal Wallis, $H_1 = 0.133$, $p > 0.05$). Outliers were assessed by converting raw data into standardized scores, where cases with a *z*-score ± 3.00 were considered potential outliers (Tabachnick and Fidell, 2006). The outlier for fecundity for *Diplostomum* sp. 4 in the mixed species treatment with *Diplostomum* sp. 1 that was greater than ± 3.00 *z*-score was retained within the analyses. Normality was tested with skew and kurtosis ($p \leq 0.01$) and homoscedasticity was tested with Levene's Test ($p \leq 0.05$: Tabachnick and Fidell, 2006). The data were skewed and heteroscedastic for intestinal distribution, normal and homoscedastic for intestinal length and linear span, and normal but heteroscedastic for fecundity. Transformations on the intestinal distribution and fecundity were unsuccessful in normalizing the data therefore appropriate statistical tests were applied.

Three approaches were used to explore the spatial distribution. First, the actual number of worms per segment was plotted to provide a visual representation of the data. Second, the central tendency was compared among single species infections based on the mean (± SD) location of worms in a generalized linear model (GLM) using a Poisson probability distribution model with both total length of the intestine and number of flukes included as covariates. Wald chi-square pairwise comparisons of the intestinal

sections between species were compared to determine if there were significant differences in preferred site of establishment. Third, the total number of flukes per intestinal segment was converted into proportion of flukes per segment and a GLM was constructed using a Normal probability distribution model with both total length of the intestine and number of flukes included as covariates. Wald chi-square tests pairwise comparisons of the proportion of flukes in each intestinal section were used to determine if where there was a significant difference in preferred site of establishment in single species treatments and when a congener was present.

Factors influencing the linear span of infection along the intestine were also explored to determine if total number of flukes or presence of a congener extended or reduced the span of infection, independent of the which sections contained flukes. A one-way analysis of variance (ANOVA) was calculated to determine whether there was a difference among treatments for the mean linear span occupied with Tukey HSD post-hoc tests calculated for pairwise comparisons of single treatments as well as between single treatments and their conspecific in the mixed treatments. Pearson correlations were calculated to determine if the total number of flukes was correlated with the total number of intestinal sections occupied within the gull.

Finally, a GLM was constructed to describe the influence of species of *Diplostomum* in single or mixed treatments on mean fecundity using a Poisson probability distribution model with the total number of worms present in the same intestinal segment entered as a covariate. Pairwise comparisons were calculated between single treatments as well as between single treatments and their conspecific in the mixed treatments to test for a numerical effect of interaction. Probabilities of ≤ 0.05 were considered significant and ≤ 0.007 when Bonferroni corrected for multiple comparisons.

RESULTS

Overall gull infections

The 11 gulls exposed to *Diplostomum* sp. 1 and the five gulls exposed to *Diplostomum* sp. 4 were infected; however only six of the 10 gulls exposed to *D. baeri* were successfully infected (Table 5). Only the infected gulls were included in the data analysis. The mean (± the SD) number of flukes recovered from the single infection treatments was 40.18 (± 54.86) for *Diplostomum* sp. 1, 26.00 (± 19.53) for *Diplostomum* sp. 4 and 2.67 (± 2.42) for *D. baeri* (Table 5).

At necropsy the five gulls exposed to *Diplostomum* sp. 1 and *Diplostomum* sp. 4 were infected with both species. However only three of the five gulls exposed to *Diplostomum* sp. 1 and *D. baeri* were infected by both species (Table 5) and only these three were included in the data analysis. The mean (± SD) numbers of flukes recovered for the mixed infection treatments were: 27.00 (± 19.48) for *Diplostomum* sp. 1 when co-infected with *Diplostomum* sp. 4 and 169.33 (± 38.73) when *D. baeri* was present; 30.25 (± 20.47) for *Diplostomum* sp. 4 when co-infected with *Diplostomum* sp. 1; and 3.67 (± 3.79) for *D. baeri* when *Diplostomum* sp. 1 was present (Table 5). As the actual number of metacercariae the gulls were exposed to was unknown, the proportions of flukes recovered relative to the infective dose could not be calculated and statistical comparisons of fluke numbers would not be meaningful for the single or mixed treatments.

Spatial distribution

The intestinal length ranged from 11 – 15 sections (55 – 75 cm) with an average length of 64.20 cm (± 6.35 cm). No flukes were recovered from sections 13 through 15. For the single species treatments, the four uninfected gulls exposed to *D. baeri* were removed from the dataset. For the mixed species treatments, the two gulls exposed to both *Diplostomum* sp. 1 and *D. baeri* which had only *Diplostomum* sp. 1 recovered at the time of necropsy were removed from the dataset. In single species treatments, *Diplostomum* sp. 1 occupied sections 1 through 12, with the highest number of flukes occupying mid-section 8 (Table 5, Figure 4). *Diplostomum* sp. 4 occupied both anterior sections 1 through 4 and mid-posterior sections 7 through 10 with the greatest number of flukes occupying the first anterior section (Table 5, Figure 4). *D. baeri* occupied mid-posterior sections 4 through 9 with the greatest number of flukes recovered from mid-section 6 (Table 5, Figure 4). There was a significant effect of species of *Diplostomum* (GLM, $W_3 = 8.842$, $p = 0.012$), segment of intestine (GLM, $W_{10} = 64.876$, $p = < 0.01$), covariates of total intestinal length (GLM, $W_1 = 8.731$, $p = < 0.01$) and the total number of flukes (GLM, $W_1 = 424.959$, $p = < 0.01$) on the number of adult flukes. Pairwise comparisons of the single treatments for each intestinal section showed significant differences in mean number of flukes for segments 1 through 8 between *Diplostomum* sp. 1 and *Diplostomum* sp. 4 ($p = < 0.01$: Table 5, Figure 4). Mean numbers in intestinal segments 2 through 5 and 7 through 10 differed significantly between *Diplostomum* sp. 1 and *D. baeri* ($0.047 < p < 0.01$: Table 5, Figure 4). Similarly, mean numbers in intestinal segments 1 through 4, 6, 9 and 10 differed significantly between *Diplostomum* sp. 4 and *D. baeri* ($0.026 < p < 0.01$: Table 5, Figure 4). In mixed treatments, while the spatial distribution of *Diplostomum* sp. 1 and *D. baeri* overlapped those

of *Diplostomum* sp. 1 and *Diplostomum* sp. 4 did not (Figures 5, 6). *Diplostomum* sp. 4 occupied the anterior sections, with at least one section of intestine devoid of flukes separating them from *Diplostomum* sp. 1, which occupied the midrange sections (Figures 5, 6). In comparison to the single species infections where *Diplostomum* sp. 4 also occupied both anterior and posterior sections (Figure 4), in the mixed infections only three flukes (from one of the five gulls) of *Diplostomum* sp. 4 were recovered from the posterior sections co-occupied by *Diplostomum* sp. 1. Similarly, there was only one occurrence of a fluke of *Diplostomum* sp. 1 in an anterior section co-occupied by *Diplostomum* sp. 4.

There was a significant effect of intestinal segment (GLM, W_{11} = 130.226, p = < 0.01) on the proportion of flukes, but no significant effect of the covariates of total intestinal length (GLM, W_1 = 0.000, p > 0.05) or the total number of flukes (GLM, W_1 = 0.000, p > 0.05) on the proportion of the flukes in each segment. The pairwise comparisons between single species treatments showed significant differences in proportions of flukes in the intestinal segments between the three species. Segments 1 and 2 along with segments 6 through 9 differed in proportion of flukes between *Diplostomum* sp. 1 and *Diplostomum* sp. 4 (0.04 < p < 0.01). Segments 6 though 9 differed in proportion of flukes between *Diplostomum* sp. 1 and *D. baeri* (p = < 0.01). Segments 1, 6 and 7 differed in proportion of flukes between *Diplostomum* sp. 4 and *D. baeri* (p = < 0.01). The pairwise comparisons of single and mixed treatments between conspecifics showed no effect on distribution for either *Diplostomum* sp. 1 or *Diplostomum* sp. 4 in the mixed treatments (p > 0.05: Table 1, Figures 2, 3). For *D. baeri*, there was a significant change in distribution for intestinal segments 5 through 8 when *Diplostomum* sp. 1 was present (p = < 0.01: Table 1, Figures 5, 6).

Linear span

The number of sections occupied was positively correlated with intensity for single infections with *Diplostomum* sp. 1 (r_{11} = 0.812, p = < 0.007, significant after Bonferroni correction) and *D. baeri* (r_6 = 0.986, p = < 0.007, significant after Bonferroni correction). However, the correlation was not significant with Bonferroni correction for *Diplostomum* sp. 4 alone (r_5 = -0.618, p > 0.007, not significant after Bonferroni correction) or for the mixed species pairs (p > 0.007, not significant after Bonferroni correction).

The mean (± the SD) number of sections occupied in single species infections was 4.73 (± 2.61) for *Diplostomum* sp. 1, 4.20 (± 1.30) for *Diplostomum* sp. 4 and 1.67 (± 1.21) for *D. baeri* (Table 6). In mixed species treatments where the mean (± the SD) number of sections occupied was 4.20 (± 1.30) for *Diplostomum* sp. 1 and 2.80 (± 0.84) for *Diplostomum* sp. 4; and 7.33 (± 1.53) for *Diplostomum* sp. 1 and

2.00 (± 1.73) for *D. baeri* (Table 6). Comparisons of mean number of sections spanned showed a significant difference among treatments (ANOVA, $F_{6, 31} = 4.557$, $p = < 0.01$). However, post-hoc comparisons using the Tukey HSD post-hoc tests indicated there to be no significant difference between single species treatments ($p > 0.007$, not significant after Bonferroni correction), nor between single-species treatments and their conspecifics in mixed treatments ($p > 0.007$, not significant after Bonferroni correction: Table 6).

Fecundity

The mean (±SD) number of eggs in the uterus for the single species infections was 1.65 ± 1.42 for *Diplostomum* sp. 1, 13.20 (± 7.45) for *Diplostomum* sp. 4 and 1.50 (± 1.65) for *D. baeri*, (Table 7, Figure 7). The mean (±SD) number of eggs in the uterus for the mixed treatments was 1.50 (± 1.10) for *Diplostomum* sp. 1 and 6.25 (± 7.15) for *Diplostomum* sp. 4; and 3.35 (± 3.05) for *Diplostomum* sp. 1 and 1.27 (± 0.79) *D. baeri* (Table 7, Figure 7).

There was a significant difference in the mean number of eggs *in utero* between treatments (GLM, $W_6 = 372.171$, $p = < 0.01$) with a significant interaction between the total number of conspecifics in the same intestinal segment and mean number of eggs in the fluke (GLM, $W_1 = 9.314$, $p = < 0.01$). Pairwise comparisons of single species treatments indicated the mean egg number *in utero* for *Diplostomum* sp. 4 was significantly greater than in *Diplostomum* sp. 1 ($p = < 0.007$, significant after Bonferroni correction) and *D. baeri* ($p = < 0.007$, significant after Bonferroni correction). There was no difference for the number of eggs *in utero* for the single infection treatments between *Diplostomum* sp. 1 and *D. baeri* ($p > 0.05$: Table 7, Figure 7).

Pairwise comparisons of single species treatments compared to conspecifics in mixed treatments indicated there was a significant decrease in the mean number of eggs *in utero* for *Diplostomum* sp. 4 ($p = < 0.007$, significant after Bonferroni correction Table 7, Figure 7). There was no difference in the mean number of eggs *in utero* for *Diplostomum* sp. 1 in the mixed treatment with *Diplostomum* sp. 4 ($p > 0.05$, Table 7, Figure 7); however, there was a significant increase in fecundity of *Diplostomum* sp. 1 in the mixed treatment with *D. baeri* ($p = < 0.007$, significant after Bonferroni correction Table 7, Figure 7). There was no significant difference in the mean number of eggs for *D. baeri* in mixed treatments in comparison to the single species treatment ($p > 0.05$, Table 7, Figure 7).

DISCUSSION

Infections of three species of *Diplostomum* in the gull definitive host were compared in single and mixed-species combinations to assess interspecific interactions affecting spatial distribution, mean linear span and fecundity. In single species infections, a higher proportion of adult flukes were recovered from the anterior portion of the gut of ring-billed gulls for *Diplostomum* sp. 4, whereas *Diplostomum* sp. 1 and *D. baeri* occupied midrange positions. The presence of a congener influenced only *D. baeri* with regards to spatial distribution. There was a positive relationship between total linear span and total number of flukes within species. However, the mean linear span remained constant in both single and mixed species treatments. *Diplostomum* sp. 4 had higher fecundity than the other species in single species infections. There was a clear interspecific interaction with regards to fecundity as there was a significant decline of eggs *in utero* for *Diplostomum* sp. 4 in the presence of *Diplostomum* sp. 1, but an increase in eggs *in utero* for *Diplostomum* sp. 1 in the presence of *D. baeri*. The fecundity data for both species pairs tested as well as the displacement of *D. baeri* when concurrently infected with *Diplostomum* sp. 1 suggest interspecific interaction.

Overall, *Diplostomum* sp. 1 had the highest mean intensity in single treatments. This corresponds to the infective dose estimated from infection levels in the fish necropsied to assess infection success. While previous studies (Dick and Rosen, 1981; Karvonen et al., 2006) counted the metacercariae in the lenses to estimate the dosage per chick and establishment success, here the lenses or whole eyes were not examined before being fed to the gull in an attempt to maximize survival of the metacercariae. Therefore, even though dosages of cercariae to infect the fish were standardized, actual infection dose is unknown and conclusive comparisons of proportions of flukes recovered relative to the number of metacercariae administered cannot be made. In mixed treatments, numbers of *Diplostomum* sp. 1 were higher when *D. baeri* was present. Although this may have been due to facilitation interaction effects through host immune suppression induced by the presence of *D. baeri*, it may also be that the dose of *Diplostomum* sp. 1 metacercariae was higher in the mixed species treatment. In addition, the low intensities of *D. baeri* in both single and mixed treatments may be a consequence of the ring-billed gull not being the preferred definitive host for this species of *Diplostomum*. For example, in a similar experimental study, Shostak et al. (1987) found a greater number of the humor-infecting species, *D. baeri bucculentum*, in herring gulls than in ring-billed gulls. Similar to our experiment, the infection levels in Shostak et al.'s (1987) study could not be ascertained; however, host specificity of the adult flukes may indeed vary among species of *Diplostomum*.

Individual parasite species are found in specific microhabitats within their host (Holmes, 1973; Rohde, 1979). Physicochemical gradients along the length of the intestine may determine the parasite's niche (Holmes, 1990). For example, the pH of the gut of vertebrates varies along its length and tolerance levels of the flukes may be a determinant for their preferred attachment site (Smyth and Halton, 1983). In single species infections, both *Diplostomum* sp. 1 and *D. baeri* occupied mid-region segments whereas *Diplostomum* sp. 4 occupied anterior segments. Preferences for anterior versus mid-regions of the gut have been reported for various species of *Diplostomum* and are not unusual (Rees, 1955; Hoffman and Hundley, 1957; Dick and Rosen, 1981; Niewiadomska, 1984; Shostak et al., 1987; Field and Irwin, 1995; McKeown and Irwin, 1995; Karvonen et al., 2006). The extreme posterior regions of the gut remained vacant, which may suggest this region was unsuitable for occupation by the species studied.

Of the two mixed species treatments, only *D. baeri* experienced changes in distribution in mixed species treatments. This makes sense given that *D. baeri* and *Diplostomum* sp. 1 shared a similar location in single species infections, whereas *Diplostomum* sp. 1 and *Diplostomum* sp. 4 infected distinct regions of the intestine. A general view in ecology is that competition would arise if species using overlapping niches occur concurrently, usually to the exclusion of one species or segregation of niche space (Holmes, 1973). A shift in niche may reduce interspecific interactions and facilitate co-existence (Wertheim et al., 2000). A shift in spatial position along the gut has previously been demonstrated in the helminth community of grebes, where interactive species avoided spatial overlaps between related species in mixed species infections (Stock and Holmes, 1988). For example, cestodes *W. paraporale* shifted posteriorly when there was an increased abundance of cestodes *Diorchis* sp. (Stock and Holmes, 1988). Similarly, the mean locations of the nematodes *C. americana* and *S. robustus* in the intestines of flying squirrels were significantly different in experimental single infections versus naturally concurrent infections, where *C. americana* shifted posteriorly and *S. robustus* shifted anteriorly (Patrick, 1991). Segregation of species' distributions has also been shown by both Dick and Rosen (1981) and Karvonen et al. (2006) among mixed infections of species of *Diplostomum.* The clear gap separating *Diplostomum* sp. 1 and *Diplostomum* sp. 4 indicated distinct fundamental niches for these two parasites and accounts for the absence of change in their realized niches in the presence of the congener. *Diplostomum* sp. 1 and *Diplostomum* sp. 4 are sympatric in nature (Locke et al., 2010a, 2010b; Désilets et al., 2013) and are considered to be negatively associated in the fish hosts (Désilets et al., 2013). Therefore, the distinct locations observed in the gull host may reflect past competition and selective pressures that would have led to spatial segregation to facilitate co-existence (Poulin, 2007).

In contrast, the fundamental niches of *Diplostomum* sp. 1 and *D. baeri* overlapped in the single species treatments. Although there was no effect on *Diplostomum* sp. 1 when *D. baeri* was present, *D. baeri* shifted out of the preferred location of *Diplostomum* sp. 1. *Diplostomum* sp. 1 and *D. baeri* are sympatric and have been collected from fishes in the same localities (Locke et al., 2010a, 2010b; Désilets et al., 2013). The significant shift for *D. baeri* suggests a functional effect in an interactive community. Some potential reasons for the shift may be due to competition for resources, changes in the physiochemical environment or to increase mating opportunities between conspecifics (Poulin, 2007). As the numbers of *Diplostomum* sp. 1 were much higher than those of *D. baeri*, competition for resources would seem to be the more likely explanation, however, further investigations into the mechanisms of interaction are needed.

Although there was no difference in mean linear span between single species treatments nor between pairwise comparisons of single species infections with their conspecifics in mixed treatments, there was a natural expansion of the linear span with increasing intensity of *Diplostomum* sp. 1 and *D. baeri* in single infections. This may be due to a decrease in the amount of space and nutrients available when infrapopulation size increases (Poulin, 2007) or may reflect a random process of establishment of flukes around the preferred location, resulting in an expanded distribution as worm numbers increase. Our results here are similar to those reported for other intestinal flukes. For example, the linear span for 13 common intestinal species of cestodes and acanthocephalans in lesser scaup ducks (*Aythya affinis*) was correlated with the number of conspecifics within the same bird (Bush and Holmes, 1986). Similarly, the mean linear span was positively correlated with infrapopulation size for both nematodes *C. americana* and *S. robustus* in the intestine of the flying squirrel (Patrick, 1991). However, it was surprising that this relationship was not observed in for the single treatment of *Diplostomum* sp. 4 nor the mixed treatments. This could be due to the low sample size for the mixed treatments of *Diplostomum* sp. 1 and *D. baeri* or may actually indicate interspecific competition whereby flukes are precluding from establishing near the congener.

Fecundity of *Diplostomum* sp. 4 was higher than both *Diplostomum* sp. 1 and *D. baeri* in single species infections. The low fecundities of both *Diplostomum* sp. 1 and *D. baeri* are similar to egg numbers reported for other species of *Diplostomum* (summarized in Lapierre et al., 2018). As *Diplostomum* sp. 4 was located in the anterior duodenum, it would have first access to nutrients possibly allowing greater fecundity than the two species of *Diplostomum* occurring in the midway along the intestine. However,

Diplostomum commutatum, with the highest reported number of eggs, was recovered from the large intestine of its definitive host (Dubois, 1970). Differences in location and resource abundance, therefore, may not be the ultimate explanation underlying differences in fecundity. As suggested by Lapierre et al. (2018), the ring-billed gull may not be the preferred host for *Diplostomum* sp. 1 and *D. baeri*, and this could also explain their lower fecundity compared with *Diplostomum* sp. 4.

Although fecundity of *D. baeri* was unaffected by co-infection with *Diplostomum* sp. 1, fecundity of *Diplostomum* sp. 4 in gulls was lower during co-infection with *Diplostomum* sp. 1, and fecundity of *Diplostomum* sp. 1 was higher in the presence of *D. baeri*. Further, there was a negative interaction between the number of conspecifics in an intestinal segment and the number of eggs *in utero*. This is a clear indication of a numerical interaction effect. Both decreases (e.g. Holland, 1984) and increases (e.g. Presidente et al., 1973) in fecundity have been demonstrated in mixed species infections for other intestinal parasites. If the distribution of a parasite shifts to less than optimal conditions, this may result reduced egg production, resulting in a reduction in fitness (Holmes, 1962). Alternatively, the presence of *Diplostomum* sp. 1 could be affecting the bacterial flora, the physicochemical characteristics of the intestine, resource acquisition or metabolism, the immune response of the host, one of any combination of which could be interfering with gamete production, fertilization, or components involved in egg shell formation such as vitelline cells or the Mehlis gland in *Diplostomum* sp. 4 (Smyth and Halton, 1983). However, in the case of *Diplostomum* sp. 1, the presence of *D. baeri* had a positive effect on mean fecundity. This could be due to a suppression of host immune responses by *D. baeri* allowing for greater egg production as was observed in *H. contortus* when concurrently infected with *F. hepatica* in sheep (Presidente et al., 1973), which may have also contributed to the enhanced intensities of *Diplostomum* sp. 1 in the presence of *D. baeri*, as mentioned above. As all three species have been reported from the same localities (Locke et al., 2010a; 2010b; Désilets et al., 2013), there could be a reduction in population levels of *Diplostomum* sp. 4 and an increase of *Diplostomum* sp. 1 in areas of sympatry. Further, no significant interactions have been reported between *Diplostomum* sp. 1 with *D. baeri* in fish hosts (Désilets et al., 2013), increasing the probability of having concurrent infections of these two species.

In conclusion, results herein reveal patterns of microhabitat distribution of three molecularly-delineated species of *Diplostomum* and evidence of interaction between them in the definitive host, with both functional (changes in distribution) and numerical (changes in fecundity) effects. Evidence of site specificity is seen in single species infections where the majority of *Diplostomum* sp. 4 is located in the

first two anterior sections of the intestine whereas the most *Diplostomum* sp. 1 and *D. baeri* are located in the mid- to posterior sections five through nine. In single species infections the parasites occupied linear spans that increased as population size increased. In mixed species infections, the intestinal distributions remained unchanged for the lens species. Further, there was no change in the mean linear span, suggesting the realized niche in co-infections was equivalent to the fundamental niche in single species infections. Although it is likely that the intensity-dependent increase in linear span of both *Diplostomum* sp. 1 and *D. baeri* resulted from the random displacement of flukes around the preferred location that would be expected with higher numbers of flukes, we cannot rule out the possibility that interspecific competition for resources may also have occurred. If we had explored whether fecundity or size of the adult worm was affected by intensity for each intestinal section for each gull, this might have provided evidence of intraspecific competition, but this analysis was not done. Our results provide evidence of interspecific competition between *Diplostomum* sp. 1 and *Diplostomum* sp. 4. The restriction of *Diplostomum* sp. 1 from anterior sections in mixed treatments with *Diplostomum* sp. 4 suggests that *Diplostomum* sp. 4 may reduce the likelihood of *Diplostomum* sp. 1 establishing in the anterior intestine. While some suggest segregation may be due primarily to active site selection in interactive communities (Holmes, 1973), others suggest that niche segregation simply reflects species differences and may facilitate mate encounter in non-interactive communities (Rohde, 1979). Karvonen et al. (2006a) concluded the segregation to be indicative of a non-interactive community between species of *Diplostomum*. However, in our study the presence of *Diplostomum* sp. 1 lowered the fecundity of *Diplostomum* sp. 4 and is indicative of an interactive community. We also provide evidence of interspecific interactions between *Diplostomum* sp. 1 and *D. baeri* based on a shift of *D. baeri* away from the preferred site of *Diplostomum* sp. 1 and on the increased fecundity of *Diplostomum* sp. 1 when the gulls were concurrently infected with *D. baeri*. Together, these data suggest that species interactions in the definitive host can affect population dynamics and community structure of *Diplostomum* spp.

