Molecular characterization of *Tubifex* populations in Alberta, Canada, and their role in the spread of whirling disease

by

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Abstract

Whirling disease is a parasitic infection caused by *Myxobolus cerebralis* Hofer (Cnidaria: Myxozoa) and is debilitating to the salmonid fish that act as hosts. As of 2016, this parasite has been confirmed in Alberta, Canada. This thesis focused on estimating how long *M. cerebralis* has been present in the province of Alberta, Canada, and the potential for this parasite to spread in the province through the oligochaete host, *Tubifex tubifex* (Müller) (Annelida: Naididae).

To discern the introduction date of *M. cerebralis* into the province I used a combination of eDNA detections utilizing quantitative polymerase chain reactions (qPCR) and soil radioisotope dating. Sediment samples from Johnson Lake in Banff National Park, the location of the first whirling disease-positive fish detected in Alberta, were taken as well as two other waterbodies in the same watershed. These sediment samples were sectioned by depth and assessed for age and *M. cerebralis* DNA. These methods agreed with the introduction of the parasite to Johnson Lake, AB, around 2016. As a control, Johnson Lake core samples were also assessed for Brook Trout and Rainbow Trout DNA through qPCR, as introduction dates are known to begin after 1900 for this waterbody. Trout DNA was not detected in sediment prior to their introduction, with low DNA detections as early as $1972_{.9}$, confirming the reliability of these molecular methods.

To increase our knowledge of the distribution of *Tubifex* spp. and *M. cerebralis* in Alberta, I used CoxI barcoding of worms combined with qPCR detections for *M. cerebralis* DNA and Whole genome sequencing (WGS). Oligochaete samples from southern watersheds in the Crowsnest region of Alberta were taken in 2019. A total of 409 worm samples were sequenced and barcoded, with 156 returning positive qPCR results for *M. cerebralis* DNA. All positive *M. cerebralis* detections with >1000 DNA copies belonged to *Tubifex* sp. 3, corresponding to individuals suspected of shedding triactinomyxons (TAM), the parasite life stage responsible for infecting new

fish. Whole genome sequencing was completed for each *Tubifex* spp. described from Alberta, which allowed mitochondrial genomes to be assembled and compared phylogenetically, expanding on the current understanding of *Tubifex* spp. and indicating new species in need of descriptions are present in Alberta, Canada. Observations of these *Tubifex* spp. suggest they differ in susceptibility to *M. cerebralis*.

Preface

Parts of the research contained within this thesis were collaborations, the first of which was headed by Dr. Patrick Hanington and Dr. Rolf Vinebrooke of the University of Alberta School of Public Health and Department of Biological Sciences, respectively. This project titled "Spatio-temporal assessment of whirling disease in Alberta using paleo-eDNA" is currently in the final review stages amongst the authors and will soon be submitted for peer-review. The analysis of the data collected as well as the official write-up of the research, was completed by myself, with sample collection and radioisotope analysis completed by Paul Drevnick of the Government of Alberta and the University of Alberta as well as Colin Cooke of the Government of Alberta and the University of Calgary Extraction of DNA from samples and *M. cerebralis* qPCR analysis were undertaken by the Molecular Biology Service Unit at the University of Alberta. The second data chapter in this thesis "Taxonomic differentiation of *Tubifex* spp. that defines *Myxobolus cerebralis* transmission in Alberta, Canada" was a collaboration between Alberta Environment and Parks (AEP) and myself, led by Dr. Patrick Hanington of the University of Alberta School of Public Health. This chapter is also in the final review stages and will be submitted for peer-review shortly to Parasites and Vectors. All samples for this second project were collected by a team from AEP. Extraction of DNA and qPCR analysis was completed by Dr. Emannuel A. Pila of AEP, with all barcoding, genome assembly, phylogenetics and downstream analysis completed by myself along with composition of the data chapter.

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

Marie Curie

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Chapter 1 Literature Review

Biology of myxozoans and placement within the phylum Cnidaria

The subphylum Myxozoa is a strictly parasitic group of cnidarians. Most myxozoans species have a two-host life cycle alternating between an annelid worm and a fish; however, members of the family Saccosporidae alternate between bryozoans and fish (Canning & Okamura, 2003). This family includes the causative parasite of proliferative kidney disease (PKD) in salmonids: *Tetracapsuloides bryosalmonae* (Canning, Curry, Feist, Longshaw & Okamura) (Canning et al., 1999). The annelid or bryozoan is the definitive host as sexual reproduction is undertaken during actinospore development, whereas the fish are the intermediate host due in which asexual reproduction of the parasite occurs (Canning & Okamura, 2003; El-Matbouli & Hoffmann, 1998).