Table 5. Summary statistics describing the total number of flukes and intestinal distribution (spatial distribution along the length of the intestine) recovered at time of necropsy of *Diplostomum* sp. 1, *Diplostomum* sp. 4 and *Diplostomum baeri* in controlled single-species experimental infections and simultaneous mixed infections of *Diplostomum* sp. 1 with *Diplostomum* sp. 4 or *Diplostomum* sp. 1 with *D. baeri* in the definitive host, the ring-billed gull (*Larus delawarensis*).

Species	Experiment	Gull sample size (n)	Number of successfully infected gulls	Mean ± SD	Range	Intestinal sections occupied (peak)
Diplostomum sp. 1	single-species	11	11	40.18 ± 54.86	3 - 185	1 - 12 (8)
Diplostomum sp. 4	single-species	5	5	26.00 ± 19.53	2 - 52	1 - 4 and 7 - 10 (1)
D. baeri	single-species	10	6	2.67 ± 2.42	1 - 7	4 - 9 (7)
Diplostomum sp. 1	concurrent with *Diplostomum* sp. 4	5	5	27.00 ± 19.48	11 - 59	4 - 9 (8)
Diplostomum sp. 1	concurrent with *D. baeri*	5	3	169.33 ± 38.73	145 - 214	2 – 11 (6)
Diplostomum sp. 4	concurrent with *Diplostomum* sp. 1	5	5	30.25 ± 20.47	4 - 52	1 - 4 and 7 – 9 (1)
D. baeri	concurrent with *Diplostomum* sp. 1	5	3	3.67 ± 3.79	1-8	5 - 8 (8)

Table 6. Summary statistics describing the mean linear span (total number of intestinal sections occupied) for *Diplostomum* sp. 1, *Diplostomum* sp. 4 and *Diplostomum baeri* in controlled single-species experimental infections and simultaneous mixed infections of *Diplostomum* sp. 1 with *Diplostomum* sp. 4 or *Diplostomum* sp. 1 with *D. baeri* in the definitive host, the ring-billed gull (*Larus delawarensis*) along with corresponding test statistic (ANOVA, $F_{6, 31} = 4.557$, $p = < 0.01$). Tukey HSD post-hoc tests comparing pairwise comparisons between single treatments and between single treatments with conspecifics in mixed treatments showed no significant differences ($p > 0.007$, not significant after Bonferroni correction).

Species	Experiment	Gull sample size (n)	Mean ± SD	Range
Diplostomum sp. 1	single-species	11	4.73 ± 2.61	1 - 9
Diplostomum sp. 4	single-species	5	4.20 ± 1.30	3 - 6
D. baeri	single-species	6	1.67 ± 1.21	1 - 4
Diplostomum sp. 1	concurrent with *Diplostomum* sp. 4	5	4.20 ± 1.30	3 - 6
Diplostomum sp. 1	concurrent with *D. baeri*	3	7.33 ± 1.53	6 - 9
Diplostomum sp. 4	concurrent with *Diplostomum* sp. 1	5	2.80 ± 0.84	2 - 4
D. baeri	concurrent with *Diplostomum* sp. 1	3	2.00 ± 1.73	1 - 4

Table 7. Summary statistics describing the mean number of eggs *in utero* for the species *Diplostomum* sp. 1, *Diplostomum* sp. 4 and *Diplostomum baeri* in controlled single-species experimental infections and simultaneous mixed infections of *Diplostomum* sp. 1 with *Diplostomum* sp. 4 or *Diplostomum* sp. 1 with *D. baeri* in the definitive host, the ring-billed gull (*Larus delawarensis*). There was a significant difference in the mean number of eggs *in utero* between treatments (GLM, $W_6 = 372.171$, $p = < 0.01$) with a significant negative interaction between the total number of conspecifics in the same intestinal segment and mean number of eggs in the fluke (GLM, $W_1 = 9.314$, $p = < 0.01$). Significant differences of pairwise comparisons between single treatments are represented by different superscript letters. Significant differences between single treatments and conspecifics in mixed treatments ($p \leq 0.007$, significant after Bonferroni correction) are indicated by *.

Species	Experiment	Fluke sample size	Mean ± SD	Range
Diplostomum sp. 1	single-species	20	1.65 ± 1.42[b]	1 - 9
Diplostomum sp. 4	single-species	20	13.20 ± 7.45[a]	0 - 31
D. baeri	single-species	14	1.50 ± 1.65[b]	0 - 4
Diplostomum sp. 1	concurrent with *Diplostomum* sp. 4	20	1.50 ± 1.10	0 - 4
Diplostomum sp. 1	concurrent with *D. baeri*	20	3.35 ± 3.05*	0 - 11
Diplostomum sp. 4	concurrent with *Diplostomum* sp. 1	20	6.25 ± 7.15*	0 - 29
D. baeri	concurrent with *Diplostomum* sp. 1	11	1.27 ± 0.79	0 - 2

Figure 4. Box and whisker plots of intestinal distribution for three molecularly-delineated species *Diplostomum* sp. 1, *Diplostomum* sp. 4 and *Diplostomum baeri* in controlled single-species experimental infections in the definitive host, the ring-billed gull (*Larus delawarensis*). The intestinal sections are in 5 cm increments beginning with the first segment directly posterior to the stomach continuing along the length of the intestine to the last section flukes were recovered. The box represents the interquartile range and the horizontal line within the box represents the median. The whiskers end at the largest and smallest value excluding any outliers, and the circles and asterisks are non-statistically significant outliers.

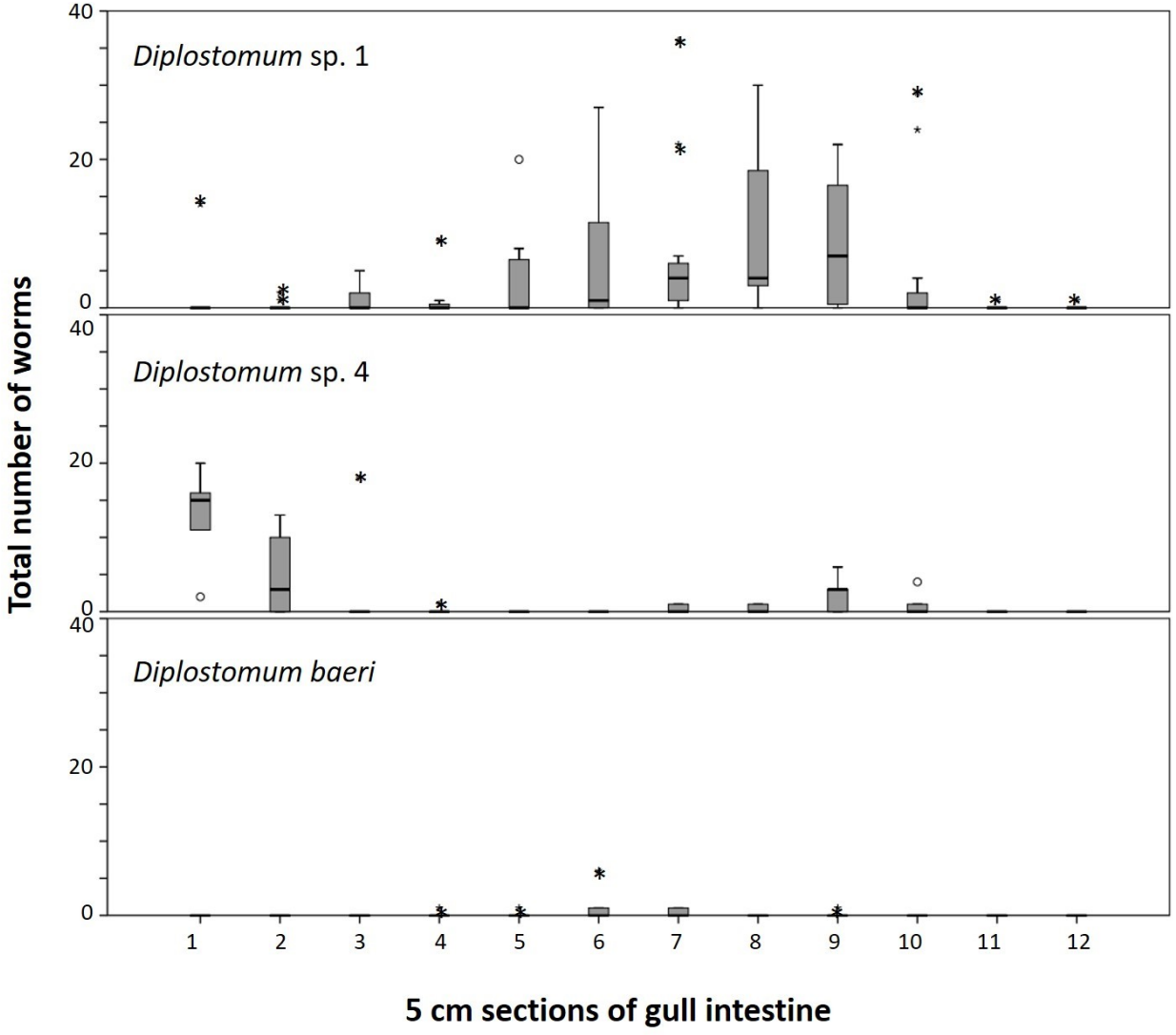
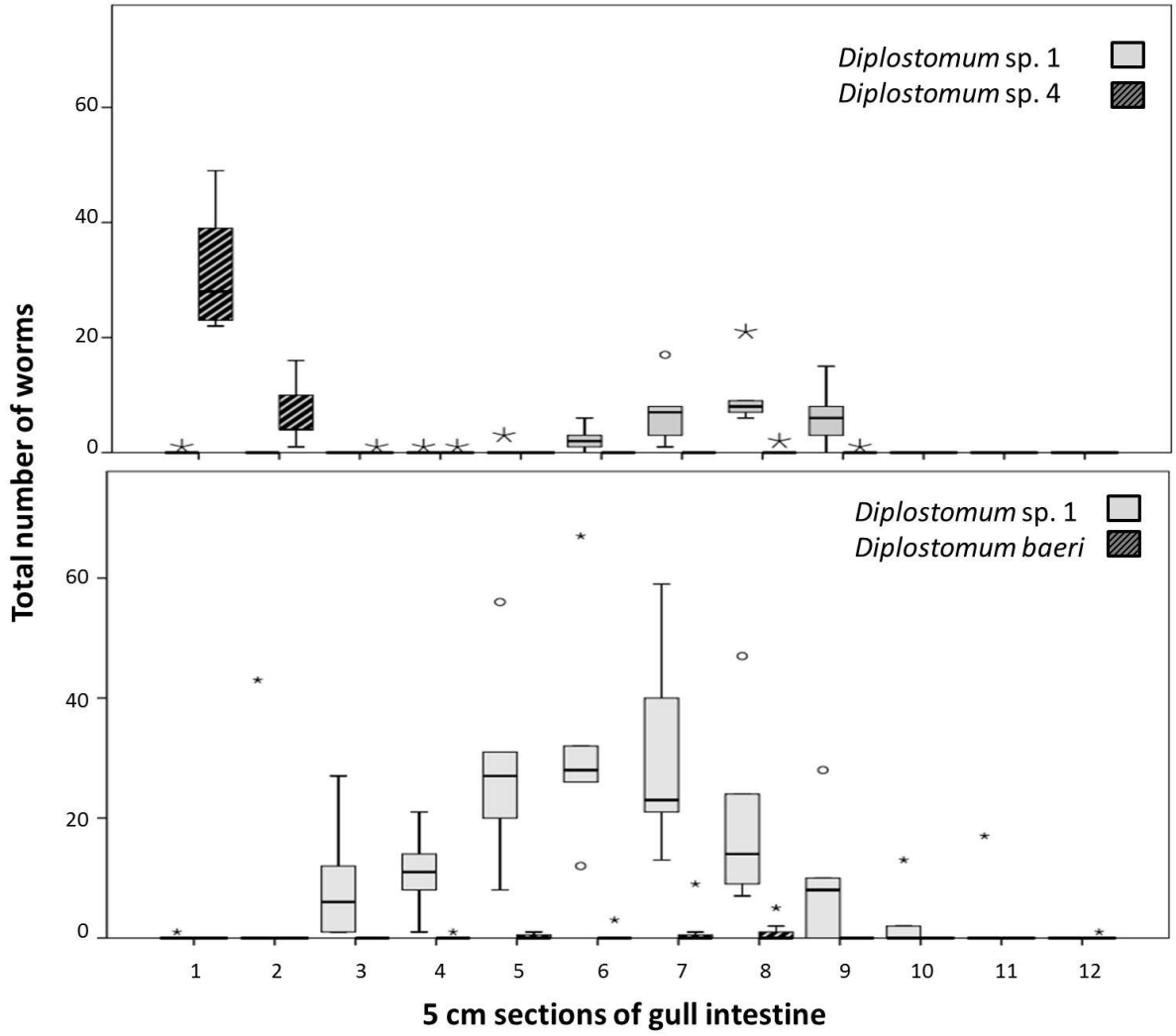


Figure 5. Box and whisker plots of intestinal distribution of three molecularly-delineated species *Diplostomum* sp. 1, *Diplostomum* sp. 4 and *Diplostomum baeri* in single infections and simultaneous mixed infections of *Diplostomum* sp. 1 with *Diplostomum* sp. 4 or *Diplostomum* sp. 1 with *D. baeri* in the definitive host, the ring-billed gull, (*Larus delawarensis*). The intestinal sections are in 5 cm increments beginning with the first segment directly posterior to the stomach continuing along the length of the intestine to the last section where flukes were recovered. The box represents the interquartile range and the horizontal line within the box represents the median. The whiskers end at the largest and smallest value excluding any outliers, and the circles and asterisks are non-statistically significant outliers.



1

Figure 6. Line graph of intestinal distribution of three molecularly-delineated species *Diplostomum* sp. 1, *Diplostomum* sp. 4 and *Diplostomum baeri* in single and simultaneous mixed infections of *Diplostomum* sp. 1 with *Diplostomum* sp. 4 or *Diplostomum* sp. 1 with *D. baeri* in the definitive host, the ring-billed gull (*Larus delawarensis*). The intestinal sections are in 5 cm increments beginning with the first section directly posterior to the stomach continuing along the length of the intestine to the last section flukes were recovered. A generalized linear model (GLM) using a Normal probability distribution was constructed to compare the mean proportion of flukes in each intestinal section (GLM, $W_{11} = 130.226$, $p = < 0.01$) controlling for total intestinal length and total conspecifics and significant differences of pairwise comparisons between single treatments and between single treatments with conspecifics in mixed treatments ($p \leq 0.007$) are indicated on the figure. Symbols for significant pairwise differences are a darkened circle (●) for single species comparisons between *Diplostomum* sp. 1 and 4, a clear circle (○) for single species comparisons between *Diplostomum* sp. 1 and *D. baeri*, and a clear diamond (◊) between *Diplostomum* sp. 4 and *D. baeri*. Only mixed treatments for *D. baeri* showed significant differences and are indicated with an asterisks (*).

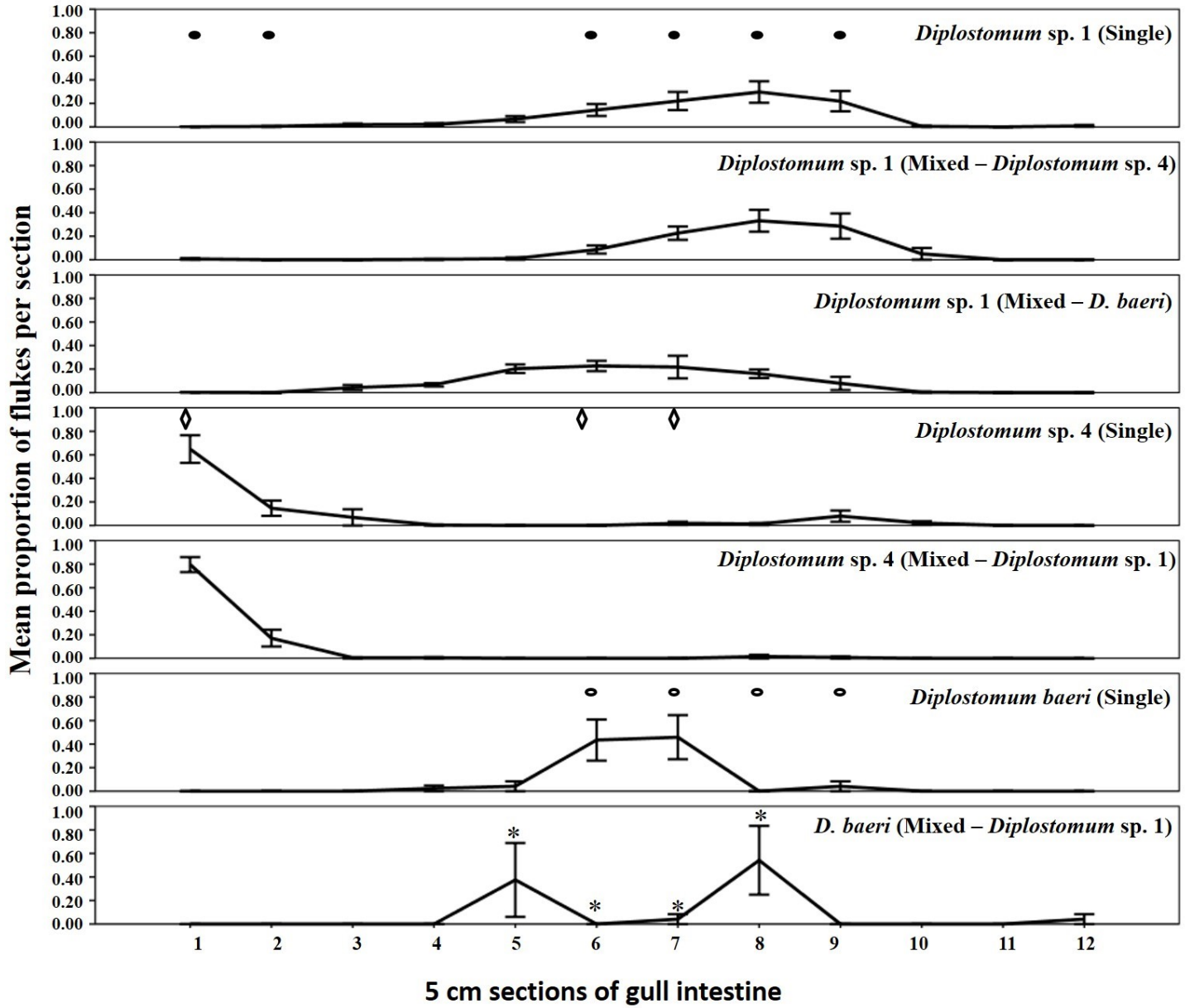
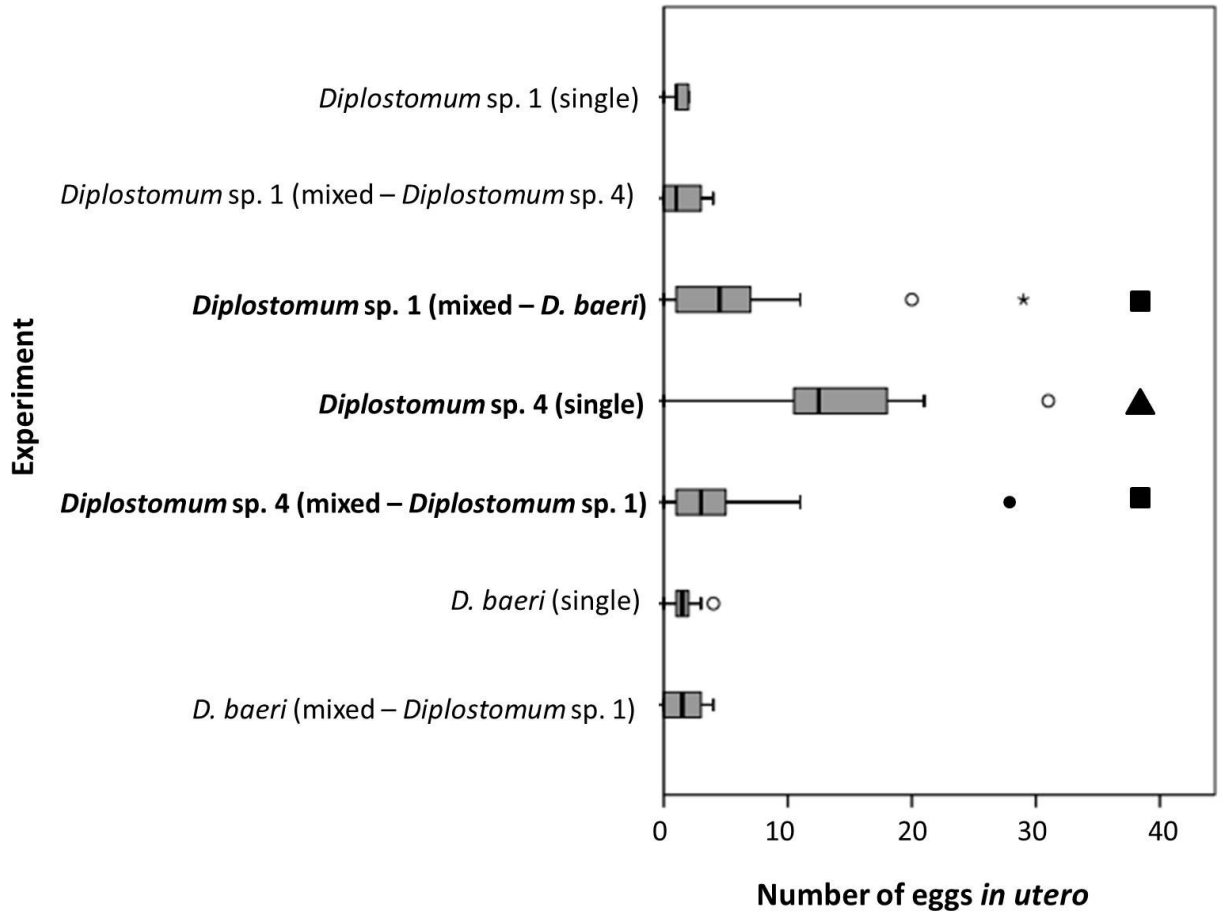

Mean proportion of flukes per section
1.00
0.80
0.60
0.40
0.20
0.00
Diplostomum sp. 1 (Single)
Diplostomum sp. 1 (Mixed – Diplostomum sp. 4)
Diplostomum sp. 1 (Mixed – D. baeri)
Diplostomum sp. 4 (Single)
Diplostomum sp. 4 (Mixed – Diplostomum sp. 1)
Diplostomum baeri (Single)
D. baeri (Mixed – Diplostomum sp. 1)
1
2
3
4
5
6
7
8
9
10
11
12
5 cm sections of gull intestine

Figure 7. Box and whisker plots of the total number of eggs *in utero* counted from stained adult specimens of 3 molecularly-delineated species, *Diplostomum* sp. 1, *Diplostomum* sp. 4 and *Diplostomum baeri*, in controlled single-species experimental infections and simultaneous mixed infections of *Diplostomum* sp. 1 with *Diplostomum* sp. 4 or *Diplostomum* sp. 1 with *D. baeri* in the definitive host, the ring-billed gull (*Larus delawarensis*). The box represents the interquartile range and the horizontal line within the box represents the median. The whiskers end at the largest and smallest value excluding any outliers, the empty circles and asterisks are non-significant outliers and the darkened circle is a statistically significant outlier. A generalized linear model (GLM) using a Poisson probability distribution was constructed to compare the mean number of eggs *in utero* between treatments (GLM, $W_6 = 372.171$, $p = < 0.01$) controlling for the total number of conspecifics in the intestinal segment. Significant differences are indicated with a triangle (▲) for single treatment comparisons and a square (■) for comparisons between single treatments with conspecifics in mixed treatments ($p \leq 0.007$)**.**



CHAPTER 4. A comparison of egg morphometrics and number of two molecularly-delineated species of *Diplostomum* (Digenea)

ABSTRACT: While it generally has been accepted that evolutionary pressures may drive parasitic worms to produce many small eggs to overcome the challenges of transmission, recent studies suggest that there is wide variability in reproductive strategies among them. Although egg morphometrics and numbers have been reported from many species of *Diplostomum*, the presence of cryptic species has resulted in uncertainty in morphometric measurements for different life history stages. Here, we examine differences between egg characteristics of two molecular-delineated species of *Diplostomum* (Digenea). We investigate whether interspecific differences exist in egg morphometrics or egg number and test if there are intraspecific relationships between size of the egg and the hindbody of adult worms or between size of the egg and fecundity. Egg measurements were obtained from both those collected in feces of experimentally-infected ring-billed gulls (*Larus delawarensis*) and those observed in stained adult specimens of *Diplostomum* spp. Regardless of the source of measurement, there was a significant difference in the length of the eggs and in egg number between the two species, while there was no difference in the widths. Larger worms did not produce significantly larger eggs in either species. Further, egg size was not correlated with the number of eggs present in the stained specimens of either species; smaller eggs were not present in greater numbers. Egg morphometrics can assist in differentiating between these two species of *Diplostomum*. Furthermore, differences in egg sizes and number may reflect other potential differences in life history traits between the two species such as miracidial longevity and infectivity and cercarial output among others which still need to be investigated.

INTRODUCTION

Variation in egg size is a central feature of the life history strategies of parasitic organisms, and yet it remains mostly unexplained (Poulin, 1995). It is generally accepted that difficulties in transmission of the parasite life cycles have driven selection towards the production of many small eggs (Price, 1980) at the expense of fewer larger ones. However, Poulin (2007) argues variation in parasite reproductive strategies would be better to ensure survival, but little is known of the patterns or causes behind trematode egg-size variability.

In terms of interspecific variation, comparisons of species of trematode indicate a positive correlation between egg size and adult worm size where larger worms produced larger eggs, but there is little evidence of a trade-off between egg size and egg number (Poulin, 1997). However, all life history traits determining reproductive success cannot be maximized simultaneously; any investment into one trait will be at the expense of another (Stearns, 1992). Energy will be invested by the adult to produce many smaller eggs or fewer larger ones, but not both. For example, species of *Schistosoma* (Digenea) that produce fewer but larger eggs release larger miracidia which develop into sporocysts with a higher asexual reproductive output than other species which produce a greater number of smaller eggs (Loker, 1983).

In this study, we compare egg morphometrics and egg number of two species of *Diplostomum* (Digenea). Species of *Diplostomum* infect fish-eating birds, primarily gulls, as their definitive hosts, lymnaeid snails and primarily fish as first and second intermediate hosts respectively (Figure 1). Within the fish, the metacercariae of most species infect the lenses, a few species infect other parts of the eye (vitreous humor, retina) and fewer still, the brain. Interest in *Diplostomum* is due mainly to the pathogenicity of the metacercariae, where large numbers within the lens can cause cataracts, impairing host vision and are a significant problem in aquaculture (e.g., Ashton et al., 1969).

Traditionally, identification of species of *Diplostomum* is based on morphology; however, at the metacercarial stage there are few diagnostic features (Gibson, 1996) and at any stage within the life cycle using morphology alone can be problematic due to overlapping morphometrics (Gibson, 1996; Niewiadomska, 1996). Recent DNA based studies on species of *Diplostomum* in North America and Europe (Locke et al., 2010a; 2015; Georgeiva et al., 2013) have confirmed the genus is far more diverse than was originally believed, with many species requiring formal taxonomic description. The inclusion of undetected cryptic species may have inflated ranges of morphometric measurements within species in previous studies (e.g., see Dubois, 1970).

The goals of this study were to first investigate whether two molecularly-delineated species of *Diplostomum* could be separated based on egg morphometrics; second, to determine if there are interspecific differences in uterine egg number; and third, to test for intraspecific correlations between egg and adult hindbody body size and egg size and egg number.

MATERIALS AND METHODS

Marsh pond snails (*Lymnaea elodes*) were collected manually from the rocky littoral zone of Wheaton Lake, Bocabec, New Brunswick, Canada (45°10'08.3"N 66°59'56.2"W) during the first week of September 2012. Each snail was placed in an individual cup of 250 ml of fresh dechlorinated water to stimulate cercarial shedding. Twenty-five of 350 *L. elodes* examined shed furcocercariae however only five snails were used in this experiment due to snail mortality.

Snails shedding furcocercariae were isolated, and a sample of cercariae was collected from each snail and killed by freezing. Their DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen)™ following the manufacturer's protocol. Amplification and sequencing of the partial internal transcribed spacer region of ribosomal DNA (ITS-rDNA) was performed using the primers, protocols and methods described by Galazzo et al. (2002) and Locke et al. (2010a). Assembly and editing were performed in Geneious R8 versions 8.0.4 (Kearse et al., 2012) and the new sequences generated were submitted to Genbank (Accession #'s: KY358236 - KY358240). The new ITS-rDNA sequences were aligned and trimmed in Geneious R8 version 8.0.4 (Kearse et al., 2012) and were compared with 28 published ITS-rDNA sequences of species of *Diplostomum* from Canada and Europe (Galazzo et al., 2002; Locke et al., 2010a; Georgieva et al., 2013; Blasco-Costa et al., 2014; Pérez-Del-Olmo et al., 2014; Locke et al., 2015; Selbach et al., 2015). The trimmed alignments were then used to construct a number-of-difference based neighbor-joining tree with 1000 bootstrap replicates and pairwise deletion of gaps using a published sequence from GenBank for *Tylodelphus scheuringi* (Moszczynska et al., 2009) for the outgroup (Blasco-Costa et al., 2014; Selbach et al., 2015) in MEGA version 6 (Tamura et al., 2013). The cercariae were delineated based on robust evidence of a neighbor-joining analysis that clustered 2 isolates with *Diplostomum* sp. 1 (GQ292522.1, Locke et al., 2010a; KT186794.1, Locke et al., 2015) and 3 isolates with *Diplostomum* sp. 4 (GQ292520.1, Locke et al., 2010a: Appendix 7). While it is rare for snails to be concurrently infected with more than one species of parasite (Soldánová et al., 2012), the trimmed alignments (Appendix 2) were analyzed for diagnostic sites (Appendix 3) and the peaks of the original chromatograms (Appendix 4) were re-examined at the diagnostic sites to validate species identification. No ambiguities were observed, confirming that each individual snail was infected with a single species of *Diplostomum*.

All the animals used in this experiment were maintained and handled in accordance with the guidelines of the Canadian Council of Animal Care. Gull chicks were collected under authority of Canadian

Wildlife Service (Quebec). The experiments were officially approved by the Concordia University Animal Research Ethics Committee (AREC, certificate #30000269).

The evening prior to the experiment, each infected snail was placed in 250 ml of fresh water. The cercariae for each species of *Diplostomum* were collected the following morning, approximately 12 hours later. Naïve rainbow trout (*Oncorhynchus mykiss*) approximately 5 cm in total length obtained from a commercial farmer in Sainte-Edwidge, Quebec, Canada, were exposed in six groups of 10 fish to 250 - 500 cercariae of *Diplostomum* sp. 1 (60 fish for each species) for one hour in an aerated tank containing approximately 5 L of water. A second group of 60 fish was exposed in a similar manner to cercariae of *Diplostomum* sp. 4. Upon termination of exposure, the fish exposed to each species were placed in separate tanks, maintained and fed commercial fish food for a minimum of two months.

Newly hatched ring-billed gull chicks (*Larus delawarensis*), used in this study, were captured from Île Deslauriers, Quebec, Canada (45°42'45.1"N 73°26'25.0"W) in May 2013. The gulls were fed wet/dry commercial dog/cat food and maintained for a period of at least eight weeks before exposure.

Prior to the experiment, two pilot studies were performed. First, in order to estimate the mean intensity of metacercariae per fish lens, 10 rainbow trout from each group were examined approximately two months post-exposure, and the lenses were examined. All of the trout in each group were infected; those exposed to *Diplostomum* sp. 1 had on average (± standard deviation, SD) 22.5 (± 11.0) metacercariae per lens and trout exposed to *Diplostomum* sp. 4 had 7.7 (± 3.2) metacercariae per lens. Second, to confirm that the metacercariae were infective one gull for each species of *Diplostomum* was infected with two-month-old metacercariae in intact lenses. The morning of the gull infection, the fish for each species of *Diplostomum* were euthanized and the lenses were removed and placed in sterile physiological saline solution (0.85%). Each gull was fed the intact lenses from 10 fish exposed to either *Diplostomum* sp. 1 or *Diplostomum* sp. 4. Each bird was isolated one-week post-infection for one hour in a wire bottom cage over a tray of water from which feces were collected and examined for eggs. Eggs were separated from fecal matter by washing with water through a series of mesh screens (120 and 70 µm), allowed to settle and the sediment was examined for eggs using a stereomicroscope. Eggs were recovered from the feces of both gulls.

Metacercariae approximately three months old were used to infect the birds in the gull exposure experiment. A total of 10 gulls were exposed, each receiving the lenses from 10 fish. Five gulls were

infected with metacercariae of *Diplostomum* sp. 1 and five gulls with metacercariae of *Diplostomum* sp. 4.

After the onset of egg production (one-week post-infection), the 10 birds were isolated independently for one hour in a wire bottom cage over trays of water and feces were collected and examined for eggs as described above. The eggs for each species were collected with a pipette, pooled and stored in water at 4°C in separate glass vials.

The gulls were euthanized and necropsied three weeks post infection. The intestines were removed, opened longitudinally from anterior to posterior and placed into a dish of saline solution. The intestine was washed repeatedly with the saline solution using a pipette to dislodge any worms. The mucosa and washings were examined with a stereomicroscope. Worms were collected, killed with hot saline and preserved in 95% ethanol.

Ten haphazardly selected adult worms from one gull for each species of *Diplostomum* were hydrated, stained in a 1% solution of acetocarmine, dehydrated through absolute ethanol, cleared in xylene and mounted in Permount™. As all the worms were killed, fixed, stained and mounted using the same procedure, the distortion effects of these techniques would be equivalent among the specimens and the measurements are comparable. Body size of trematodes is usually estimated by calculating the product of the length by the width (μm^2) (Poulin, 1997). However, unlike most digeneans, species of *Diplostomum* have a distinct forebody and hindbody. As the forebody is more labile, the size of the adult worm is less accurately estimated by including the forebody measurement. Further, the gonad reproductive organs are located in the hindbody of the adult worm. Therefore, from the 10 stained specimens for each species, the size of the adult worm was estimated as the product of the hindbody length by its width.

Egg measurements of length and width (µm) were collected from both those recovered from the gull feces and those measured from the stained adult specimens. The eggs collected from the gull feces were measured approximately one-month post-collection using a glass depression slide with a compound microscope.

The egg size was estimated as the product of its length by its width (μm^2) (Poulin and Hamilton, 2000). If more than one egg was present, then the average of up to five eggs was calculated to represent

the mean egg size for each worm. The numbers of eggs present in the uterus of each stained adult specimen were used as an estimate of fecundity (Poulin and Hamilton, 2000).

Descriptive statistics of mean ± SD and range values for the estimated size of the hindbody of adult worms and length, width, and number of eggs were calculated for each species of *Diplostomum*. Outliers were assessed by converting raw data into standardized scores, where cases with a *z*-score ± 3.00 were considered potential outliers (Tabachnick and Fidell, 2006). Normality was tested with skew and kurtosis ($p < 0.01$) and homoscedasticity was tested with Levene's Test ($p < 0.05$: Tabachnick and Fidell, 2006).

Two-tailed independent samples t-tests were calculated to determine if there were differences between the species of *Diplostomum* in mean: adult hindbody size and the dimensions of eggs measured in either water or in stained specimens. In the case of the egg lengths from stained adults, overall egg sizes and egg number where the data were heteroscedastic and the appropriate transformations were unsuccessful, t-tests assuming unequal variances were performed. Transformations on the width data from eggs measured in water were unsuccessful and a non-parametric Mann-Whitney U test was calculated to determine if there were significant differences between the mean widths of the eggs for each species of *Diplostomum* measured in water.

For the intraspecific comparisons, the data were normal and heteroscedastic, therefore Spearman's *rho* correlations were calculated for each species independently: the first to assess the intraspecific relationship between the mean egg size and estimated adult hindbody size to determine if larger worms are producing larger eggs; and the second to assess the intraspecific relationship between mean egg size and egg number to determine if greater egg number was correlated with smaller eggs in individual worms. Probabilities of ≤ 0.05 were considered significant. Statistical analyses were conducted using the SPSS® 24.0 software package.

RESULTS

All gulls were successfully infected. There was no difference in the mean hindbody size of the adult for *Diplostomum* sp. 1 and *Diplostomum* sp. 4 (Independent t-test; $p = 0.073$: Table 8). There was a significant difference in the mean length, but not width, of the eggs in the two species regardless of whether they were measured *in utero* or in water. Eggs of *Diplostomum* sp. 4 were consistently longer than those of *Diplostomum* sp. 1 independent of the method by which the eggs were measured (water:

Independent t-test; $p < 0.001$; stained: Independent t-test for unequal variances; $p = 0.001$: Table 8). There was no significant difference between the species in egg width for either method (water: Mann-Whitney U test; $p = 0.767$; stained: Independent t-test; $p = 0.796$: Table 8). Overall, the mean egg size for *Diplostomum* sp. 4 was larger than that of *Diplostomum* sp. 1 (Independent t-test for unequal variances; $p = 0.005$: Table 8). Further, the mean number of eggs produced by *Diplostomum* sp. 4 was significantly greater than *Diplostomum* sp. 1 (Independent t-test for unequal variances; $p < 0.001$: Table 8).

There was no relationship between the egg size and the hindbody size for either species (*Diplostomum* sp. 1: Spearman's *rho*, r = 0.006, $p = 0.987$; *Diplostomum* sp. 4: Spearman's *rho*, r = -0.040, $p = 0.913$). Larger worms did not produce larger eggs. Nor was there any significant intraspecific relationship between egg size and egg number for either species (*Diplostomum* sp. 1: Spearman's *rho*, r = 0.475, $p = 0.165$; *Diplostomum* sp. 4: Spearman's *rho*, r = 0.056, $p = 0.879$). The eggs of worms that produced a greater number of eggs were not smaller in size than those that produced fewer eggs for either species, potentially indicating no trade-off between egg size and number within species.

DISCUSSION

This is the first study to examine interspecific differences of egg morphology, morphometrics and egg number between two molecularly-delineated species of *Diplostomum* and to explore the intraspecific relationships between egg size and adult worm hindbody size and egg number. Our findings show a significant difference in length, but not in width, of the eggs between the species, where mean egg size for *Diplostomum* sp. 4 was larger than that of *Diplostomum* sp. 1. To date, egg morphometrics have been reported from 38 species of *Diplostomum* (Table 9). However, the degree of overlap between reported ranges in egg size makes species identification using this measure problematic, unless means and variances are presented so that *post hoc* comparisons can be made. Despite the ranges overlapping between the two species compared here, the mean length and overall egg sizes do differ, permitting morphological discrimination between these two species. Final taxonomic descriptions of these putative species may reveal additional morphological criteria to discriminate between them.

The morphometric egg ranges may vary depending on the medium, as shown here between eggs measured in water versus those measured *in utero* from stained adults. Eggs measured from stained specimens are often collapsed, distorting their natural dimensions. The range for all morphometric characteristics must be regarded with caution as preservation and staining techniques may impact the measurements of those specimens. Nevertheless, the overall mean difference in egg size between

Diplostomum sp. 1 and *Diplostomum* sp. 4 might reflect further differences in life history characteristics between these two species of *Diplostomum*, despite their similar life cycles. For example, in a discriminant analysis comparing the morphometrics of eight adult characteristics of two species of *Sphaeridiotrema* (Digenea), egg length accounted for the highest variability and 96% of the specimens could be correctly identified based on egg length alone and where they overlapped, uterine and cirrus characteristics could reliably identify most specimens (McLaughlin et al., 1992).

With respect to the interspecific pattern among trematodes that larger worms produce larger eggs (Poulin, 1997), there was no significant difference in adult hindbody sizes for the two species of *Diplostomum* examined herein. Although *Diplostomum* sp. 1 specimens had slightly longer hindbodies, their eggs were smaller than those of *Diplostomum* sp. 4 and there was no intraspecific correlation for either species between egg size and adult hindbody size. Similarly, to the pattern reported by Poulin (1997) for trematodes, there does not seem to be a trade-off between egg size and fecundity. *Diplostomum* sp. 4 produced not only larger eggs but more numerous eggs, much like those of *D. commutatum* which have the highest maximum egg length reported and also the highest egg number (Table 9). With regards to egg number, *Diplostomum* sp. 1 had few eggs *in utero*, similar to egg numbers reported from many other species of *Diplostomum* (Table 9).

Egg production can be continuous over the life span of the worm or partitioned by fluctuating egg production into discrete clutches (Poulin, 2007). In this case, one hypothesis could be that *Diplostomum* sp. 1 produces smaller eggs at a slower rate but over a longer life span (a bet-hedging strategy), reflecting prolonged egg production over time. Given the worms were collected at the same age, no such pattern could be observed.

Alternatively, ring-billed gulls may not be the preferred definitive host for *Diplostomum* sp. 1 and this species may be better adapted to other larid hosts, where they perform better. Adults of both species have been reported from only a single study in either European herring gulls (*L. argentatus*) or ring-billed gulls (Locke et al., 2015). Therefore, there are insufficient molecular data to determine if there are preferences between the two species of *Diplostomum* for different definitive hosts. All these and more questions remain to be elucidated regarding these two as well as other species of *Diplostomum*.

The results presented here provide evidence of differences between these two species of *Diplostomum* with regards to their reproductive parameters. Further studies into the egg production

patterns, hatching dynamics, miracidial size, longevity and infectivity and cercarial output would be of interest to obtain a better understanding of the extent of the variability allowing further understanding of the driving forces behind egg size and number differences and potential resource partitioning between these two species of *Diplostomum*.

Table 8. Summary statistics describing the distributions of overall mean intensity of infection success, estimated size of the adult hindbody (length X width), egg morphometrics of length and width measured in water or from stained adults, estimated egg size (length X width) and egg number present in the uterus of stained specimens for molecularly-delineated species: *Diplostomum* sp. 1 and *Diplostomum* sp. 4 along with corresponding test statistic values and *p* values for species comparisons for each variable. Significant differences are indicated in bold.

	***Diplostomum* sp. 1**			***Diplostomum* sp. 4**				
	Sample size (n)	Mean ± SD	Range	Sample size (n)	Mean ± SD	Range	Test statistic	*p* value
Adult hindbody size (mm^2)	10	403.80 ± 47.89	311.05 – 466.58	10	356.28 ± 62.64	274.76 – 505.46	t_{18} = 1.905	0.073
Egg length (µm) measured in water	30	89.01 ± 5.33	80.92 – 99.96	30	99.80 ± 5.23	85.68 – 104.72	t_{58} = -7.920	< 0.001
Egg length (µm) measured from stained adults	30	90.12 ± 7.70	71.40 – 104.72	30	95.53 ± 2.48	90.44 – 99.96	$t_{34.95}$ = -3.664	0.001
Egg width (µm) measured in water	30	63.33 ± 2.25	62.00 – 67.00	30	63.17 ± 2.15	62.00 – 67.00	*Z* = 435	0.767
Egg width (µm) measured from stained adults	30	52.97 ± 5.02	43.00 – 62.00	30	53.27 ± 3.85	48.00 – 57.00	t_{58} = -0.260	0.796
Egg size (mm^2)	10	0.94 ± 0.12	0.76 – 1.14	10	1.08 ± 0.04	1.01 – 1.12	$t_{10.91}$ = -3.495	0.005

Egg number	10	1.80 ± 0.79	1.00 – 3.00	10	16.80 ± 6.34	11.00 – 31.00	$t_{9.28}$ = -7.426	< 0.001

Table 9. Summary of egg morphometrics (length and width) and egg number reported for species of *Diplostomum*. The species list was created based on Dubois (1970) and Niewiadomska (1996).

	Length (µm) mean (range)	**Width (µm) mean (range)**	**Egg number mean (range)**	**Location**	**Reference**
D. adamsi	99 (95 – 109)	65 (63 – 68)	9 (6 – 11)	North America	Lester and Huizinga (1977)
D. amygdalum	(78 – 90)	(47 – 59)	NA	Australia	Dubois (1970)
D. ardeae	(90 – 96)	(57 – 66)	2	North America	Dubois (1970)
D. baeri (syn. *D. volvens*)	(96 – 113)	(60 – 77)	(5 – 20)	Europe	Dubois (1970)
D. baeri bucculentum	(100 – 115)	(63-75)	(up to 15)	North America	Dubois (1970)
D. baeri eucaliae	102 (92 – 111)	59 (54 – 64)	(0 – 6)	North America	Hoffman and Hundley (1957)
D. commutatum (syn. *D. rutili*)	(101 – 132)	(57 – 80)	(up to 44)	Europe, Asia, North America	Dubois (1970)
D. crassum	(95 – 120)	(61 – 78)	(up to 27)	North America	Dubois (1970)
D. gasterostei	111	64	NA	Europe	Dubois (1970)
	111	64	(3 – 12)		Williams (1966)
D. gavium	(90 – 115)	(60 – 77)	(up to 30)	North America, Asia, Europe	Dubois (1970)
D. gobiorum	99 (85 – 105)	63 (57 – 70)	*	Europe	Shigin (1969)
D. huronense	(90 – 110)	(53 – 73)	(up to 34)	North America	Dubois (1970)
D. indistinctum	(91 – 115)	(57-72)	(up to 26)	North America	Dubois (1970)

D. kronschnepi	(90 – 110)	(43 – 77)	NA	Asia	Dubois (1970)
D. mahonae	(78 – 93)	(45 – 68)	(up to 20)	Europe	Dubois (1970)
D. marshalli	(94 – 104)	(58 – 68)	(up to 15)	North America	Dubois (1970)
D. mergi alascense	(94 – 110)	(55 – 65)	(up to 4)	North America	Dubois (1970)
D. mergi mergi	(80 – 110)	(50 – 67)	(few in number)	Europe, Asia	Dubois (1970)
D. micradenum	NA	NA	(few in number)	North America	Dubois (1970)
D. minutum	110	70	(2 – 7)	South America	Dubois (1970)
D. murrayense	(72 – 100)	(40 – 63)	(up to 16)	Australia	Dubois (1970)
D. nemachili†					
D. nordmanni†					
D. oedicnemum	(96 – 104)	(55 – 60)	2	Asia	Dubois (1970)
D. paracaudum	98 (85 – 120)	58 (55 – 70)	*	Europe	Shigin (1977)
D. paraspathaceum†					
D. parviventosum	(91 – 105)	(58 – 67)	(up to 10)	Europe	Dubois (1970)
D. pelmatoides	(87 – 93)	(48 – 62)	(1 – 4)	Europe	Rees (1955)
D. petromyzifluviatilis	103	51	9	Europe	Sweeting (1976)
D. phoxini	103	64	(2 – 4)	Europe	Williams (1966)
	(84 – 96)	(48 – 70)	(up to 9)		Dubois (1970)
D. pseudobaeri	99 (91 – 100)	61 (55 – 70)	10	Europe	Field and Irwin (1995)
D. pseudospathaceum (syn. *D. chromatophorum*)	NA	NA	NA	Europe	Niewiadomska (1984)

D. pungitii	119 (93 – 130)	70 (65 – 72)	(2 – 4)	Europe	Sitko and Rzad (2014)
D. pusillum	(77 – 115)	(55 – 78)	(up to 10)	Europe and Asia	Dubois (1970)
D. repandum	(90 – 115)	(60 – 72)	(many in number)	North America	Dubois (1970)
D. scheuringi	NA	NA	NA	North America	Hughes (1929)
D. scudderi	103	59	(few in number)	North America	Dubois (1970)
D. sobolevi	(77 – 89)	(51 – 64)	(1 – 6)	Europe	Dubois (1970)
D. spathaceum (syn. *D. helveticum* and *D. paracaudum*)	84 – 115	52 - 76	(up to 36)	Europe	Dubois (1970)
D. sternae	(95 – 110)	(45 – 79)	(up to 4)	Asia	Dubois (1970)
D. sudarikovi	(109 – 122)	(55 – 83)	NA	Europe	Dubois (1970)
D. vanelli	(93 – 108)	(54 – 63)	(few in number)	Asia	Dubois (1970)
D. yogenum	104 (95 – 110)	67 (60 – 75)	*	Europe	Shigin (1977)

*Unknown information, only the data available in the tables were translated from Russian to English.

†Adult species named in Niewiadomska (1996) for which no egg information was found.

CHAPTER 5. A comparison of the egg development and hatching success of two molecularly-delineated species of *Diplostomum* (Digenea)

ABSTRACT: The life cycles of many species of *Diplostomum* (Digenea) have been elucidated; however, few studies include the details of egg development and hatching success. Here the eggs of two molecularly-delineated sympatric species of *Diplostomum* were observed for differences in developmental parameters. These parameters included prepatent period, time required for first visible eyespot formation, hatching time, and hatching success. There was no significant difference in the mean prepatent period, total number of miracidia that developed eyespots or number of days to hatch between the two species. There was a significant difference in the average time for first visible eyespots to appear and hatching success. These data highlight the need for further studies investigating sympatric species of *Diplostomum* to document and understand differences in life history traits during the various phases of their life cycles and their role in transmission success.

INTRODUCTION

Digenetic trematodes (Platyhelminthes) have complex life cycles and many species require three different hosts for completion. The eggs of most species are unembryonated when passed from the adult fluke in the feces and, require a further period of development in an aquatic environment before the miracidium develops, hatches and disperses to infect the first intermediate host, a gastropod snail (Smyth and Halton, 1983). The complexity of digenean life cycles presents transmission challenges that begin when the egg is passed by the definitive host, including the effect of environmental conditions on the rate and development and hatching success of the egg, the short life span of the free-living stages and the difficulty of finding suitable hosts to continue development.

Different abiotic factors, such as temperature, oxygen tension, presence of definitive feces, and pH are known to influence the embryonation rate of fluke eggs (Smyth and Halton, 1983). For those species whose eggs hatch in water, the overall rate of embryonation and hatching success will determine when and how many miracidia enter the environment (Smyth and Halton, 1983). One adaptation to reduce the impact of this vulnerable stage is for parasites to produce many eggs (Price, 1980). Another may be phenotypic plasticity, where a flexible developmental schedule would permit successful transmission under various environmental conditions (Stearns, 1992).

Generally, an individual snail is infected by a single species of parasite at a time (Soldánová et al., 2012). Even though prevalence of parasitized snails is low, the use of the same species of snail host by species of parasite in the same geographic locality may increase the selective pressure on life history traits such as embryonation rate and/or hatching success to increase the successful transmission of one species over another.

Trematodes belonging to the genus *Diplostomum* (Trematoda: Digenea) have a three-host life cycle that includes fish-eating birds, primarily gulls, as their definitive hosts, and lymnaeid snails and primarily fish as first and second intermediate hosts, respectively (Figure 1). The fertilized eggs require a period of development in water to embryonate before they hatch, releasing a miracidium, which will infect a lymnaeid snail. If environmental factors are held constant, eggs from a particular species should embryonate and hatch within a narrow time period (Field and Irwin, 1995; McKeown and Irwin, 1995). Field and Irwin (1995) further hypothesize that this time period may vary among species.

Although the developmental details of the cycles of many species of *Diplostomum* are known (Niewiadomska, 1996), few studies include data of egg hatching success. Therefore, one of the basic aspects of the life history of many species of *Diplostomum* remains largely unstudied. In the few studies that provide data on egg hatching in *Diplostomum* spp., two potential confounding issues exist. First, in some studies gulls were fed infected lenses obtained from wild fish (e.g. Hoffman and Hundley, 1957; Lester and Huizinga, 1976; Field and Irwin, 1995; McKeown and Irwin, 1995). Implicit in this approach is the presumption of a single species of *Diplostomum* present in the lenses. However recent molecular studies have shown the presence of morphologically indistinguishable unidentified larval forms (Locke et al., 2010a, 2010b; Georgeiva et al., 2013) and mixed-species infections occur in the lenses of wild fish (Désilets et al., 2013). Thus, undetected mixed infections in exposed gulls may be a confounding factor in earlier studies not only by inflating what was assumed to be intraspecific variability but also potentially masking interspecific differences.

Further, it has long been recognized that many species of *Diplostomum* have overlapping morphometrics which has led to uncertainty as to whether morphology alone is sufficient for species level identification in many cases (Chappell et al., 1994; Niewiadomska, 1996). This has been further brought into question more recently by the revelation of multiple undescribed species within the genus (Locke et al., 2010a; Georgeiva et al., 2013). Second, it is impossible to know without further experimentation if the effect of temperature is equivalent across species of *Diplostomum*; therefore, the differing incubation

temperatures used in previous studies (e.g. Hoffman and Hundley, 1957; Dönges, 1969; Lester and Huizinga, 1977; Field and Irwin, 1995) only allow for limited interspecific comparisons to be made (Morley and Lewis, 2014). Overall, the problematic taxonomy and varying experimental conditions leave little conclusive data on basic reproductive information such as prepatent period, time required for eyespot development, hatching time and hatching success for individual species of *Diplostomum*. Such data either by themselves or in combination with other factors are essential to better understand the abundance and seasonal dynamics of particular species in a given habitat.

The goal of this chapter is to seek evidence of divergence in life history characteristics by comparing the prepatent period, time for first visible eyespots to appear, hatching time and hatching success of two sympatric molecularly-delineated species of *Diplostomum*, both of which use *Lymnaea elodes* as the first intermediate host in our study system, and to quantify the extent of intra- and interspecific variation between them. Closely related species with similar evolutionary pressures may maintain similar life history traits; however, the use of common resources may also promote divergence in order to reduce the overlap of life-history characteristics (Karvonen et al., 2006b).

MATERIALS AND METHODS

Naturally-infected *L. elodes* which were collected manually from the shoreline of Wheaton Lake, Bocabec, New Brunswick, Canada (45°10'08.3"N 66°59'56.2"W) for experimental work described in chapter 4 were also used in this experiment. Naturally infected snails were individually isolated in cups containing 250 ml of dechlorinated water. The cercariae were isolated, DNA extracted, amplified for the partial internal transcribed spacer region of ribosomal DNA (ITS-rDNA) and sequenced following primers, protocols and methods described in chapter 4. While there are more base pair differences in the mitochondrial cytochrome c oxidase subunit 1 barcode region (Locke et al., 2010b), the more conserved ITS-rDNA provides more successful amplification results and is adequate for species delineation. The ITS-rDNA sequences (Genbank Accession #'s: KY358236 - KY358240) were aligned, trimmed to 884-base pairs using Geneious R8 version 8.0.4 (Kearse et al., 2012) and compared in a number-of-difference based neighbor-joining tree with 1000 bootstrap replicates and pairwise deletion of gaps using a published sequence from GenBank for *Tylodelphys scheuringi* (Moszczynska et al., 2009) for the outgroup (Blasco-Costa et al., 2014; Selbach et al., 2015) in MEGA version 6 (Tamura et al., 2013) with 28 published ITS-rDNA sequences of *Diplostomum* spp. from Canada and Europe (Galazzo et al., 2002; Locke et al., 2010a; Georgieva et al., 2013; Blasco-Costa et al., 2014; Pérez-Del-Olmo et al., 2014; Locke et al., 2015; Selbach

et al., 2015). Robust evidence of the neighbor-joining analysis clustered two isolates with *Diplostomum* sp. 1 (GQ292522.1, Locke et al., 2010a; KT186794.1, Locke et al., 2015) and three isolates with *Diplostomum* sp. 4 (GQ292520.1, Locke et al., 2010a) and the cercariae were identified to species as *Diplostomum* sp. 1 and *Diplostomum* sp. 4 using provisional nomenclature based on Locke et al. (2010a) (Appendix 7). While it is rare for snails to be concurrently infected with more than one species of parasite (Soldánová et al., 2012), the trimmed alignments (Appendix 2) were analyzed for diagnostic sites (Appendix 3) and the peaks of the original chromatograms (Appendix 4) were re-examined at the diagnostic sites to validate species identification. No ambiguities were observed, confirming that each individual snail was infected with a single species of *Diplostomum*.

Experimental infections of each species of *Diplostomum* were established in the laboratory concurrently with the experiments described in chapter 4. All the animals used in this experiment were maintained and handled in accordance with the guidelines of the Canadian Council of Animal Care. Gull chicks were collected under authority of Canadian Wildlife Service (Quebec, Canada). The experiments were officially approved by the Concordia University Animal Research Ethics Committee (AREC, certificate #30000269).

One hundred naïve rainbow trout (*Oncorhynchus mykiss*) approximately 5 cm in total length, obtained from a commercial hatchery in Sainte-Edwidge, Quebec, were separated into two groups of 50 fish. The first set of fish was each exposed in five groups of 10 fish to 250 - 500 cercariae, estimated using a stereomicroscope, of *Diplostomum* sp. 1 for one hour in an aerated tank containing approximately five liters of water. A second group of 50 fish was exposed in a similar manner to cercariae of *Diplostomum* sp. 4. Upon termination of exposure, the fish exposed to each species were placed in separate tanks, maintained and fed commercial fish food for three months.

Newly hatched ring-billed gull chicks (*Larus delawarensis*) were collected from Île Deslauriers, Quebec (45°42'45.1"N 73°26'25.0"W). The gulls were fed wet/dry commercial dog/cat food and maintained for a period of at least eight weeks before initial exposure. The feces of each gull chick were examined one day prior to the commencement of the experiment to check for a previously established infection. Each gull was isolated for one hour in a wire bottom cage over a tray of water from which feces were collected and examined for eggs. The feces were examined by washing the fecal matter with water through a series of mesh screens (120 and 70 µm), allowing the sediment to settle and subsequently examined for eggs using a stereomicroscope. No eggs were recovered from the feces of the gull chicks.

The morning of the gull infection, the fish exposed to each species of *Diplostomum* were euthanized and the eyes removed in a physiological saline solution (0.85%). Each gull was fed the whole lenses from 10 fish. Five gulls were infected with metacercariae of *Diplostomum* sp. 1 and five gulls with metacercariae of *Diplostomum* sp. 4.

Beginning one day post-exposure (PE), each gull was isolated for one hour /day in a wire bottom cage over a tray of water. The feces were collected and examined daily for eggs as described above to determine the prepatent period (first recovery of eggs after exposure) of each species.

For each of the 10 birds, at one-week PE, 150 eggs were haphazardly collected for the egg observations. The 150 eggs were distributed into three groups of 50 eggs, and each group of eggs was placed in a plastic petri dish (60 X 15 mm) filled with water. The dishes were kept at room temperature (approximately 18 ° C) and exposed daily to nine hours of artificial ambient light (9:00 am – 6:00 pm). Water temperatures are normally 18 ° C or higher from early June to mid-September at these latitudes (D. J. Marcogliese, unpublished observations). The eggs were examined under a stereomicroscope daily to determine the time required (in days) for first visible eyespots to appear and hatching to occur and to quantify the number of miracidia to develop eyespots and hatch in each sample. Egg development was categorized as undeveloped when the eggs were undifferentiated; embryonated when the eyespots of the miracidium were first visible; or hatched when the operculum was open and the egg shell was empty. Observations continued for three weeks based on the maximum hatching times reported from previous studies on egg hatching dynamics of species of *Diplostomum* (Table 10) and the observations of previous studies noting relatively synchronous hatching of two different species held under identical conditions (Field and Irwin, 1995). Eggs that failed to hatch during this time period nor exhibiting development were considered non-hatched.

Descriptive statistics of mean (± standard deviation, SD) and range values for both species of *Diplostomum* studied for the following variables were calculated: prepatent period, first appearance of eyespot formation, hatching time and total number of miracidia to develop eyespots and hatch. Outliers were assessed by converting raw data into standardize scores, where cases with a *z*-score ± 3.00 were considered potential outliers (Tabachnick and Fidell, 2006). Normality was tested with skew and kurtosis ($p < 0.01$) and homoscedasticity was tested with Levene's Test ($p < 0.05$: Tabachnick and Fidell, 2006).

There were no outliers but transformations were required for two variables. The first, a square root (K – X) transformation was performed for the negatively skewed variable of days for hatching to occur, where K was equal to the largest score plus 1 (Tabachnick and Fidell, 2006). For the second, a square root transformation was required for the positively skewed total number of eggs to hatch.

Two-tailed independent samples t-tests were calculated to determine if there were significant differences between *Diplostomum* sp. 1 and *Diplostomum* sp. 4 for each of the following variables; mean prepatent periods, mean number of days for first visible eyespots to appear and hatching to occur and mean number of total eggs to hatch. In the case of total number of miracidia to develop eyespots where the data were heteroscedastic, therefore a two-tailed independent samples t-test not assuming heterogeneity was calculated. Probabilities of ≤ 0.05 were considered significant. Statistical analyses were conducted using the SPSS® 24.0 software package.

RESULTS

There was no significant difference in the mean prepatent period between the two species of *Diplostomum* studied herein (Independent t-test, $p = 0.580$: Table 11). Both species began producing eggs either four- or five-days PE.

There was a significant difference in the average number of days it took for first visible eyespots to appear between the species (Independent t-test, $p = 0.033$). On average *Diplostomum* sp. 4 developed eyespots approximately one day before those of *Diplostomum* sp. 1 (Table 11). However, there was no significant difference between the species in the number of days required for eggs to hatch (Independent t-test, $p = 0.330$). Both species began hatching on average 20 days after being passed in the feces of the gull (Table 11).

There was no significant difference in the mean number of miracidia to develop eyespots between the species (Independent t-test, $p = 0.177$: Table 11). However, there was a significant difference in the mean number of eggs that hatched between the species (Independent t-test, $p = 0.004$), where on average *Diplostomum* sp. 4 had a higher hatching success rate than *Diplostomum* sp. 1 (Table 11). Overall, out of a total of 750 eggs for each species, there were only 5 (0.67%) and 28 (3.73%) of the eggs successfully hatched for *Diplostomum* sp.1 and *Diplostomum* sp. 4 respectively.

DISCUSSION

To the best of my knowledge this is the first study to examine interspecific differences in prepatent period, eyespot formation and hatching characteristics between two molecularly-delineated sympatric species of *Diplostomum.* These findings show that while there was no difference in mean prepatent period or the total number of miracidia to develop eyespots or timing of hatch, there was a significant difference in mean length of time required for first visible eyespots to appear and overall hatching success. The life cycles of 22 species of *Diplostomum* have been experimentally completed (Niewiadomska, 1996). Of these, only eight species have details of egg hatching dynamics (Table 10), However, these results may be an amalgamation of interspecific differences due the recent discovery of the presence of several morphologically indistinguishable unidentified larval forms (Locke et al., 2010a) and should be interpreted with caution.

In the case of the two species studied here, both use *L. elodes* as the first intermediate host. Natural selection can potentially favor differing prepatent periods. A faster prepatent period has eggs being released sooner into the environment, whereas a slower prepatent period could allow for the parasite to reach a larger size and produce eggs at a higher rate (Poulin, 2007). In a parallel study, adults of *Diplostomum* sp. 1 were shown to have equivalent hindbody size but smaller and fewer eggs than those of *Diplostomum* sp. 4 (Chapter 4; Lapierre et al., 2018); however, this did not translate to differences in prepatent period. The two species of *Diplostomum* examined here did not differ on the time it took to reach sexual maturity; both started to produce eggs between four to five days PE, which is similar to reported prepatent periods for other species in the genus (Table 10). One of these studies also infected definitive hosts by feeding only fish eyes (McKeown and Irwin, 1995). Feeding the gulls solely the lens may have decreased the digestion period and shorted the prepatent period than would be observed in natural conditions as gulls would likely ingest whole fish, increasing the digestion period.

The first occurrence of eyespots for *Diplostomum* sp. 1 and *Diplostomum* sp. 4 was later than the reported ranges for other *Diplostomum* spp. (Table 10). However, the first occurrence of hatching was similar to other species of *Diplostomum* (Table 10). Only Field and Irwin (1995) present experimental data comparing both eyespot development and hatching from two species held under identical conditions, where the eggs of *D. spathaceum* developed eyespots and hatched quicker than those of *D. pseudospathaceum* (Table 10). Here however, even though *Diplostomum* sp. 4 had faster eyespot

formation than *Diplostomum* sp. 1, this was not translated into faster hatching. It is unclear whether faster development time leads to a fitness advantage, given that the hatching times did not differ.

The two species studied herein differed in hatching success, with *Diplostomum* sp. 4 having greater hatching success than *Diplostomum* sp. 1. The hatching success seen here however, was much lower than that of *D. pseudospathaceum* (22.8 - 34.6%) reported by Rieger et al. (2013) or the 90% hatching success reported by Field et al. (1994) for *D. spathaceum*, *D. parviventosum*, *D. pseudobaeri* and *D. volvens*.

McKeown and Irwin (1995) reported synchronous hatching upon light stimulation. With only means and not ranges reported from other previous studies characterizing egg hatching in *Diplostomum* spp., it is assumed the time frame was relatively synchronous (Table 10). However, the low hatching success may be due to dormancy of some eggs that would hatch at a later date, reflecting a possible bet-hedging strategy (Poulin, 2007), which could mean the data here would be underestimating hatching success for *Diplostomum* sp. 1. Longer time-frame studies would be needed, with controlled water maintenance, different incubation temperatures and sterile conditions to decrease potential bacterial or fungal growth.

Lastly, as suggested by Lapierre et al. (2018) (Chapter 4), the ring-billed gull may not be the best definitive host for either species in this study, especially *Diplostomum* sp. 1. However, in a coinciding experiment, both *Diplostomum* sp. 1 and *Diplostomum* sp. 4 established successfully in the ring-billed gull and produced eggs in the ring-billed gull (Chapter 3, Table 7, Figure 7).

The results provide evidence of limited differences between egg development and hatching success of these two species of *Diplostomum*. These data highlight the need for further studies of sympatric species of *Diplostomum* to understand how differences in life history traits may lead to possible partitioning of life cycle characteristics to avoid competition within different hosts throughout the parasites' life cycles.

Table 10. Summary of experimental life cycle studies examining egg hatching dynamics of *Diplostomum* spp.

Reference	Species	Snail host	Experimental bird host	Prepatent period (d)	Incubation temperature (°C)	First eyespot appearance (d)	First hatching (d)
Hoffman and Hundley (1957)	*D. baeri eucaliae*	*Stagnicola palustris, S. palustris elodes*	Chicks	3	23	10	12
Dönges (1969)	*D. phoxini*	*Lymnaea peregra ovata*	*Anas platyrhynchos*	4 - 6	19.5 - 20	NA	17
					20.0 - 21		19
Lester and Huizinga (1977)	*D. adamsi*	*L. elodes*, *L. stagnalis*	*Larus argentatus*	NA	22	NA	22*
Yurlova and Fedorov (1989)	*D. chromatophorum* (syn. *D. pseudospathaceum*)	*L. stagnalis*, *L. draverti*, *L. tumida*	*L. ridibundus*	8	NA	NA	17
Field and Irwin (1995)	*D. spathaceum*	*L. peregra*	*Gallus gallus domesticus*	NA	30	6	10
	D. pseudobaeri					9	13
	D. spathaceum	*L. peregra,*		4†	30	6	‡

McKeown and	*D. parviventosum*	*L. stagnalis*	*G. gallus*	8	‡
Irwin (1995)	*D. volvens*		*domesticus*	11	‡

* Eggs were maintained in the dark and exposed to light on the 22nd day and hatching occurred.

† Feces collection began 4 days post-infection.

‡ Hatching values were not reported, values reported were when the miracidium was fully formed and ready to hatch, not when they were free from the shell.

Table 11. Summary statistics describing the distributions of mean (± standard deviation, SD) and range for the prepatent periods, timing for eyespot formation, and hatching time and success for *Diplostomum* sp. 1 and *Diplostomum* sp. 4, along with corresponding test statistic (two-tailed independent samples t-test) and *p* values for species comparisons for each variable. Significant differences between species for the specified variables are indicated in bold.

	***Diplostomum* sp. 1**			***Diplostomum* sp. 4**			**Species comparisons**	
	Sample size (n)*	Mean ± SD	Range	Sample size (n)*	Mean ± SD	Range	Test statistic	*p*
Prepatent period (days)	5 gulls	4.33 ± 0.52	4 - 5	5 gulls	4.60 ± 0.55	4 - 5	$t_8 = -0.577$	0.580
First appearance of eyespots (days)	13	15.38 ± 1.21	14 - 17	15	14.40 ± 1.18	13 - 17	$t_{26} = 2.250$	0.033
First occurrence of hatching (days)	4	20.75 ± 0.50	20 - 21	13	20.31 ± 0.86	18 - 21	$t_{15} = -1.008$	0.330
Total number of eggs to develop eyespots	15	8.87 ± 6.50	0 - 26	15	6.40 ± 1.92	3 - 9	$t_{16.423} = 1.409$	0.177
Total number of hatched eggs	15	0.40 ± 0.74	0 - 2	15	1.80 ± 2.04	0 - 7	$t_{28} = -3.152$	0.004

*Sample size for the egg variables is the total number of eggs that developed eyespots and hatched out of a total sample of 150 eggs.

GENERAL DISCUSSION AND CONCLUSION

Species of *Diplostomum* have a complex life cycle involving three hosts, a snail (first intermediate host), fish (second intermediate host) and piscivorous bird (definitive host) (Figure 1). Species of *Diplostomum* infect the eyes of the fish host and have received much attention as they can be pathogenic due to potential vision loss from cataract formation, reduced growth, and changes in appearance and behaviour (Chappell et al., 1994; Karvonen, 2012). Until recently the majority of research has focused on *Diplostomum spathaceum*, a lens-infecting species. Unfortunately, the taxonomy of species of *Diplostomum* remains unresolved. Previous field work involving species of *Diplostomum* revealed cryptic diversity and lens-infecting species were reported from an array of phylogenetically diverse fish hosts (Locke et al., 2010a, 2010b; Georgieva et al., 2013) with negative interactions between species in the fish host (Désilets et al., 2013). This suggests that our current understanding of the life history and community dynamics of *Diplostomum* spp. may be an amalgamation of data from multiple cryptic species that overestimates species diversity.

These recent developments prompted the series of experimental studies within this dissertation. The goal of this thesis was to elucidate differences in life history characteristics and interactions in the fish and gull host of sympatric cryptic species. The research design included the establishment of the life cycle of three species of *Diplostomum* using naturally infected marsh pond snails (*Lymnaea* spp.) to establish laboratory infections on various species of fish and in ring-billed gulls (*Larus delawarensis*). Use of morphology-based methods of identification of species of *Diplostomum* is problematic, so I applied DNA based methods to identify larval and adult stages in this dissertation. Here, molecular-delineation to species level allowed me to distinguish species of *Diplostomum* using DNA at different stages of the life cycle and provided a unique opportunity to compare life history parameters and to manipulate interactions at the level of the fish and gull hosts. The species were designated as *Diplostomum* sp. 1 and *Diplostomum* sp. 4, both of which are found in the lens, using provisional nomenclature based on Locke et al. (2010a) and *D. baeri*, which infects the humor, using nomenclature based on Georgieva et al. (2013) (Appendix 5).

Field data have reported *Diplostomum* sp. 1 from various fish species (Locke et al., 2010a; 2010b; Désilets et al., 2013). This study provides the first experimental exposures to five phylogenetically diverse species of fish and shows *Diplostomum* sp. 1 established in cichlids (*Amatitlania nigrofasciata*), dace (*Chrosomus eos*), guppies (*Poecilia* sp.) and rainbow trout (*Oncorhynchus mykiss*), but not walleye (*Sander*

vitreus) (Chapter 1). The intensity of infections was low for each species of fish indicating low specificity, corroborating field data (Locke et al., 2010a; 2010b; Désilets et al., 2013) and were similar with regards to low host specifity with the reported experimental data on *D. spathaceum* (Betterton, 1974; Sweeting 1974; Speed and Pauley, 1984). The benefit of low host specificity for *Diplostomum* sp. 1 is that it can exploit multiple host species; however, the cost being low establishment success. This cost was evident in the significant decline in establishment success for *Diplostomum* sp. 1 in challenge infections to both conspecific or congener primary exposures (Chapter 2). These are the first results to indicate the importance of the role of priority in establishment success for *Diplostomum* sp. 1. Further, as the challenge exposures were performed before adaptive immune responses would have been mounted (Chappell et al., 1994), these results raise a whole new series of questions to be investigated such as interspecific differences in timing of cercarial release in natural conditions, time for cercariae to reach the lens and innate immune responses initiated upon cercarial penetration of naïve and pre-exposed fish.

Once the infected fish has been ingested by the gull definitive host, adult parasites normally occupy a specific and predictable site within the host where conditions are optimal for growth and reproduction (Holmes and Price, 1986). *Diplostomum* sp. 1 occupied a midrange position within the gut of the ring-billed gull, with a positive relationship between total linear span and total number of flukes (Chapter 3). *Diplostomum* sp. 1 began to produce a few small eggs (Chapter 3 and 4) approximately four to five days PE (Chapter 5), much like many other reported species of *Diplostomum* (Table 10). These are the first data for species of *Diplostomum* to indicate there to be no relationship between the egg size and the hindbody size nor between egg size and number of eggs (Chapter 4). The lack of relationship between hindbody size and egg size was unexpected as previous comparisons of species of trematode indicate a positive correlation between egg size and adult worm size (Poulin, 1997). However, the absence of a correlation between egg size and number of eggs was not surprising as there has been little evidence of a trade-off between egg size and egg number in trematodes (Poulin, 1997). Experimental observations of development and hatching indicates that *Diplostomum* sp. 1 has a developmental period similar to other reported species of *Diplostomum* (Table 10 and 11) but with very low hatching success (Chapter 5). The extremely low hatching success brought up two main questions: first, should the experiment have lasted longer to verify if the eggs of *Diplostomum* sp. 1 would hatch at a later date; second, were the experimental conditions adequate? Further studies are required to clarify the life history characteristics of *Diplostomum* sp. 1 with regards to egg development and hatching.

Field data have also reported *Diplostomum* sp. 4 from various fish species (Locke et al., 2010a; 2010b; Désilets et al., 2013). *Diplostomum* sp. 4 was successful in establishing in only dace, guppies and rainbow trout, further, they had a much higher mean intensity in rainbow trout in comparison to *Diplostomum* sp. 1 (Chapter 1). These are the first tests to experimentally infect dace, guppies and rainbow trout with *Diplostomum* sp. 4. While the index of host specificity classified both *Diplostomum* sp. 1 and *Diplostomum* sp. 4 as equivalent generalists, there are subtle differences in that *Diplostomum* sp. 4 has a higher structural and phylogenetic specificity than *Diplostomum* sp. 1. These are the first data to show differences in structural and phylogenetic specificity in lens-infecting species of *Diplostomum*. The benefit for *Diplostomum* sp. 4 to have a higher structural and phylogenetic specificity is that in rainbow trout it was able to achieve higher intensities, which would potentially lead to greater numbers of conspecific mates in the gull host leading to an increased genetic diversity in future generations if rainbow trout are naturally infected. Further, the higher structural specificity of *Diplostomum* sp. 4 is revealed in similar establishment success in challenge infections in rainbow trout whether the primary exposure was to a conspecific or a congener (Chapter 2). In Chapter 2 however, naïve rainbow trout were equally susceptible to infection by either *Diplostomum* sp. 1 or *Diplostomum* sp. 4 upon initial exposure, indicating little difference between these two species of *Diplostomum* with regards to their ability to successfully attach, penetrate and migrate to the lens. This may have been due to smaller sample sizes which were unable to elucidate the interspecific differences. Unlike previous challenge experiments (e.g. Scharsack and Kalbe, 2014), these data showed no reduction in establishment success for *Diplostomum* sp. 4 in challenge infections, which would be beneficial as it would potentially increase transmission success (Karvonen, 2012).

Transmission to the gull host revealed *Diplostomum* sp. 4 to occupy an anterior intestinal distribution in the gut of the ring-billed gull, also with a positive relationship between total linear span and total number of flukes (Chapter 3). *Diplostomum* sp. 4 began to produce many large eggs (Chapter 3 and 4) approximately four to five days PE (Chapter 5). These are the first data to show significant egg size differences between *Diplostomum* sp. 1 and *Diplostomum* sp. 4. Here also, there was no relationship between the egg size and the hindbody size nor between egg size and number of eggs (Chapter 4). Differences in egg sizes and number may reflect other potential differences in life history traits between the two species such as miracidial longevity and infectivity and cercarial output among others which still need to be investigated. For example, larger eggs producing bigger miracidia may have more energy reserves allowing them a longer life span. Further, larger miracidia may develop into mother sporocysts

capable of increased cercarial output. Experimental observations of development and hatching would indicate for *Diplostomum* sp. 4 to have faster eyespot development and higher hatching success than *Diplostomum* sp. 1 (Chapter 5), but still very low in comparison to other reported results. The laboratory conditions produced low hatching success and the relative differences between *Diplostomum* sp. 1 and *Diplostomum* sp. 4 may not represent inherent differences in life history characteristics.

Limited information was gathered for *D. baeri*. Transmission to the gull host revealed *D. baeri* to occupy a midrange intestinal distribution in the gut of the ring-billed gull, produced few eggs, and a positive relationship between total linear span and total number of flukes (Chapter 3). As naturally infected snails were collected from the field, the sampling efforts or low infection rates are potential reasons as to why *D. baeri* were only collected during one season.

Comparatively, examining only single infections based on the information gathered here, due to the low number of eggs and hatching success of *Diplostomum* sp. 1, it would be expected for fewer snails to be infected with *Diplostomum* sp. 1 and more species of fish to be infected at low intensities of *Diplostomum* sp. 1 compared to *Diplostomum* sp. 4. However, these parasites do not exist in isolation. All three species examined herein have been reported from the same localities (Locke et al., 2010a, 2010b; Désilets et al., 2013). The infracommunity within the fish host can potentially be transferred to the bird host.

Chapter 3 investigated the effects of intraspecific and interspecific interactions on the intestinal distribution and range and fecundity for *Diplostomum* sp. 1, *Diplostomum* sp. 4 and *D. baeri* in gulls in mixed species infections (*Diplostomum* sp. 1 and *D. baeri*; *Diplostomum* sp. 1 and *Diplostomum* sp. 4). In mixed infections, significant spatial displacement was observed only for *D. baeri* when *Diplostomum* sp. 1 was also present. Intensity was directly correlated to the number of occupied intestinal segments, and there was no significant difference in mean linear span for each species between single and mixed species infections. In mixed species infections, *Diplostomum* sp. 4 experienced a dramatic decline in fecundity in the presence of *Diplostomum* sp. 1 whereas fecundity of *Diplostomum* sp. 1 increased in mixed infections with *D. baeri*. A limitation herein (Chapter 3) was the inability to control for infection levels in the gull host and it should be noted that *Diplostomum* sp. 1 had the greatest intensity in the mixed infections with *D. baeri* which could have led to the increase in fecundity. These results highlight evidence of interspecific interactions which may play a role in population dynamics of *Diplostomum* spp. and community structure.

Taking the data on mixed species into consideration, the presence of *D. baeri* would seemingly benefit *Diplostomum* sp. 1 as the changes in the microenvironment may lead to greater egg production. Whether or not the shift in spatial distribution of *D. baeri* has positive or negative consequences has yet to be explored. However, the presence of *Diplostomum* sp. 1 in conjunction with *Diplostomum* sp. 4 would be costly for *Diplostomum* sp. 4 due to the decrease in fecundity. Further studies examining interspecific differences of cercarial release and infectivity would be of interest to further elucidate the interactions between these two species.

Overall, this dissertation provides novel experimental evidence of host specificity, intra- and interspecific interaction at the level of both the fish and gull host, along with life history characteristic differences between species with respect to egg size and number, rate of eye spot development and potentially hatching success. As many previous studies examining lens infecting species were questionably identified as *D. spathaceum*, the evidence of interspecific differences between the lens infecting species studied herein lend to the suggestion of an overestimation of the plasticity of *D. spathaceum* and the need for re-evaluation. Such discoveries are important because they elucidate similarities and differences of the life cycle and ecological differences between species of *Diplostomum* to showcase their uniqueness and interspecific interactions. Laboratory experiments provide the opportunity to manipulate variables and to better understand transmission ecology, host specificity, life history, and many other aspects of parasite biology (Poulin, 2007). Conducting similar experiments with other species would allow for a comparative approach to be applied and to be able to look for patterns across species, which is only recently being applied to parasites (Morand and Poulin, 2003). Application of ecological theories to parasitic systems allows for an increase in understanding of interactions among parasites as well as between parasites and their hosts and environments in a wider ecological context.

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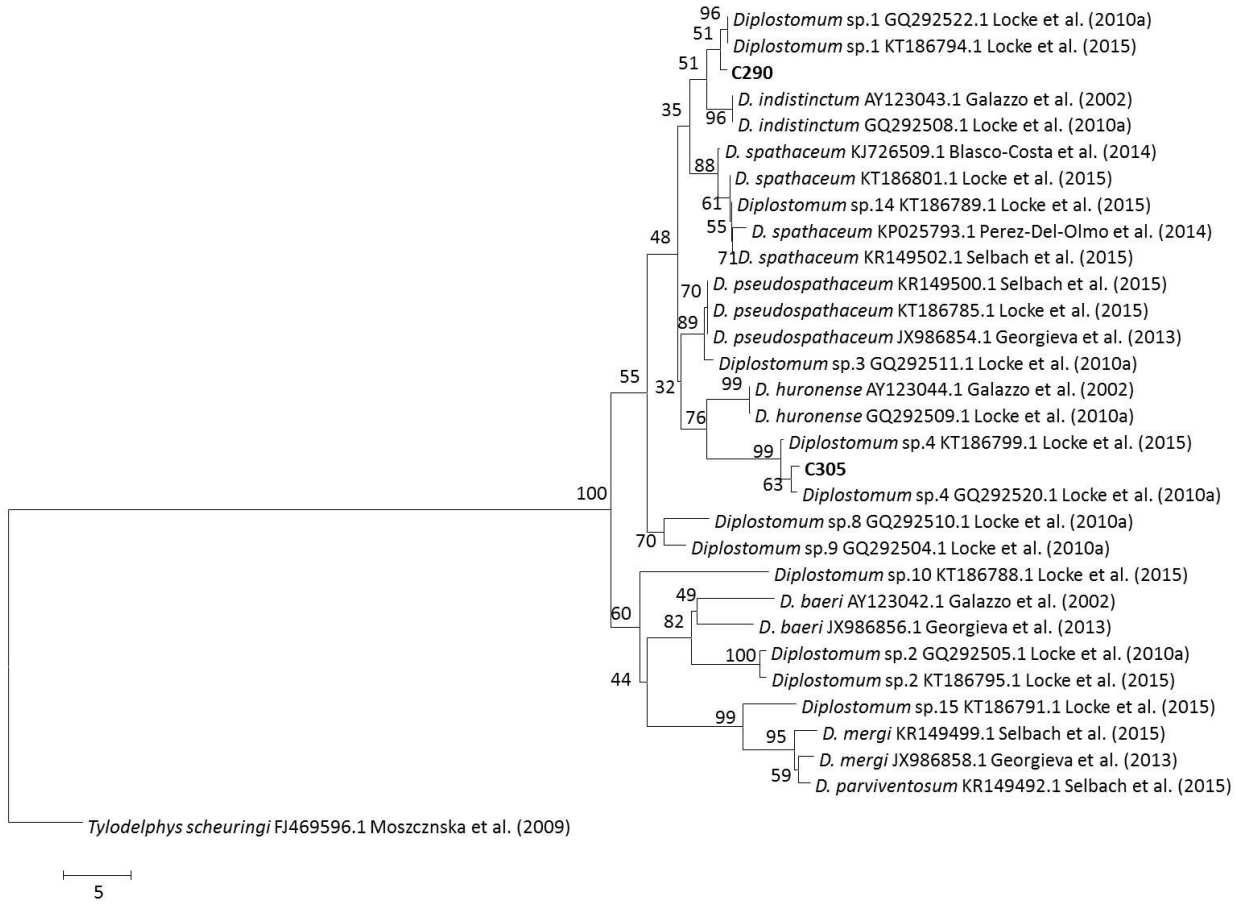
APPENDICES

Appendix 1. Neighbor-joining consensus tree for species of *Diplostomum* reconstructed using two newly generated (indicated in bold as C290 and C305, personal reference numbers) and 28 ITS-rDNA sequences retrieved from GenBank. Outgroup: *Tylodelphys scheuringi*. Nodal support is based on 1000 bootstrap replicates. The scale bar indicates the number of base pair differences.



Appendix 2. Trimmed multiple alignment of 23 new specimens (bolded) of *Diplostomum* spp. cercariae collected from *Lymnaea elodes* snail hosts in Wheaton Lake, Bocabec, New Brunswick from an 884-base pair (bp) alignment of the internal transcribed spacer region of rDNA. Diagnostic sites are shaded between comparisons of the sister clade of the closest congener based on neighbor-joining analyses (Appendix 1 and 5). Comparisons are between *Diplostomum* sp. 1 with *Diplostomum indistinctum*, *Diplostomum* sp. 4 with *Diplostomum huronense* and *Diplostomum baeri* with *Diplostomum* sp. 2. A hyphen indicates a gap in the position.

GenBank sequence ID	**Base Pair**	**Sequence**
***D. baeri* MH108185**	1	TTATCGAACTCGGTCTCGGCCGGGTTTGGAAATAATTGGCGCGTTGGGTTAGCAA
***D. baeri* MH108185**	1	TTATCGAACTCGGTCTCGGCCGGGTTTGGAAATAATTGGCGCGTTGGGTTAGCAA
***D. baeri* MH108187**	1	TTATCGAACTCGGTCTCGGCCGGGTTTGGAAATAATTGGCGCGTTGGGTTAGCAA
***D. baeri* MH108188**	1	TTATCGAACTCGGTCTCGGCCGGGTTTGGAAATAATTGGCGCGTTGGGTTAGCAA
***D. baeri* MH108189**	1	TTATCGAACTCGGTCTCGGCCGGGTTTGGAAATAATTGGCGCGTTGGGTTAGCAA
***D. baeri* MH108201**	1	TTATCGAACTCGGTCTCGGCCGGGTTTGGAAATAATTGGCGCGTTGGGTTAGCAA
***D. baeri* MH108202**	1	TTATCGAACTCGGTCTCGGCCGGGTTTGGAAATAATTGGCGCGTTGGGTTAGCAA
D. baeri AY123042.1 Galazzo et al. (2002)	1	TTATCGAACTCGGTTTCGGCCGGGTTCGGAATTAATTGGCGCGTTGGGTTAGCAA
D. baeri JX986856.1 Georgieva et al. (2013)	1	TTATCGAACTCGGTCTCGGCCGGGTTTGGAAATAATTGGCGCGTTGGGTTAGCAA
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	1	TTATCGCSCTCGGTTTCGACCGGGTTCGGAAATAATTGGCGCGTTGGGTTAGCAA
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	1	TTATCGAGCTCGGTTTCGACCGGGTTCGGAAATAATTGGCGCGTTGGGTTAGCAA
D. mergi KR149499.1 Selbach et al. (2015)	1	TTATCGAACTCGGTTTCGACCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
D. parviventosum KR149492.1 Selbach et al. (2015)	1	TTATCGAACTCGGTTTCGACCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA

D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
D. pseudospathaceum KT186785.1 Locke et al. (2015)	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	1	TTATCGAACTCGGTTTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	1	TTATCGAACTCGGTTTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
D. spathaceum KR149502.1 Selbach et al. (2015)	1	TTATCGAACTCGGTTTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
***Diplostomum* sp. 1 KY358239**	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
***Diplostomum* sp. 1 KY358240**	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
***Diplostomum* sp. 1 MH108191**	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
***Diplostomum* sp. 1 MH108192**	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
***Diplostomum* sp. 1 MH108193**	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
***Diplostomum* sp. 1 MH108194**	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
***Diplostomum* sp. 1 MH108195**	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
***Diplostomum* sp. 1 MH108196**	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
Diplostomum sp. 1 MH108197	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
***Diplostomum* sp. 1 MH108198**	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
***Diplostomum* sp. 1 MH108199**	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
***Diplostomum* sp. 1 MH108200**	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
D. indistinctum AY123043.1 Galazzo et al. (2002)	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
D. indistinctum GQ292508.1 Locke et al. (2010a)	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA

Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	1	TTATCGAACTCGGTCTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	1	TTATCGAACTCGGTCTCGGCCGGGTTTGGAATTAATTGGCGCGTTGGGTTGGCAA
***Diplostomum* sp. 4 KY358236**	1	TTATCGAACTCGGTCTCGGCCGGGTTTGGAATTAATTGGCGCGTTGGGTTGGCAA
***Diplostomum* sp. 4 KY358237**	1	TTATCGAACTCGGTCTCGGCCGGGTTTGGAATTAATTGGCGCGTTGGGTTGGCAA
***Diplostomum* sp. 4 KY358238**	1	TTATCGAACTCGGTCTCGGCCGGGTTTGGAATTAATTGGCGCGTTGGGTTGGCAA
***Diplostomum* sp. 4 MH108190**	1	TTATCGAACTCGGTCTCGGCCGGGTTTGGAATTAATTGGCGCGTTGGGTTGGCAA
D. huronense AY123044.1 Galazzo et al. (2002)	1	TTATCGAACTCGGTCTCGGCCGG-TTCGGATTTAATTGGCGCGTTGGGTTGGCAA
D. huronense GQ292509.1 Locke et al. (2010a)	1	TTATCGAACTCGGTCTCGGCCGG-TTCGGATTTAATTGGCGCGTTGGGTTGGCAA
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	1	TTATCGAACTCGGTTTCGGCCGG-TTCGGATTTAATTGGCGCGTTGGGTTGGCAA
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	1	TTATCGAACTCGGTTTCGGCCGG-TTCGGATTTAATTGGCGCGTTGGGTTGGCAA
Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	1	TTATCGGATTCGGTTTCGGCCGGGTTCGGATTTAATTGGCGCGTTGGGTTGGCAA
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	1	TTATCGAACTCGGTTTCGGCCGGGTTYGGATTTAATTGGCGCGTTGGGTTGGCAA
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	1	TTATCGAACTCGGTTTCGACCGGGTTCGGATTTAATTGGCGCGTTAGGTTGGCAA
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	1	TTATCGGGNNCGGNCTCGGCCGGGTTCGGAACTAATTGGCGCGTTNNNNNNGCAA
***D. baeri* MH108185**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGCAAGGGACCC
***D. baeri* MH108185**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGCAAGGGACCC
***D. baeri* MH108187**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGCAAGGGACCC
***D. baeri* MH108188**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGCAAGGGACCC
***D. baeri* MH108189**	56	TTGAGTTAACCTACCGTGTCAAGGAATAGACGGATGGGCTTCCCGCAAGGGACCC
***D. baeri* MH108201**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGCAAGGGACCC
***D. baeri* MH108202**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGCAAGGGACCC

D. baeri AY123042.1 Galazzo et al. (2002)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGCAAGGGACCC
D. baeri JX986856.1 Georgieva et al. (2013)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGCAAGGGACCC
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGCAAGGGACCC
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGCAAGGGACCC
D. mergi KR149499.1 Selbach et al. (2015)	56	TTGAGTTAACCTGGCGTGTCAAGGAATTGACGGATGGGCTTCCCGCAAGGGACCC
D. parviventosum KR149492.1 Selbach et al. (2015)	56	TTGAGTTAACCTGGCGTGTCAAGGAATTGACGGATGGGCTTCCCGCAAGGGACCC
D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
D. pseudospathaceum KT186785.1 Locke et al. (2015)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
D. spathaceum KR149502.1 Selbach et al. (2015)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
***Diplostomum* sp. 1 KY358239**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
***Diplostomum* sp. 1 KY358240**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
***Diplostomum* sp. 1 MH108191**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
***Diplostomum* sp. 1 MH108192**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
***Diplostomum* sp. 1 MH108193**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
***Diplostomum* sp. 1 MH108194**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
***Diplostomum* sp. 1 MH108195**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
***Diplostomum* sp. 1 MH108196**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC

Diplostomum sp. 1 MH108197	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
***Diplostomum* sp. 1 MH108198**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
***Diplostomum* sp. 1 MH108199**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
***Diplostomum* sp. 1 MH108200**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
D. indistinctum AY123043.1 Galazzo et al. (2002)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
D. indistinctum GQ292508.1 Locke et al. (2010a)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
***Diplostomum* sp. 4 KY358236**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
***Diplostomum* sp. 4 KY358237**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
***Diplostomum* sp. 4 KY358238**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
***Diplostomum* sp. 4 MH108190**	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
D. huronense AY123044.1 Galazzo et al. (2002)	55	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
D. huronense GQ292509.1 Locke et al. (2010a)	55	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGAKGGGCTTCCCGTAAGGGACCC
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	55	TTGAGTTAGCCCAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGCAAGGGACCC
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	55	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	56	TTGAGTTAACCTAGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	56	TTGAGTTAACCTAGCRTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	56	TTGAGTTAACCTGGCGTGTCAAGGAATTGACGGATGGGCTTCCCGTAAGGGACCC
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	56	TTGAGTTGGTCAGGCGCGTCAAGGAATTGACGGATGGGCT-CCCATAGAGGACCC
***D. baeri* MH108185**	111	GCGAATTACAGTGCATATG--AACGGGATTGACGGACGAACTCTTCACTTGTGGA

***D. baeri* MH108185**	111	GCGAATTACAGTGCATATG--AACGGGATTGACGGACGAACTCTTCACTTGTGGA
***D. baeri* MH108187**	111	GCGAATTACAGTGCATATG--AACGGGATTGACGGACGAACTCTTCACTTGTGGA
***D. baeri* MH108188**	111	GCGAATTACAGTGCATATG--AACGGGATTGACGGACGAACTCTTCACTTGTGGA
***D. baeri* MH108189**	111	GCGAATTACAGTGCATATG--AACGGGATTGACGGACGAACTCTTCACTTGTGGA
***D. baeri* MH108201**	111	GCGAATTACAGTGCATATG--AACGGGATTGACGGACGAACTCTTCACTTGTGGA
***D. baeri* MH108202**	111	GCGAATTACAGTGCATATG--AACGGGATTGACGGACGAACTCTTCACTTGTGGA
D. baeri AY123042.1 Galazzo et al. (2002)	111	GCGAATTACAGTGCATAT--AAACGGGATTGACGGACGAACTCTCCACTTGTGGA
D. baeri JX986856.1 Georgieva et al. (2013)	111	GCGAATTACAGTGCATAT--AAACGGGATTGACGGACGAACTCTTCACTTGTGGA
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	111	GCGAATAACAGTGCATAT--AAACGGGATTGACGGACGAACTCTTCACTTGTGGA
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	111	GCGAATAACAGTGCATAT--AAACGGGATTGACGGACGAACTCTTCACTTGTGGA
D. mergi KR149499.1 Selbach et al. (2015)	111	GCACATAACAGTGCATATACAAACGGGATTGACGGACGAACTCTTTACTTGTGAA
D. parviventosum KR149492.1 Selbach et al. (2015)	111	GCACATAACAGTGCATATATAAACGGGATTGACGGACGAACTCTTTACTTGTGAA
D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	111	GCGAATTACAGTGCAGA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGAA
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	111	GCGAATTACAGTGCAGA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGAA
D. pseudospathaceum KT186785.1 Locke et al. (2015)	111	GCGAATTACAGTGCAGA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGAA
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
D. spathaceum KR149502.1 Selbach et al. (2015)	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
***Diplostomum* sp. 1 KY358239**	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
***Diplostomum* sp. 1 KY358240**	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA

***Diplostomum* sp. 1 MH108191**	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
***Diplostomum* sp. 1 MH108192**	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
***Diplostomum* sp. 1 MH108193**	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
***Diplostomum* sp. 1 MH108194**	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
***Diplostomum* sp. 1 MH108195**	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
***Diplostomum* sp. 1 MH108196**	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
Diplostomum sp. 1 MH108197	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
***Diplostomum* sp. 1 MH108198**	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
***Diplostomum* sp. 1 MH108199**	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
***Diplostomum* sp. 1 MH108200**	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
D. indistinctum AY123043.1 Galazzo et al. (2002)	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
D. indistinctum GQ292508.1 Locke et al. (2010a)	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	111	GCGAATTACAGTGCAG--TCAAACGGGATTGACGGACGAACTCTTCACTTGTGAA
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	111	GCGAATTAAAGTGCAG--TCAAACGGGATTGACGGACGAACCCTTCACTTGTGGA
***Diplostomum* sp. 4 KY358236**	111	GCGAATTAAAGTACAG--TCAAACGGGATTGACGGACGAACCCTTCACTTGTGGA
***Diplostomum* sp. 4 KY358237**	111	GCGAATTAAAGTACAG--TCAAACGGGATTGACGGACGAACCCTTCACTTGTGGA
***Diplostomum* sp. 4 KY358238**	111	GCGAATTAAAGTACAG--TCAAACGGGATTGACGGACGAACCCTTCACTTGTGGA
***Diplostomum* sp. 4 MH108190**	111	GCGAATTAAAGTACAG--TCAAACGGGATTGACGGACGAACCCTTCACTTGTGGA
D. huronense AY123044.1 Galazzo et al. (2002)	110	GCGAATTACAGTGCAGA--CACACGGGATTGACGGACGAACTCTTCACTTGTGGA
D. huronense GQ292509.1 Locke et al. (2010a)	110	GCGAATTACAGTGCAGA--CACACGGGATTGACGGACGAACTCTTCACTTGTGGA
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	110	GCGAATTACAGTGCAGA-CCAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	110	GCGAATTACAGTGCAGA-CCAAACGGGATTGACGGACGAACTCTTCACTTGTGGA

Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	111	GCGAATAACAGTGCATAT--AAACGGGATTGACGGACGAGCTCTTCACTTGTGGA
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	111	GCGAATTACAGTGCAAA--CAAACGGGATTGACGGACGAACTCTTCACTTGTGGA
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	111	GCGAATAACAGTGCATAT--AAACGGGATTGACGGACGAACTCTTTACTTGTGAA
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	110	GCGAATTACAGTGCAGT----AACGGAATTGACGGATGAGCCCTTTACTTGTAGA
***D. baeri* MH108185**	164	GGG-TTCGCG--ATACTATTGGCCATACCTGAGACGTG--------GTTCTGCTG
***D. baeri* MH108185**	164	GGG-TTCGCG--ATACTATTGGCCATACCTGAGACGTG--------GTTCTGCTG
***D. baeri* MH108187**	164	GGG-TTCGCG--ATACTATTGGCCATACCTGAGACGTG--------GTTCTGCTG
***D. baeri* MH108188**	164	GGG-TTCGCG--ATACTATTGGCCATACCTGAGACGTG--------GTTCTGCTG
***D. baeri* MH108189**	164	GGG-TTCGCG--ATACTATTGGCCATACCTGAGACGTG--------GTTCTGCTG
***D. baeri* MH108201**	164	GGG-TTCGCG--ATACTATTGGCCATACCTGAGACGTG--------GTTCTGCTG
***D. baeri* MH108202**	164	GGG-TTCGCG--ATACTATTGGCCATACCTGAGACGTG--------GTTCTGCTG
D. baeri AY123042.1 Galazzo et al. (2002)	164	GGG-TTCGCG-AAT-CTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
D. baeri JX986856.1 Georgieva et al. (2013)	164	GGG-TTCGCG--ATACTATTGGCCATACCTGAGACGTG--------GTTCTGCTG
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	164	GGG-TTCGCG--ATTCTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	164	GGG-TTCGCG--ATTCTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
D. mergi KR149499.1 Selbach et al. (2015)	166	GGG-TTCGCG--ATTCTATTGGCCATACCTGAGACGTG--------GTTCTACTG
D. parviventosum KR149492.1 Selbach et al. (2015)	166	GGG-TTCGCG--ATTCTATTGGCCATACCTGAGACGTG--------GTTCTACTG
D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	164	GGG-TTCGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	164	GGG-TTCGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
D. pseudospathaceum KT186785.1 Locke et al. (2015)	164	GGG-TTCGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	164	GGGTTTGGC--AATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG

D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	164	GGGTTTGGC--AATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
D. spathaceum KR149502.1 Selbach et al. (2015)	164	GGGTTTGGC--AATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
***Diplostomum* sp. 1 KY358239**	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
***Diplostomum* sp. 1 KY358240**	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
***Diplostomum* sp. 1 MH108191**	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
***Diplostomum* sp. 1 MH108192**	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
***Diplostomum* sp. 1 MH108193**	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
***Diplostomum* sp. 1 MH108194**	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
***Diplostomum* sp. 1 MH108195**	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
***Diplostomum* sp. 1 MH108196**	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
Diplostomum sp. 1 MH108197	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
***Diplostomum* sp. 1 MH108198**	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
***Diplostomum* sp. 1 MH108199**	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
***Diplostomum* sp. 1 MH108200**	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
D. indistinctum AY123043.1 Galazzo et al. (2002)	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
D. indistinctum GQ292508.1 Locke et al. (2010a)	164	GGGTTTGGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	164	GGG-TTCGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	164	GGG-TTCGCG--ATTCTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
***Diplostomum* sp. 4 KY358236**	164	GGG-TTCGCG--ATTCTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
***Diplostomum* sp. 4 KY358237**	164	GGG-TTCGCG--ATTCTATTGGCCATACCTGAGACGTA--------GTTCTGCTG

***Diplostomum* sp. 4 KY358238**	164	GGG-TTCGCG--ATTCTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
***Diplostomum* sp. 4 MH108190**	164	GGG-TTCGCG--ATTCTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
D. huronense AY123044.1 Galazzo et al. (2002)	163	GGG-TTCGCG--ATTCTATTGGCCATACCTGCGACGTA--------GTTCTGCTG
D. huronense GQ292509.1 Locke et al. (2010a)	163	GGG-TTCGCG--ATTCTATTGGCCATACCTGCGACGTA--------GTTCTGCTG
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	164	GGG-TTCGCG--ATTCTATTGGCCATACCTGAGACGTA--------GTTCTACTG
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	164	GGG-TTCGCG--ATACTATTGGCCATACCTGAGACGTA--------GTTCTACTG
Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	164	GGG-TTCGCG--ATTCTATTGGCCATACCTGAGACGTG--------GTT-TACTG
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	164	GGGTTTGGC--AATACTATTGGCCATACCTGAGACGTA--------GTTCTGCTG
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	164	GGG-TTCGCG--ATTCTATTGGCCATACCTGAGACGTG--------GTTCTACTG
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	161	GGGAT-CGCGGTATA--ATTGGCCATACCTGAGGCGTAGTGGACTTGTTCTGCTG
***D. baeri* MH108185**	208	CGTTTCACAAGTACGGTCCGTATTGTGGTGGGGTGCCTATCCTGTCTGATACCCT
***D. baeri* MH108185**	208	CGTTTCACAAGTACGGTCCGTATTGTGGTGGGGTGCCTATCCTGTCTGATACCCT
***D. baeri* MH108187**	208	CGTTTCACAAGTACGGTCCGTATTGTGGTGGGGTGCCTATCCTGTCTGATACCCT
***D. baeri* MH108188**	208	CGTTTCACAAGTACGGTCCGTATTGTGGTGGGGTGCCTATCCTGTCTGATACCCT
***D. baeri* MH108189**	208	CGTTTCACAAGTACGGTCCGTATTGTGGTGGGGTGCCTATCCTGTCTGATACCCT
***D. baeri* MH108201**	208	CGTTTCACAAGTACGGTCCGTATTGTGGTGGGGTGCCTATCCTGTCTGATACCCT
***D. baeri* MH108202**	208	CGTTTCACAAGTACGGTCCGTATTGTGGTGGGGTGCCTATCCTGTCTGATACCCT
D. baeri AY123042.1 Galazzo et al. (2002)	208	CGTTTCACAAGTACGGTCCGTATTGTGGTGGGGTGCCTATCCTGTCTGATACCCT
D. baeri JX986856.1 Georgieva et al. (2013)	208	CGTTTCACAAGTACGGTCCGTATTGTGGTGGGGTGCCTATCCTGTCTGATACCCT
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	208	CGTTTCACAAGTACGGTCCGTATTGTGGTGGGGTGCCTATCCTGTCTGATACCCT
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	208	CGTTTCACAAGTACGGTCCGTATTGTGGTGGGGTGCCTATCCTGTCTGATACCCT

D. mergi KR149499.1 Selbach et al. (2015)	210	CGTTTCATAAGTACGGTCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
D. parviventosum KR149492.1 Selbach et al. (2015)	210	CGTTTCATAAGTACGGTCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	208	CGTTTCACAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	208	CGTTTCACAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
D. pseudospathaceum KT186785.1 Locke et al. (2015)	208	CGTTTCACAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	209	CGTTTCACAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	209	CGTTTCACAAGTACGGCCCGAATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
D. spathaceum KR149502.1 Selbach et al. (2015)	209	CGTTTCACAAGTACGGCCCGAATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
***Diplostomum* sp. 1 KY358239**	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
***Diplostomum* sp. 1 KY358240**	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
***Diplostomum* sp. 1 MH108191**	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
***Diplostomum* sp. 1 MH108192**	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
***Diplostomum* sp. 1 MH108193**	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
***Diplostomum* sp. 1 MH108194**	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
***Diplostomum* sp. 1 MH108195**	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
***Diplostomum* sp. 1 MH108196**	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
Diplostomum sp. 1 MH108197	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
***Diplostomum* sp. 1 MH108198**	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
***Diplostomum* sp. 1 MH108199**	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
***Diplostomum* sp. 1 MH108200**	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT

D. indistinctum AY123043.1 Galazzo et al. (2002)	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
D. indistinctum GQ292508.1 Locke et al. (2010a)	209	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	208	CGTTTCACAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	208	CGTTTCATAAGTACGGCCCGTATGTTGGTGGGGTGCCTATCCTGTCTGATACCCT
***Diplostomum* sp. 4 KY358236**	208	CGTTTCATAAGTACGGCCCGTATGTTGGTGGGGTGCCTATCCTGTCTGATACCCT
***Diplostomum* sp. 4 KY358237**	208	CGTTTCATAAGTACGGCCCGTATGTTGGTGGGGTGCCTATCCTGTCTGATACCCT
***Diplostomum* sp. 4 KY358238**	208	CGTTTCATAAGTACGGCCCGTATGTTGGTGGGGTGCCTATCCTGTCTGATACCCT
***Diplostomum* sp. 4 MH108190**	208	CGTTTCATAAGTACGGCCCGTATGTTGGTGGGGTGCCTATCCTGTCTGATACCCT
D. huronense AY123044.1 Galazzo et al. (2002)	207	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
D. huronense GQ292509.1 Locke et al. (2010a)	207	CGTTTCATAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	208	CGTTTCACAAGTACGGCCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	208	CGTTTCACAAGTACGGTCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	207	CGTTTCACAAGTACGGTCCGTATTTCGGTGGGGTGCCTATCCTGTCTGATACTCT
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	209	YGTTTCACAAGTACGGCCCGAATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	208	CGTTTCATAAGTACGGTCCGTATTTTGGTGGGGTGCCTATCCTGTCTGATACCCT
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	213	CATCTCATAAGTACGGCCCGTATTTAGGTGGGGTGCCTATCCTGTCTGATACTCT
***D. baeri* MH108185**	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***D. baeri* MH108185**	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***D. baeri* MH108187**	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***D. baeri* MH108188**	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***D. baeri* MH108189**	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA

***D. baeri* MH108201**	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***D. baeri* MH108202**	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
D. baeri AY123042.1 Galazzo et al. (2002)	263	GATGGTTGACTTGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
D. baeri JX986856.1 Georgieva et al. (2013)	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
D. mergi KR149499.1 Selbach et al. (2015)	265	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
D. parviventosum KR149492.1 Selbach et al. (2015)	265	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
D. pseudospathaceum KT186785.1 Locke et al. (2015)	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	264	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	264	GATGGTTGGCTCGTGGCTTGGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
D. spathaceum KR149502.1 Selbach et al. (2015)	264	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	264	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	264	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***Diplostomum* sp. 1 KY358239**	264	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***Diplostomum* sp. 1 KY358240**	264	GATGGTTGGCTCGTGGCTCCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***Diplostomum* sp. 1 MH108191**	264	GATGGTTGGCTCGTGGCTCCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***Diplostomum* sp. 1 MH108192**	264	GATGGTTGGCTCGTGGCTCCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***Diplostomum* sp. 1 MH108193**	264	GATGGTTGGCTCGTGGCTCCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***Diplostomum* sp. 1 MH108194**	264	GATGGTTGGCTCGTGGCTCCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA

***Diplostomum* sp. 1 MH108195**	264	GATGGTTGGCTCGTGGCTCCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***Diplostomum* sp. 1 MH108196**	264	GATGGTTGGCTCGTGGCTCCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
Diplostomum sp. 1 MH108197	264	GATGGTTGGCTCGTGGCTCCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***Diplostomum* sp. 1 MH108198**	264	GATGGTTGGCTCGTGGCTCCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***Diplostomum* sp. 1 MH108199**	264	GATGGTTGGCTCGTGGCTCCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***Diplostomum* sp. 1 MH108200**	264	GATGGTTGGCTCGTGGCTCCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
D. indistinctum AY123043.1 Galazzo et al. (2002)	264	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
D. indistinctum GQ292508.1 Locke et al. (2010a)	264	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***Diplostomum* sp. 4 KY358236**	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***Diplostomum* sp. 4 KY358237**	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***Diplostomum* sp. 4 KY358238**	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
***Diplostomum* sp. 4 MH108190**	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
D. huronense AY123044.1 Galazzo et al. (2002)	262	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
D. huronense GQ292509.1 Locke et al. (2010a)	262	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	262	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	264	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	263	GATGGTTGGCTCGTGGCTTCGGCTGCCTTGTCATGCCAAGGGTGATGGGAAAGTA
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	268	GATGGTTGACTCGTGGCCTCGGCTGCTTTGTTATGCCAGGAGTGATGGGACAGTA

***D. baeri* MH108185**	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTAATA-GTCCAG
***D. baeri* MH108185**	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTAATA-GTCCAG
***D. baeri* MH108187**	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTAATA-GTCCAG
***D. baeri* MH108188**	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTAATA-GTCCAG
***D. baeri* MH108189**	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTAATA-GTCCAG
***D. baeri* MH108201**	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTAATA-GTCCAG
***D. baeri* MH108202**	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTAATA-GTCCAG
D. baeri AY123042.1 Galazzo et al. (2002)	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTAATA-GTCCAG
D. baeri JX986856.1 Georgieva et al. (2013)	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTAATA-GTCCAG
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	318	CTGTATCTATCTCAGTGCAAGGCTCAATGAG-GGT-CTCGGACTAATA-GTCCAG
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	318	CTGTATCTATCTCAGTGCAAGGCTCAATGAG-GGT-CTCGGACTAATA-GTCCAG
D. mergi KR149499.1 Selbach et al. (2015)	320	CTGTATCTATCTCAGTGCAAGGCTCAAAGAGA-GTGCT-GGACTATTA-GTTCAG
D. parviventosum KR149492.1 Selbach et al. (2015)	320	CTGTATCTATCTCAGTGCAAGGCTCAAAGAGA-GTGCT-GGACTAATA-GTTCAG
D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
D. pseudospathaceum KT186785.1 Locke et al. (2015)	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
D. spathaceum KR149502.1 Selbach et al. (2015)	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG

***Diplostomum* sp. 1 KY358239**	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
***Diplostomum* sp. 1 KY358240**	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
***Diplostomum* sp. 1 MH108191**	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
***Diplostomum* sp. 1 MH108192**	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
***Diplostomum* sp. 1 MH108193**	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
***Diplostomum* sp. 1 MH108194**	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
***Diplostomum* sp. 1 MH108195**	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
***Diplostomum* sp. 1 MH108196**	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
Diplostomum sp. 1 MH108197	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
***Diplostomum* sp. 1 MH108198**	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
***Diplostomum* sp. 1 MH108199**	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
***Diplostomum* sp. 1 MH108200**	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
D. indistinctum AY123043.1 Galazzo et al. (2002)	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
D. indistinctum GQ292508.1 Locke et al. (2010a)	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
***Diplostomum* sp. 4 KY358236**	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
***Diplostomum* sp. 4 KY358237**	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
***Diplostomum* sp. 4 KY358238**	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
***Diplostomum* sp. 4 MH108190**	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
D. huronense AY123044.1 Galazzo et al. (2002)	317	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCA-
D. huronense GQ292509.1 Locke et al. (2010a)	317	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG

Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	317	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTAATA-GTCCAG
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	319	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTCGGACTACTATGTCCAG
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	318	CTGTATCTATCTCAGTGCAAGGCTCAAAGAG-GGT-CTTGGACTAATA-GTCCAG
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	323	CTGTACTTATCTCAGTGCAAGGCTCCAAGAGAGGTG-T-GGACTACT-TGTCCTA
***D. baeri* MH108185**	370	CCTCCGCCCCATCTTGTTGTTTCTACTACTATTTTTACACTGTTTAAGTTGGTTA
***D. baeri* MH108185**	370	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
***D. baeri* MH108187**	370	CCTCCGCCCCATCTTGTTGTTTCTACTACTATTTTTACACTGTTTAAGTTGGTTA
***D. baeri* MH108188**	370	CCTCCGCCCCATCTTGTTGTTTCTACTACTATTTTTACACTGTTTAAGTTGGTTA
***D. baeri* MH108189**	370	CCTCCGCCCCATCTTGTTGTTTCTACTACTATTTTTACACTGTTTAAGTTGGTTA
***D. baeri* MH108201**	370	CCTCCGCCCCATCTTGTTGTTTCTACTACTATTTTTACACTGTTTAAGTTGGTTA
***D. baeri* MH108202**	370	CCTCCGCCCCATCTTGTTGTTTCTACTACTATTTTTACACTGTTTAAGTTGGTTA
D. baeri AY123042.1 Galazzo et al. (2002)	370	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTCTTACACTGTTTAAGTTAGTTA
D. baeri JX986856.1 Georgieva et al. (2013)	370	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	370	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	370	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
D. mergi KR149499.1 Selbach et al. (2015)	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
D. parviventosum KR149492.1 Selbach et al. (2015)	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	371	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	371	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA

D. pseudospathaceum KT186785.1 Locke et al. (2015)	371	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTCTTACACTGTTTAAGTTGGTTA
D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTCTTACACTGTTTAAGTTGGTTA
D. spathaceum KR149502.1 Selbach et al. (2015)	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTCTTACACTGTTTAAGTTGGTTA
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
***Diplostomum* sp. 1 KY358239**	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
***Diplostomum* sp. 1 KY358240**	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
***Diplostomum* sp. 1 MH108191**	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
***Diplostomum* sp. 1 MH108192**	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
***Diplostomum* sp. 1 MH108193**	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
***Diplostomum* sp. 1 MH108194**	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
***Diplostomum* sp. 1 MH108195**	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
***Diplostomum* sp. 1 MH108196**	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
Diplostomum sp. 1 MH108197	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
***Diplostomum* sp. 1 MH108198**	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
***Diplostomum* sp. 1 MH108199**	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
***Diplostomum* sp. 1 MH108200**	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
D. indistinctum AY123043.1 Galazzo et al. (2002)	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
D. indistinctum GQ292508.1 Locke et al. (2010a)	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	371	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	371	CCTCCGCCCCATCTTGTTGTTTCTACTACCATT-TTACACTGTTTAAGTTGGTTA

***Diplostomum* sp. 4 KY358236**	371	CCTCCGCCCCATCTTGTTGTTTCTACTACCATT-TTACACTGTTTAAGTTGGTTA
***Diplostomum* sp. 4 KY358237**	371	CCTCCGCCCCATCTTGTTGTTTCTACTACCATT-TTACACTGTTTAAGTTGGTTA
***Diplostomum* sp. 4 KY358238**	371	CCTCCGCCCCATCTTGTTGTTTCTACTACCATT-TTACACTGTTTAAGTTGGTTA
***Diplostomum* sp. 4 MH108190**	371	CCTCCGCCCCATCTTGTTGTTTCTACTACCATT-TTACACTGTTTAAGTTGGTTA
D. huronense AY123044.1 Galazzo et al. (2002)	369	CCTCCGCCCCATCTTGTTGTTTCTACTACCATT-TTACACTGTTTAAGTTGGTTA
D. huronense GQ292509.1 Locke et al. (2010a)	370	CCTCCGCCCCATCTTGTTGTTTCTACTACCATT-TTACACTGTTTAAGTTGGTTA
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	371	CCTCCGCCCCATCTTGTTGTTTCTACTACCATT-TTACACTGTTTAAGTTGGTTA
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	371	CCTCCGCCCCATCTTGTTGTTTCTACTACCATT-TTACACTGTTTAAGTTGGTTA
Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	369	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	372	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTCTTACACTGTTTAAGTTGGTTA
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	370	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTA
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	375	CCTCCGCCCCATCTTGTTGTTTCTACTACCATTTTTACACTGTTTAAGTTGGTTG
***D. baeri* MH108185**	425	GGTCGGCTTG-CCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***D. baeri* MH108185**	425	GGTCGGCTTG-CCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***D. baeri* MH108187**	425	GGTCGGCTTG-CCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***D. baeri* MH108188**	425	GGTCGGCTTG-CCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***D. baeri* MH108189**	425	GGTCGGCTTG-CCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***D. baeri* MH108201**	425	GGTCGGCTTG-CCGGTCTAGCTAGCTGCCCAAAGCATGCCTCCAGACATCTTGTA
***D. baeri* MH108202**	425	GGTCGGCTTG-CCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
D. baeri AY123042.1 Galazzo et al. (2002)	425	GGTCGGCTTG-CCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
D. baeri JX986856.1 Georgieva et al. (2013)	425	GGTCGGCTTG-CCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA

Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	425	GGTCGGCTTG-CCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	425	GGTCGGCTTG-CCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
D. mergi KR149499.1 Selbach et al. (2015)	427	GTTCGGCTTGTCCGATCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
D. parviventosum KR149492.1 Selbach et al. (2015)	427	GGTCGGCTTGTCCGATCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	426	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	426	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
D. pseudospathaceum KT186785.1 Locke et al. (2015)	426	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
D. spathaceum KR149502.1 Selbach et al. (2015)	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***Diplostomum* sp. 1 KY358239**	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***Diplostomum* sp. 1 KY358240**	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***Diplostomum* sp. 1 MH108191**	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***Diplostomum* sp. 1 MH108192**	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***Diplostomum* sp. 1 MH108193**	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***Diplostomum* sp. 1 MH108194**	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***Diplostomum* sp. 1 MH108195**	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***Diplostomum* sp. 1 MH108196**	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
Diplostomum sp. 1 MH108197	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***Diplostomum* sp. 1 MH108198**	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA

***Diplostomum* sp. 1 MH108199**	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***Diplostomum* sp. 1 MH108200**	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
D. indistinctum AY123043.1 Galazzo et al. (2002)	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
D. indistinctum GQ292508.1 Locke et al. (2010a)	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	426	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	425	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***Diplostomum* sp. 4 KY358236**	425	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***Diplostomum* sp. 4 KY358237**	425	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***Diplostomum* sp. 4 KY358238**	425	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
***Diplostomum* sp. 4 MH108190**	425	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
D. huronense AY123044.1 Galazzo et al. (2002)	423	GGTCGGCTTGTCCGATCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
D. huronense GQ292509.1 Locke et al. (2010a)	424	GGTCGGCTTGTCCGATCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	425	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	425	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	424	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	427	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	425	GGTCGGCTTGTCCGGTCTAGCTAGCTGCCCATAGCATGCCTCCAGACATCTTGTA
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	430	GGTCGGTTCGTCCGGTCTGGCTAGCTGCCCATAGCATGCCTCTGGTCGTCTTGTA
***D. baeri* MH108185**	479	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***D. baeri* MH108185**	479	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***D. baeri* MH108187**	479	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA

***D. baeri* MH108188**	479	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***D. baeri* MH108189**	479	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***D. baeri* MH108201**	479	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***D. baeri* MH108202**	479	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
D. baeri AY123042.1 Galazzo et al. (2002)	479	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
D. baeri JX986856.1 Georgieva et al. (2013)	479	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	479	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	479	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
D. mergi KR149499.1 Selbach et al. (2015)	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
D. parviventosum KR149492.1 Selbach et al. (2015)	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	481	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	481	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
D. pseudospathaceum KT186785.1 Locke et al. (2015)	481	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
D. spathaceum KR149502.1 Selbach et al. (2015)	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***Diplostomum* sp. 1 KY358239**	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***Diplostomum* sp. 1 KY358240**	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***Diplostomum* sp. 1 MH108191**	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***Diplostomum* sp. 1 MH108192**	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA

***Diplostomum* sp. 1 MH108193**	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***Diplostomum* sp. 1 MH108194**	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***Diplostomum* sp. 1 MH108195**	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***Diplostomum* sp. 1 MH108196**	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
Diplostomum sp. 1 MH108197	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***Diplostomum* sp. 1 MH108198**	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***Diplostomum* sp. 1 MH108199**	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***Diplostomum* sp. 1 MH108200**	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
D. indistinctum AY123043.1 Galazzo et al. (2002)	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
D. indistinctum GQ292508.1 Locke et al. (2010a)	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	481	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	480	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***Diplostomum* sp. 4 KY358236**	480	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***Diplostomum* sp. 4 KY358237**	480	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***Diplostomum* sp. 4 KY358238**	480	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***Diplostomum* sp. 4 MH108190**	480	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
D. huronense AY123044.1 Galazzo et al. (2002)	478	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
D. huronense GQ292509.1 Locke et al. (2010a)	479	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	480	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	480	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	479	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	482	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA

Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	480	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	485	TGACTGGTCCGCATGTACAGTCGCCTGGCGGTGCCTCATCCTGGGCTAGACTGCA
***D. baeri* MH108185**	534	AACCTATATGTTC---TCATTGACTCGGTTTACCGGGTTGGTGGGTGCATTACAG
***D. baeri* MH108185**	534	AACCTATATGTTC---TCATTGACTCGGTTTACCGGGTCGGTGGGTGCATTACAG
***D. baeri* MH108187**	534	AACCTATATGTTC---TCATTGACTCGGTTTACCGGGTTGGTGGGTGCATTACAG
***D. baeri* MH108188**	534	AACCTATATGTTC---TCATTGACTCGGTTTACCGGGTTGGTGGGTGCATTACAG
***D. baeri* MH108189**	534	AACCTATATGTTC---TCATTGACTCGGTTTACCGGGTTGGTGGGTGCATTACAG
***D. baeri* MH108201**	534	AACCTATATGTTC---TCATTGACTCGGTTTACCGGGTTGGTGGGTGCATTACAG
***D. baeri* MH108202**	534	AACCTATATGTTC---TCATTGACTCGGTTTACCGGGTTGGTGGGTGCATTACAG
D. baeri AY123042.1 Galazzo et al. (2002)	534	AACCTATATGTTC---TCATTGACTCGGTTTACCGGGTTGGTGGGTGCATTACAG
D. baeri JX986856.1 Georgieva et al. (2013)	534	AACCTATATGTTC---TCATTGACTCGGTTTACCGGGTCGGTGGGTGCATTACAG
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	534	AACCTATATGTTC---TCATTGACTCGGTTTACCGGGTTGGTGGGTGCATTACAG
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	534	AACCTATATGTTC---TCATTGACTCGGTTTACCGGGTTGGTGGGTGCATTACAG
D. mergi KR149499.1 Selbach et al. (2015)	537	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTCGGTGGGTGCATTACAG
D. parviventosum KR149492.1 Selbach et al. (2015)	537	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTCGGTGGGTGCATTACAG
D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	536	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	536	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG
D. pseudospathaceum KT186785.1 Locke et al. (2015)	536	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	537	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG
D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	537	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG
D. spathaceum KR149502.1 Selbach et al. (2015)	537	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG

Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	537	AACCTATATGTAC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTAAAG
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	537	AACCTATATGTAC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTAAAG
***Diplostomum* sp. 1 KY358239**	537	AACCTATATGTAC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTAAAG
***Diplostomum* sp. 1 KY358240**	537	AACCTATATGTAC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATANAAG
***Diplostomum* sp. 1 MH108191**	537	AACCTATATGTAC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTAAAG
***Diplostomum* sp. 1 MH108192**	537	AACCTATATGTAC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTAAAG
***Diplostomum* sp. 1 MH108193**	537	AACCTATATGTAC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATAAAAG
***Diplostomum* sp. 1 MH108194**	537	AACCTATATGTAC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTAAAG
***Diplostomum* sp. 1 MH108195**	537	AACCTATATGTAC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTAAAG
***Diplostomum* sp. 1 MH108196**	537	AACCTATATGTAC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTAAAG
Diplostomum sp. 1 MH108197	537	AACCTATATGTAC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATAAAAG
***Diplostomum* sp. 1 MH108198**	537	AACCTATATGTAC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTAAAG
***Diplostomum* sp. 1 MH108199**	537	AACCTATATGTAC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTAAAG
***Diplostomum* sp. 1 MH108200**	537	AACCTATATGTAC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATWAAAG
D. indistinctum AY123043.1 Galazzo et al. (2002)	537	AACCTATATGTCC---TCATTGGCTCGGTTTACCGGGTTAGTGGGTGCATTACAG
D. indistinctum GQ292508.1 Locke et al. (2010a)	537	AACCTATATGTCC---TCATTGGCTCGGTTTACCGGGTTAGTGGGTGCATTACAG
Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	536	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	535	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG
***Diplostomum* sp. 4 KY358236**	535	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG
***Diplostomum* sp. 4 KY358237**	535	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG
***Diplostomum* sp. 4 KY358238**	535	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG
***Diplostomum* sp. 4 MH108190**	535	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG

D. huronense AY123044.1 Galazzo et al. (2002)	533	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG
D. huronense GQ292509.1 Locke et al. (2010a)	534	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	535	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	535	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTGGTGGGTGCATTACAG
Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	534	AACCTATATGTTC---TCATTGACTCGGTTTACCGGGTTGGTGGGTGCATTACAG
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	537	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTTRRTGGGTGCATTACAG
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	535	AACCTATATGTTC---TCATTGGCTCGGTTTACCGGGTCAGTGGGTGCATTACAG
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	540	AACCTATATGTTCCTGTC-TT--CTCGGTTTACCGGGTTGGTGGG-GCATTACAG
***D. baeri* MH108185**	586	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***D. baeri* MH108185**	586	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***D. baeri* MH108187**	586	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***D. baeri* MH108188**	586	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***D. baeri* MH108189**	586	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***D. baeri* MH108201**	586	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***D. baeri* MH108202**	586	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
D. baeri AY123042.1 Galazzo et al. (2002)	586	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
D. baeri JX986856.1 Georgieva et al. (2013)	586	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	586	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	586	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
D. mergi KR149499.1 Selbach et al. (2015)	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
D. parviventosum KR149492.1 Selbach et al. (2015)	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA

D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	588	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	588	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
D. pseudospathaceum KT186785.1 Locke et al. (2015)	588	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
D. spathaceum KR149502.1 Selbach et al. (2015)	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***Diplostomum* sp. 1 KY358239**	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***Diplostomum* sp. 1 KY358240**	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***Diplostomum* sp. 1 MH108191**	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***Diplostomum* sp. 1 MH108192**	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***Diplostomum* sp. 1 MH108193**	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***Diplostomum* sp. 1 MH108194**	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***Diplostomum* sp. 1 MH108195**	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***Diplostomum* sp. 1 MH108196**	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
Diplostomum sp. 1 MH108197	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***Diplostomum* sp. 1 MH108198**	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***Diplostomum* sp. 1 MH108199**	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***Diplostomum* sp. 1 MH108200**	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGNGCAGCCA
D. indistinctum AY123043.1 Galazzo et al. (2002)	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
D. indistinctum GQ292508.1 Locke et al. (2010a)	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA

Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	588	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	587	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***Diplostomum* sp. 4 KY358236**	587	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***Diplostomum* sp. 4 KY358237**	587	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***Diplostomum* sp. 4 KY358238**	587	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***Diplostomum* sp. 4 MH108190**	587	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
D. huronense AY123044.1 Galazzo et al. (2002)	585	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
D. huronense GQ292509.1 Locke et al. (2010a)	586	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	587	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	587	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	586	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	589	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	587	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAGGAGTGCAG-CA
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	591	TACAACTCTGAGCGGTGGATCACTCGGCTCGTGTGTCGATGAAG-AGTGCAGCCA
***D. baeri* MH108185**	640	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***D. baeri* MH108185**	640	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***D. baeri* MH108187**	640	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***D. baeri* MH108188**	640	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***D. baeri* MH108189**	640	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***D. baeri* MH108201**	640	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***D. baeri* MH108202**	640	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC

D. baeri AY123042.1 Galazzo et al. (2002)	640	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
D. baeri JX986856.1 Georgieva et al. (2013)	640	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	640	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	640	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
D. mergi KR149499.1 Selbach et al. (2015)	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
D. parviventosum KR149492.1 Selbach et al. (2015)	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	642	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	642	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
D. pseudospathaceum KT186785.1 Locke et al. (2015)	642	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
D. spathaceum KR149502.1 Selbach et al. (2015)	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***Diplostomum* sp. 1 KY358239**	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***Diplostomum* sp. 1 KY358240**	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***Diplostomum* sp. 1 MH108191**	643	ACTGTGTGAATTAATGTGAACTGCGTANTGCTTTGAACATCGACATCTTGAACGC
***Diplostomum* sp. 1 MH108192**	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***Diplostomum* sp. 1 MH108193**	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***Diplostomum* sp. 1 MH108194**	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***Diplostomum* sp. 1 MH108195**	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***Diplostomum* sp. 1 MH108196**	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC

Diplostomum sp. 1 MH108197	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***Diplostomum* sp. 1 MH108198**	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***Diplostomum* sp. 1 MH108199**	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***Diplostomum* sp. 1 MH108200**	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
D. indistinctum AY123043.1 Galazzo et al. (2002)	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
D. indistinctum GQ292508.1 Locke et al. (2010a)	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	642	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	641	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***Diplostomum* sp. 4 KY358236**	641	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***Diplostomum* sp. 4 KY358237**	641	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***Diplostomum* sp. 4 KY358238**	641	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
***Diplostomum* sp. 4 MH108190**	641	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
D. huronense AY123044.1 Galazzo et al. (2002)	639	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
D. huronense GQ292509.1 Locke et al. (2010a)	640	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	641	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	641	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	640	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	643	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	641	ACTGT-TGAATTAATGTGAACTGCGTACTGCTTTGAACATCGACATCTTGAACGC
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	645	ACTGTGTGAATTAATGTGAACTGCGTACTGCTTTGAGCATCGACATCTTGAACGC
***D. baeri* MH108185**	695	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA

***D. baeri* MH108185**	695	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***D. baeri* MH108187**	695	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***D. baeri* MH108188**	695	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***D. baeri* MH108189**	695	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***D. baeri* MH108201**	695	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***D. baeri* MH108202**	695	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
D. baeri AY123042.1 Galazzo et al. (2002)	695	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
D. baeri JX986856.1 Georgieva et al. (2013)	695	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	695	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	695	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
D. mergi KR149499.1 Selbach et al. (2015)	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
D. parviventosum KR149492.1 Selbach et al. (2015)	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	697	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	697	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
D. pseudospathaceum KT186785.1 Locke et al. (2015)	697	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
D. spathaceum KR149502.1 Selbach et al. (2015)	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***Diplostomum* sp. 1 KY358239**	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***Diplostomum* sp. 1 KY358240**	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA

***Diplostomum* sp. 1 MH108191**	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***Diplostomum* sp. 1 MH108192**	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***Diplostomum* sp. 1 MH108193**	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***Diplostomum* sp. 1 MH108194**	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***Diplostomum* sp. 1 MH108195**	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***Diplostomum* sp. 1 MH108196**	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
Diplostomum sp. 1 MH108197	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***Diplostomum* sp. 1 MH108198**	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***Diplostomum* sp. 1 MH108199**	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***Diplostomum* sp. 1 MH108200**	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
D. indistinctum AY123043.1 Galazzo et al. (2002)	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
D. indistinctum GQ292508.1 Locke et al. (2010a)	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	697	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	696	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***Diplostomum* sp. 4 KY358236**	696	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***Diplostomum* sp. 4 KY358237**	696	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***Diplostomum* sp. 4 KY358238**	696	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***Diplostomum* sp. 4 MH108190**	696	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
D. huronense AY123044.1 Galazzo et al. (2002)	694	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
D. huronense GQ292509.1 Locke et al. (2010a)	695	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	696	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	696	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA

Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	695	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	698	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	695	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	700	ATATTGCGGCCGCGGGATATCCCGTGGCCACGTCTGGCCGAGGGTCGGCTTATCA
***D. baeri* MH108185**	750	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***D. baeri* MH108185**	750	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***D. baeri* MH108187**	750	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***D. baeri* MH108188**	750	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***D. baeri* MH108189**	750	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***D. baeri* MH108201**	750	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***D. baeri* MH108202**	750	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
D. baeri AY123042.1 Galazzo et al. (2002)	750	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
D. baeri JX986856.1 Georgieva et al. (2013)	750	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	750	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	750	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
D. mergi KR149499.1 Selbach et al. (2015)	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
D. parviventosum KR149492.1 Selbach et al. (2015)	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	752	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	752	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
D. pseudospathaceum KT186785.1 Locke et al. (2015)	752	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT

D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
D. spathaceum KR149502.1 Selbach et al. (2015)	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***Diplostomum* sp. 1 KY358239**	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***Diplostomum* sp. 1 KY358240**	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***Diplostomum* sp. 1 MH108191**	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTNCCAGCTGGCGTGATT
***Diplostomum* sp. 1 MH108192**	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***Diplostomum* sp. 1 MH108193**	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***Diplostomum* sp. 1 MH108194**	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***Diplostomum* sp. 1 MH108195**	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***Diplostomum* sp. 1 MH108196**	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
Diplostomum sp. 1 MH108197	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***Diplostomum* sp. 1 MH108198**	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***Diplostomum* sp. 1 MH108199**	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***Diplostomum* sp. 1 MH108200**	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
D. indistinctum AY123043.1 Galazzo et al. (2002)	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
D. indistinctum GQ292508.1 Locke et al. (2010a)	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	752	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	751	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCCGGCGTGATT
***Diplostomum* sp. 4 KY358236**	751	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCCGGCGTGATT
***Diplostomum* sp. 4 KY358237**	751	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCCGGCGTGATT

***Diplostomum* sp. 4 KY358238**	751	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCCGGCGTGATT
***Diplostomum* sp. 4 MH108190**	751	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCCGGCGTGATT
D. huronense AY123044.1 Galazzo et al. (2002)	749	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCCGGCGTGATT
D. huronense GQ292509.1 Locke et al. (2010a)	750	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCCGGCGTGATT
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	751	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	751	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	750	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	753	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	750	TTTATCACGACGCCCTAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	755	TTTATCACGACGCCCAAAAAGTCGTGGCCTGGAAGTTGTGCCAGCTGGCGTGATT
***D. baeri* MH108185**	805	TCCCCATCT-GATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***D. baeri* MH108185**	805	TCCCCATCT-GATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***D. baeri* MH108187**	805	TCCCCATCT-GATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***D. baeri* MH108188**	805	TCCCCATCT-GATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***D. baeri* MH108189**	805	TCCCCATCT-GATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***D. baeri* MH108201**	805	TCCCCATCT-GATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***D. baeri* MH108202**	805	TCCCCATCT-GATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
D. baeri AY123042.1 Galazzo et al. (2002)	805	TCCCCATCTA-ATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
D. baeri JX986856.1 Georgieva et al. (2013)	805	TCCCCATCT-GATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	805	TCCCCATTTC-ATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	805	TCCCCATTTC-ATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA

D. mergi KR149499.1 Selbach et al. (2015)	808	TCCCCATTT-GATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
D. parviventosum KR149492.1 Selbach et al. (2015)	808	TCCCCATTT-GATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	807	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	807	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
D. pseudospathaceum KT186785.1 Locke et al. (2015)	807	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
D. spathaceum KR149502.1 Selbach et al. (2015)	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***Diplostomum* sp. 1 KY358239**	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***Diplostomum* sp. 1 KY358240**	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***Diplostomum* sp. 1 MH108191**	808	TCCCCATTTNGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***Diplostomum* sp. 1 MH108192**	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***Diplostomum* sp. 1 MH108193**	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***Diplostomum* sp. 1 MH108194**	808	TCCCCATTTNGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***Diplostomum* sp. 1 MH108195**	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***Diplostomum* sp. 1 MH108196**	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
Diplostomum sp. 1 MH108197	808	TCCCCATTTNGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***Diplostomum* sp. 1 MH108198**	808	TCCCCATTTNGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***Diplostomum* sp. 1 MH108199**	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***Diplostomum* sp. 1 MH108200**	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA

D. indistinctum AY123043.1 Galazzo et al. (2002)	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
D. indistinctum GQ292508.1 Locke et al. (2010a)	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	807	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	806	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***Diplostomum* sp. 4 KY358236**	806	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***Diplostomum* sp. 4 KY358237**	806	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***Diplostomum* sp. 4 KY358238**	806	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***Diplostomum* sp. 4 MH108190**	806	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
D. huronense AY123044.1 Galazzo et al. (2002)	804	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
D. huronense GQ292509.1 Locke et al. (2010a)	805	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	806	TCCCCATTT-GATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	806	TCCCCATTTA-ATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	805	TCCCCATTT-GATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	808	TCCCCATTTCGATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	805	TCCCCATCTA-ATGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	810	TCTCCACTTTG-TGGGGTGCTGTGCTATGGCTCCTTCCTAATGTGTCCGGTTGCA
***D. baeri* MH108185**	859	CCCGGCCTGGAGCTTGGTTTC
***D. baeri* MH108185**	859	CCCGGCCTGGAGCTTGGTTTC
***D. baeri* MH108187**	859	CCCGGCCTGGAGCTTGGTTTC
***D. baeri* MH108188**	859	CCCGGCCTGGAGCTTGGTTTC
***D. baeri* MH108189**	859	CCCGGCCTGGAGCTTGGTTTC

***D. baeri* MH108201**	859	CCCGGCCTGGAGCTTGGTTTC
***D. baeri* MH108202**	859	CCCGGCCTGGAGCTTGGTTTC
D. baeri AY123042.1 Galazzo et al. (2002)	859	CCCGGCCTGGAGCTTGGTTTC
D. baeri JX986856.1 Georgieva et al. (2013)	859	CCCGGCCTGGAGCTTGGTTTC
Diplostomum sp. 2 GQ292505.1 Locke et al. (2010a)	859	CCCGGCCTGGAGCTTGGTTTC
Diplostomum sp. 2 KT186795.1 Locke et al. (2015)	859	CCCGGCCTGGAGCTTGGTTTC
D. mergi KR149499.1 Selbach et al. (2015)	862	CCCGGCCTGGAGCTTGGTTTC
D. parviventosum KR149492.1 Selbach et al. (2015)	862	CCCGGCCTGGAGCTTGGTTTC
D. pseudospathaceum JX986854.1 Georgieva et al. (2013)	862	CCCAGCCTGGAGCTTGATTTC
D. pseudospathaceum KR149500.1 Selbach et al. (2015)	862	CCCAGCCTGGAGCTTGATTTC
D. pseudospathaceum KT186785.1 Locke et al. (2015)	862	CCCAGCCTGGAGCTTGATTTC
D. spathaceum KJ726509.1 Blasco-Costa et al. (2014)	863	CCCAGCCTGGAGCTTGATTTC
D. spathaceum KP025793.1 Perez-Del-Olmo et al. (2014)	863	CCCAGCCTGGAGCTTGATTTC
D. spathaceum KR149502.1 Selbach et al. (2015)	863	CCCAGCCTGGAGCTTGATTTC
Diplostomum sp. 1 GQ292522.1 Locke et al. (2010a)	863	CCCAGCCTGGAGCTTGATTTC
Diplostomum sp. 1 KT186794.1 Locke et al. (2015)	863	CCCAGCCTGGAGCTTGATTTC
***Diplostomum* sp. 1 KY358239**	863	CCCAGCCTGGAGCTTGATTTC
***Diplostomum* sp. 1 KY358240**	863	CCCAGCCTGGAGCTTGATTTC
***Diplostomum* sp. 1 MH108191**	863	CCCAGCCTGGAGCTTGATTTC
***Diplostomum* sp. 1 MH108192**	863	CCCAGCCTGGAGCTTGATTTC
***Diplostomum* sp. 1 MH108193**	863	CCCAGCCTGGAGCTTGATTTC
***Diplostomum* sp. 1 MH108194**	863	CCCAGCCTGGAGCTTGATTTC

***Diplostomum* sp. 1 MH108195**	863	CCCAGCCTGGAGCTTGATTTC
***Diplostomum* sp. 1 MH108196**	863	CCCAGCCTGGAGCTTGATTTC
Diplostomum sp. 1 MH108197	863	CCCAGCCTGGAGCTTGATTTC
***Diplostomum* sp. 1 MH108198**	863	CCCAGCCTGGAGCTTGATTTC
***Diplostomum* sp. 1 MH108199**	863	CCCAGCCTGGAGCTTGATTTC
***Diplostomum* sp. 1 MH108200**	863	CCCAGCCTGGAGCTTGATTTC
D. indistinctum AY123043.1 Galazzo et al. (2002)	863	CCCAGCCTGGAGCTTGATTTC
D. indistinctum GQ292508.1 Locke et al. (2010a)	863	CCCAGCCTGGAGCTTGATTTC
Diplostomum sp. 3 GQ292511.1 Locke et al. (2010a)	862	CCCAGCCTGGAGCTTGATTTC
Diplostomum sp. 4 GQ292520.1 Locke et al. (2010a)	861	CCCAGCCTGGAGCTTGATTTC
***Diplostomum* sp. 4 KY358236**	861	CCCAGCCTGGAGCTTGATTTC
***Diplostomum* sp. 4 KY358237**	861	CCCAGCCTGGAGCTTGATTTC
***Diplostomum* sp. 4 KY358238**	861	CCCAGCCTGGAGCTTGATTTC
***Diplostomum* sp. 4 MH108190**	861	CCCAGCCTGGAGCTTGATTTC
D. huronense AY123044.1 Galazzo et al. (2002)	859	CCCAGCCTGGAGCTTGATTTC
D. huronense GQ292509.1 Locke et al. (2010a)	860	CCCAGCCTGGAGCTTGATTTC
Diplostomum sp. 8 GQ292510.1 Locke et al. (2010a)	860	CCCAGCCTGGGACTTGATTTC
Diplostomum sp. 9 GQ292504.1 Locke et al. (2010a)	860	CCCAGCCTGGGACTTGATTTC
Diplostomum sp. 10 KT186788.1 Locke et al. (2015)	859	CCCGGCCTGGGACTTGGTTTC
Diplostomum sp. 14 KT186789.1 Locke et al. (2015)	863	CCCAGCCTGGAGCTTGATTTC
Diplostomum sp. 15 KT186791.1 Locke et al. (2015)	859	CCCGGCCTGGAGCTTGGTTTC
Tylodelphys scheuringi FJ469596.1 Mosczynska et al. (2009)	864	CCCAGCCTGGGATTTGGTTTC

Appendix 3. Diagnostic base pair differences between 23 new specimens of *Diplostomum* spp. cercariae collected from *Lymnaea elodes* snail hosts in Wheaton Lake, Bocabec, New Brunswick from an 884-base pair (bp) alignment of the internal transcribed spacer region of rDNA compared with the sister clade of with the closest congener based on neighbor-joining analyses (Appendix 1 and 5). The comparisons are between *Diplostomum* sp. 1 with *Diplostomum indistinctum*, *Diplostomum* sp. 4 with *Diplostomum huronense* and *Diplostomum baeri* with *Diplostomum* sp. 2. A hyphen indicates a gap in the position.

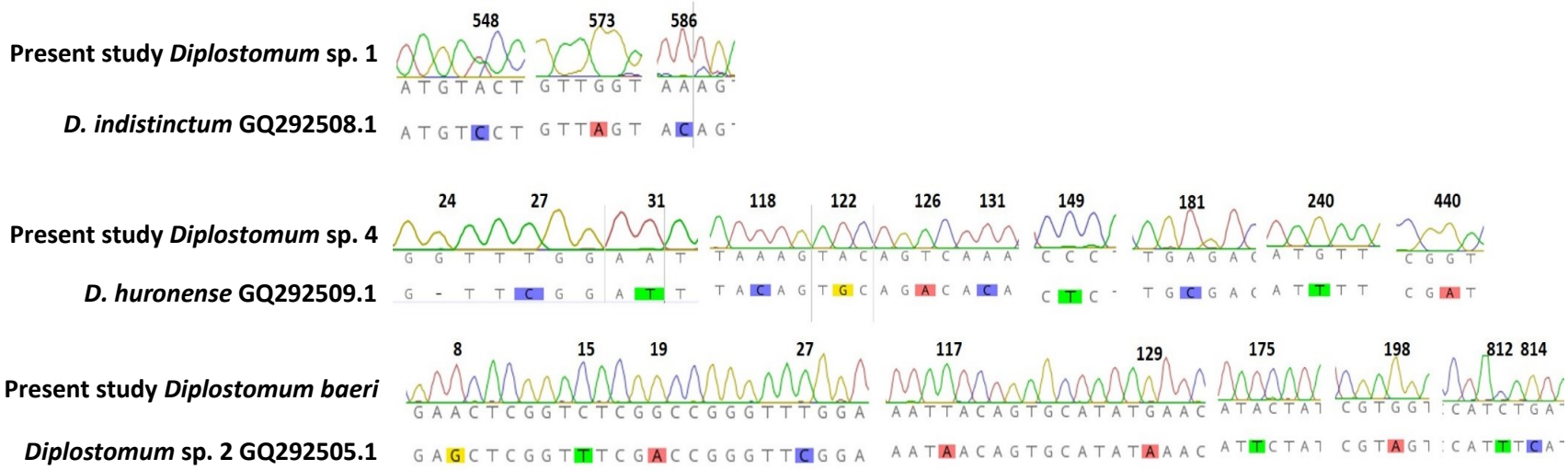
5' bp position

Specimen	**548**	**573**	**586**
Diplostomum sp.1 present study	A	G	A
Diplostomum sp.1 GQ292522.1	A	G	A
D. indistinctum GQ292508.1	C	A	C

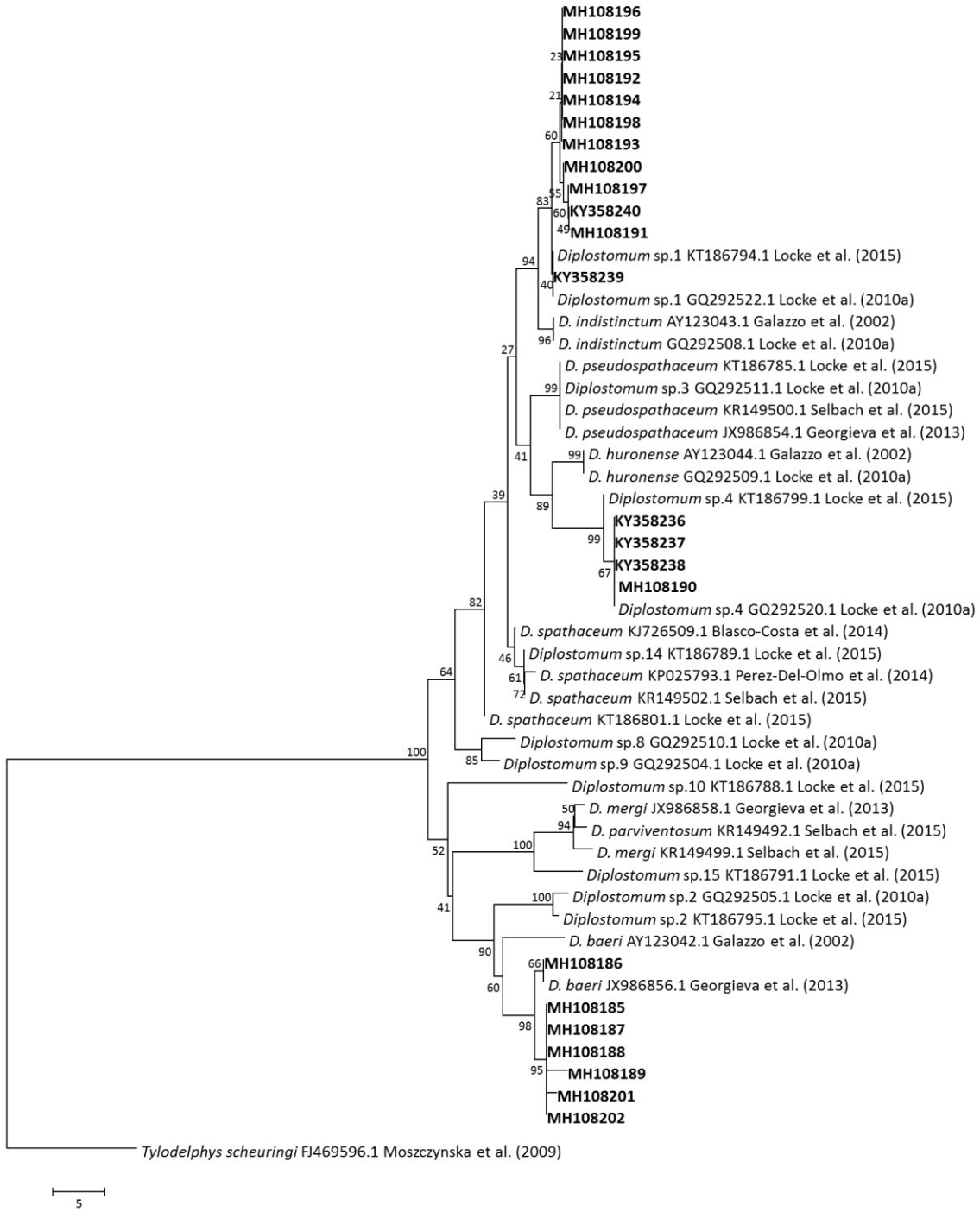
	24	**27**	**31**	**118**	**122**	**126 - 128**	**131**	**149**	**181**	**240**	**440**
Diplostomum sp. 4 present study	G	T	A	A	A	- - T	A	C	A	G	G
Diplostomum sp. 4 GQ292520.1	G	T	A	A	G	- - T	A	C	A	G	G
D. huronense GQ292509.1	-	C	T	C	G	A - -	C	T	C	T	A

	8	**15**	**19**	**27**	**117**	**129**	**175**	**198**	**812**	**814**
D. baeri present study	A	C	G	T	T	G	A	G	C	G
D. baeri JX986856.1	A	C	G	T	T	G	A	G	C	G
Diplostomum sp. 2 GQ292505.1	G	T	A	C	A	A	T	A	T	C

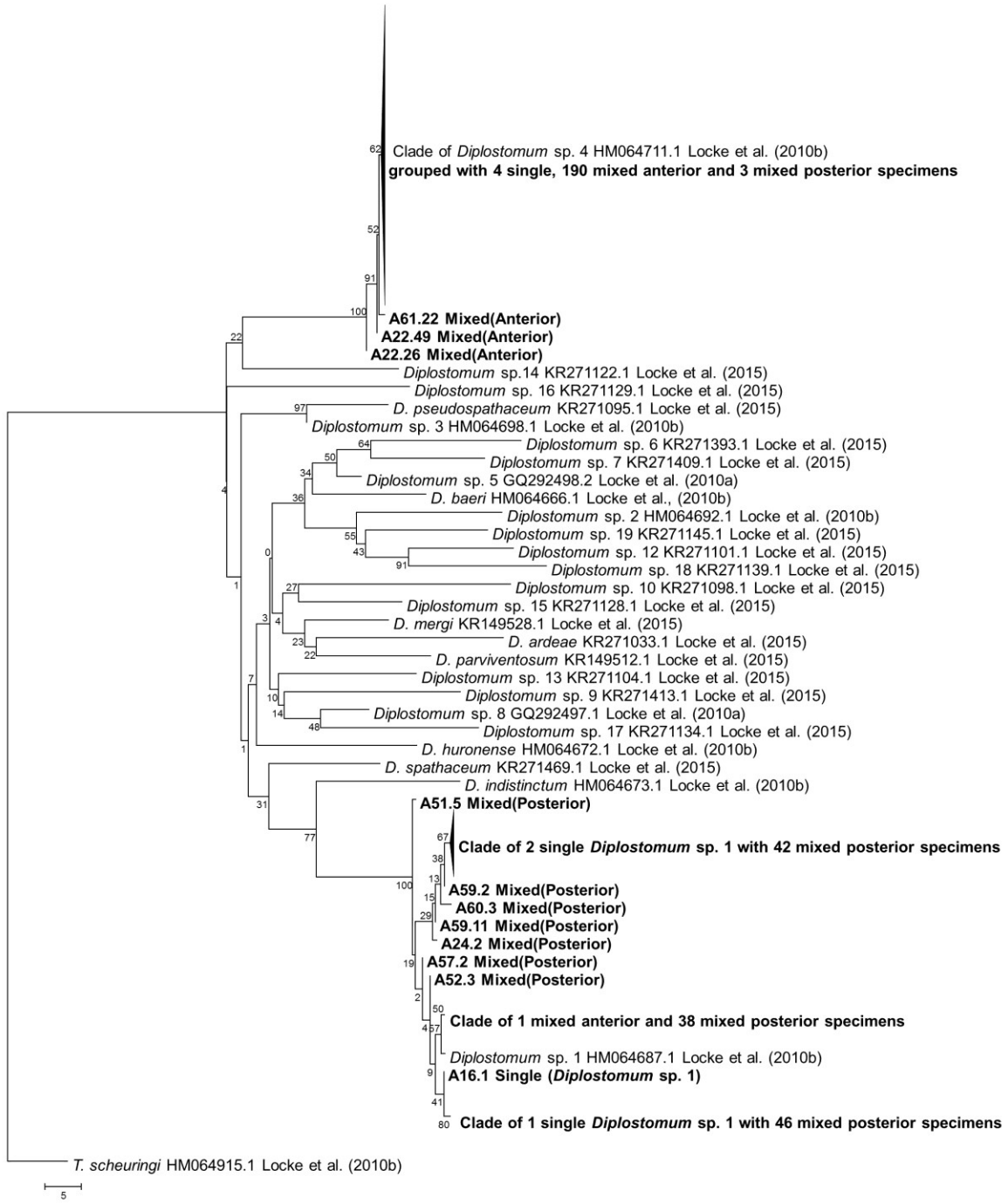
Appendix 4. Chromatogram peaks of diagnostic base pair differences between the new specimens of *Diplostomum* spp. cercariae collected from *Lymnaea elodes* snail hosts in Wheaton Lake, Bocabec, New Brunswick from an 884-base pair (bp) alignment of the internal transcribed spacer region of rDNA compared with the sister clade of with the closest congener based on a neighbor-joining analysis (Appendix 1 and 5). The comparisons are between *Diplostomum* sp. 1 with *Diplostomum indistinctum*, *Diplostomum* sp. 4 with *Diplostomum huronense* and *Diplostomum baeri* with *Diplostomum* sp. 2. Sample chromatograms are presented for chromatogram peak verification and numbers above the peaks represent the 5' base-pair position of the trimmed alignment (Appendix 2).



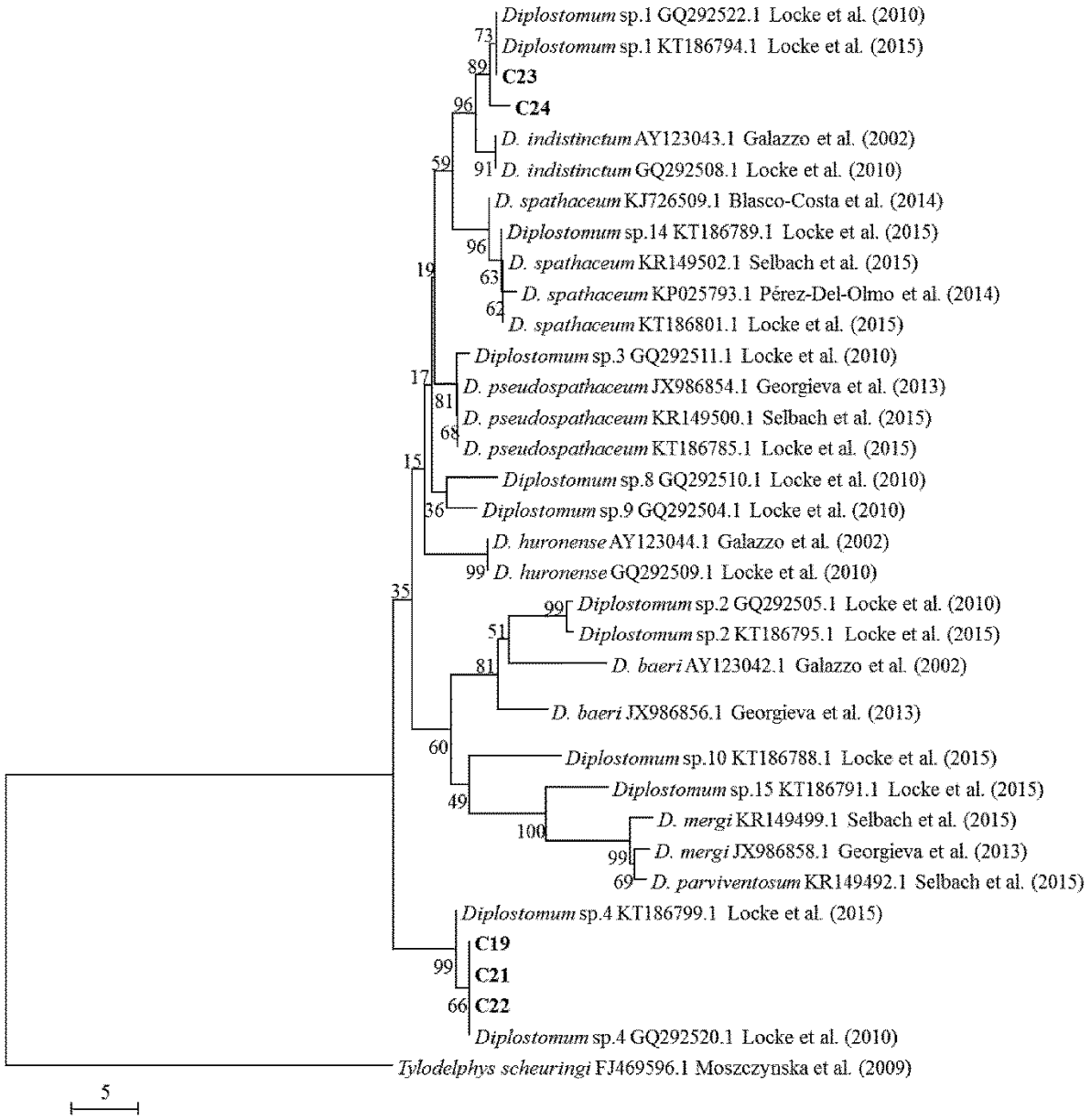
Appendix 5. Neighbor-joining consensus tree for species of *Diplostomum* reconstructed using 23 newly generated (Genbank numbers indicated in bold) and 29 ITS-rDNA sequences retrieved from GenBank. Outgroup: *Tylodelphys scheuringi.* Nodal support is based on 1000 bootstrap replicates. The scale bar indicates the number of base pair differences.



Appendix 6. Neighbor-joining consensus tree for species of *Diplostomum* reconstructed using 342 newly generated (numbered with a personal reference; indicated in bold) and 26 *cox*1 sequences retrieved from GenBank. Outgroup: *Tylodelphys scheuringi.* Nodal support is based on 1000 bootstrap replicates. The scale bar indicates the number of base pair differences.



Appendix 7. Neighbor-joining consensus tree for species of *Diplostomum* reconstructed using five newly generated (bolded and labelled with a personal reference number: C19 and C21 – C24) and 28 ITS-rDNA sequences retrieved from GenBank. Outgroup: *Tylodelphys scheuringi.* Nodal support is based on 1000 bootstrap replicates. The scale bar indicates the number of base pair differences.



Appendix 8. Trimmed 460-base pair (bp) multiple alignment of the barcode region of cytochrome oxidase 1 of mitochondrial DNA of one new sample specimen of *Diplostomum* sp. 1 collected from experimental infections of ring-billed gulls (*Larus delawarensis*) with a conspecific from GenBank and a congener (*Diplostomum indistinctum*) from the sister clade of the closest congener based on a neighbor-joining analyses (Appendix 6). Diagnostic sites are shaded. A hyphen indicates a gap in the position.

Specimen	**5' bp**	**Sequence**
Diplostomum sp. 1 HM064687.1	1	TTATTTTAATTTAATTGCTCCCGAGGTGTACAATTATATTATTACCAGTC
Diplostomum sp. 1 (new sequence)	1	TTATTTTAATTTAATTGCTCCTGAGGTGTACAATTATATTATTACCAGTC
D. indistinctum HM064673.1	1	TTATTTTAATTTGATTGCTCCGGAGGTTTATAATTATATTATTACTAGTC
Diplostomum sp. 1 HM064687.1	51	ATGGTTTAGCTATGATTTTTTTCTTTTTGATGCCGGTGTTGATAGGCGGG
Diplostomum sp. 1 (new sequence)	51	ATGGTTTAGCTATGATCTTTTTCTTTTTGATGCCTGTGTTGATAGGCGGG
D. indistinctum HM064673.1	51	ATGGTTTAGCCATGATTTTTTTCTTTTTGATGCCAGTGTTGATAGGCGGT
Diplostomum sp. 1 HM064687.1	101	TTTGGTAAATTTTTATTGCCTTTGTTGTTAGGTATGCCTGATTTGAGTTTA
Diplostomum sp. 1 (new sequence)	101	TTTGGTAAATTTTTATTGCCTTTGTTGTTAGGTATGCCTGATTTGAGTTTA
D. indistinctum HM064673.1	101	TTTGGCAAATTCTTGTTGCCTTTGTTGTTGGGTATGCCTGATTTAAGTTTA
Diplostomum sp. 1 HM064687.1	151	CCTCGTTTAAATGCTTTAAGTGCTTGATTAATGTTGCCTTCTGCTGTTTGT
Diplostomum sp. 1 (new sequence)	151	CCTCGTTTAAATGCTTTAAGTGCTTGATTAATGTTGCCTTCTGCTGTTTGT
D. indistinctum HM064673.1	151	CCCCGTTTAAATGCTTTGAGTGCTTGACTAATGTTGCCTTCGGCTGTTTGT

Diplostomum sp. 1 HM064687.1	201	TTTATAATTAGGTTATGAATTGGTTCAGGTGTTGGTTGAACCTTTTATCCG
Diplostomum sp. 1 (new sequence)	201	TTCATAATTAGGTTATGAATTGGTTCAACACTCGTGTTTCAATTATTGTTT
D. indistinctum HM064673.1	201	TTTATAATTAGTCTTTGAATTGGTTCAGGTGTTGGTTGAACCTTTTATCCG
Diplostomum sp. 1 HM064687.1	251	CCATTATCAAGGTTTCCATACAGCGGGATAGGTGTAGATTATTTGATGTT
Diplostomum sp. 1 (new sequence)	251	GGGCGTATTTATTTACTTCTATTCTTTTGTT ATCTTCGTTACCTGTTTT A
D. indistinctum HM064673.1	251	CCATTATCAAGGTTTCCATACAGCGGGATAGGTGTAGATTATTTGATGTT
Diplostomum sp. 1 HM064687.1	301	TTCTTTGCATTTAGCGGGTTTGTCGAGTGTTTTTGGTTCTTTAAATTTTA
Diplostomum sp. 1 (new sequence)	301	- - - - -TGCATTTAGCGGGTTTGTCGAGTGTTTTTGGTTCTTTAAATTTTA
D. indistinctum HM064673.1	301	TTCTTTGGAGTTGGTTGAACTTTTTATCCTCCATTATCAAGGTTTCCTTA
Diplostomum sp. 1 HM064687.1	351	TTACTACGATTTTTTCTTCTATTTTTTATTTTATTAACACTCGTGTTTCA
Diplostomum sp. 1 (new sequence)	351	TTACTACAATTTTTTCTTCTATTTTTTATTTTATTTACATTTAGCAGGTT
D. indistinctum HM064673.1	351	TACGGGTATAGGTGTTGATTATTTGATGTTTTCTTAACACTCGTGTTTC
Diplostomum sp. 1 HM064687.1	401	ATTATTGTTTGGGCGTATTTATTTACTTCTATTCTTTTGTTATCTTCGTT
Diplostomum sp. 1 (new sequence)	401	TGTCTAGTGTTTTTGGTTCGTTAAATTTTATTACTACTATCTTTTCTTC
D. indistinctum HM064673.1	401	TATAATTGTTTGGGCGTATTTATTTACTTCAATTCTTTTGTTATCTTCTTT

Diplostomum sp. 1 HM064687.1	451	ACCTGTTTTA
Diplostomum sp. 1 (new sequence)	451	ATTTTCTATT
D. indistinctum HM064673.1	451	GCCAGTTTTA

Appendix 9. Trimmed 460-base pair (bp) multiple alignment of the barcode region of cytochrome oxidase 1 of mitochondrial DNA of one new sample specimen of *Diplostomum* sp. 4 collected from experimental infections of ring-billed gulls (*Larus delawarensis*) with a conspecific from GenBank and a congener (*Diplostomum huronense*) from the sister clade of the closest congener based on a neighbor-joining analyses (Appendix 6). Diagnostic sites are shaded. A hyphen indicates a gap in the position.

Specimen	**5' bp**	**Sequence**
Diplostomum sp.4 HM064711.1	1	TTATTTTAATTTAATAGCTCCGGAGGTTTATAATTATATTATTACTAGT
Diplostomum sp. 4 (new sequence)	1	TTATTTTAATTTAATAGCTCCGGAGGTTTATAATTATATTATTACTAGT
D. huronense HM064672.1	1	TTATTTCAAATTAATAGCTCCGGAGGTTTATAATTATATTATCACTAGT
Diplostomum sp.4 HM064711.1	51	CACGGTTTGGCTATGATTTTTTTCTTTTTGATGCCTGTTTTAATTGGGGGG
Diplostomum sp. 4 (new sequence)	51	CACGGTTTGGCTATGATTTTTTTCTTTTTGATGCCTGTTTTAATTGGGGGG
D. huronense HM064672.1	51	CATGGTTTAGCTATGATTTTTTTCTTTTTAATGCCAGTTTTAATAGGTGG
Diplostomum sp.4 HM064711.1	101	GTTTGGTAATTTTTTGTTGCCTTTATTGTTGGGTATGCCTGATTTAAGTT
Diplostomum sp. 4 (new sequence)	101	GTTTGGTAATTTTTTGTTGCCTTTATTGTTGGGTATGCCTGATTTAAGTT
D. huronense HM064672.1	101	TTTTGGTAAATTTTTGTTGCCTTTATTGTTAGGTATGCCTGATTTAAGTT
Diplostomum sp.4 HM064711.1	151	TACCTCGTTTAAACGCTTTAAGTGCTTGGTTAATGTTACCATCTGCGGTT
Diplostomum sp. 4 (new sequence)	151	TACCTCGTTTAAACGCTTTAAGTGCTTGGTTAATGTTACCATCTGCGGTT
D. huronense HM064672.1	151	TACCTCGTTTAAATGCTTTAAGTGCTTGGTTAATGTTGCCGTCGGCTGTT
Diplostomum sp.4 HM064711.1	201	TGTTTTATAATTAGTTTGTGGATTGGTTCTGGGGTGGGTTGAACTTTCTA

Diplostomum sp. 4 (new sequence)	201	TGTTTTATAATTAGTTTGTGGATTGGTTCTGGGGTGGGTTGAACTTTCTA
D. huronense HM064672.1	201	TGTTTTATTATTAGTTTATGAATTGGTTCAGGAGTTGGTTGGACATTTTA
Diplostomum sp.4 HM064711.1	251	TCCCCCTTTATCTAGTTTTCCCTATACTGGTATAGGTGTTGATTATTTGAT
Diplostomum sp. 4 (new sequence)	251	TCCCCCTTTATCTAGTTTTCCCTATACTGGTATAGGTGTTGATTATTTGAT
D. huronense HM064672.1	251	TCCTCCATTATCTAGTTTTCCTTATACTGGGATAGGTGTTGATTATTTAAT
Diplostomum sp.4 HM064711.1	301	GTTTTCTTTACATTTAGCCGGATTATCTAGAGTTTTTGGTTCTTTGAATTT
Diplostomum sp. 4 (new sequence)	301	GTTTTCTTTACATTTAGCCGGATTATCTAGAGTTTTTGGTTCTTTGAATTT
D. huronense HM064672.1	301	GTTTTCATTACATTTGGCTGGTTTGTCTAGTGTTTTTGGCTCTTTGAATTT
Diplostomum sp.4 HM064711.1	351	TATAACTACTATTTTTTCTTCTATTTTTTATTTTATTAATACTCGTGTTTCT
Diplostomum sp. 4 (new sequence)	351	TATAACTACTATTTTTTCTTCTATTTTTTATTTTATTAATACTCGTGTTTCT
D. huronense HM064672.1	351	TATTACTACTATTTTTTCTTCTATTTTTTATTTTATTAATACTCGTGTCTCT
Diplostomum sp.4 HM064711.1	401	ATTATTGTTTGAGCATACTTATTTACTTCAATTCTTTTGTTGTCTTCATT
Diplostomum sp. 4 (new sequence)	401	ATTATTGTTTGAGCATACTTATTTACTTCAATTCTTTTGTTGTCTTCATT
D. huronense HM064672.1	401	ATTATTGTTTGGGCATACTTATTTACTTCAATATTATTATTGTCTTCGTT

Diplostomum sp.4 HM064711.1	451	ACCTGTTTTA
Diplostomum sp. 4 (new sequence)	451	ACCTGTTTTA
D. huronense HM064672.1	451	GCCAGTTTTA