Taxonomists long debated where myxozoan taxa belonged in the tree of life, with complications attributed to the original separation of the myxospore and actinospore life-stages as different organisms (Foox & Siddall, 2015). Until the mid-1990s myxozoans were described as protists, though taxonomists had noted the similarities of polar capsules to cnidarian nematocysts since the late 1800s (Bütschli & Schwager, 1880; Foox & Siddall, 2015). It would be the beginnings of phylogenetic analyses of sequencing data in the 1990s that would begin to place the myxozoans within the Metazoa (Smothers et al., 1994), but disputes occurred as to where within Metazoa they belonged. Conflicting research was published in favour of Bilateria (Schlegel et al., 1996; Smothers et al., 1994) versus Cnidaria (Siddall et al., 1995). Confidence in the placement of myxozoan taxa within the phylum Cnidaria would happen later upon the discovery of mini-collagen genes in *T. bryosalmonae* (Holland et al., 2011). Mini-collagens are unique to Cnidaria, as they are proteins responsible for structures in the nematocysts (Kurz et al., 1991). These mini-collagens as well as additional Cnidaria-specific genes were found within multiple myxozoan species (Shpirer et al., 2014) furthering the evidence that myxozoans belong to the phylum Cnidaria. In 2018 a research group focusing on phylogenetics and molecular clock dating placing myxozoans within the phylum Cnidaria (Holzer et al., 2018). These combined efforts cemented myxozoan species within the phylum Cnidaria, as sister taxa to *Polypodium hydriforme* Ussow (Holzer et al., 2018; Ussow, 1887).

Initial Description of *Myxobolus cerebralis*:

Myxobolus cerebralis Hofer is a myxozoan parasite described by German scientist Bruno Hofer when observing abnormal behaviour in imported American Rainbow Trout, *Oncorhynchus mykiss* (Walbaum) (Hofer, 1903). These fish were seen laying on the bottom of their enclosures and displaying erratic thrashing when attempting to swim or feed (Hofer, 1903). Upon further investigation and dissection of the fish it was determined that the parasite was residing within the brain tissues and was thus named upon this location: *cerebralis* (Hofer, 1903). Further research found the parasite also to be found within the skeletal and connective tissues of the host fish (Plehn, 1904), which further indicates the cause of high morbidity and infection severity found in juvenile salmonids when compared to adult salmonids due to the low ossification of cartilage into bone, allowing the parasite to reproduce as it localizes within the cartilage (El-Matbouli et al., 1999; Hedrick, McDowell, Gay, et al., 1999; Hoffman, 1962, 1966; Hoffman et al., 1962; Hoffman & Byrne, 1974a, 1974b; Markiw, 1991; Putz & Hoffman, 1966; Rose et al., 2000; Ryce et al., 2005). The severity of infection is also variable between different species of salmonid fish, with Brown Trout, *Salmo trutta* Linnaeus, exhibiting the lowest susceptibility through reduced internal parasitic lesions and lack of thrashing swimming behaviour (Baldwin et al., 2000; R. Hedrick et al., 1999; R. P. Hedrick et al., 1999; Yoder, 1972).

Lifecycle:

Myxobolus cerebralis relies on two hosts to complete the life-cycle: the tubificid worm *Tubifex tubifex* (Müller, 1773) and a salmonid fish such as a Rainbow Trout (Figure 1). The myxospores from *M. cerebralis* are released into the water column from damaged or decaying infected fish tissues, where they may settle into the sediment of the freshwater environment. Within the sediment, *T. tubifex* consumes the myxospores of the parasite, which then divides and matures within the gut and intestinal lumen of the worm (El-Matbouli & Hoffmann, 1998). Due to the trifid shape of the actinospore life stage, it is called a triactinomyxon (TAM). TAMs are released from the *T. tubifex* worm through fecal packets (El-Matbouli & Hoffmann, 1998). These TAMs then inflate due to osmotic gradients(Fiala et al., 2015), which gives higher buoyancy allowing the actinospore to float in the water column where the salmonid fish may be infected again (El-Matbouli et al., 1999; El-Matbouli & Hoffmann, 1998). The TAMs attach onto the epidermis of the fish and inject the sporoplasm through the skin as well as through the operculum and gills

(El-Matbouli et al., 1999). Within the fish host, the parasite multiplies and attacks the cartilage by travelling through the central nervous system (CNS), which leads to skeletal deformities and constrictions along the spinal cord and brain stem (El-Matbouli et al., 1995, 1999; El-Matbouli & Hoffmann, 1998; Rose et al., 2000). The two-host life-cycle of *M. cerebralis* was not fully elucidated for over 80 years after the initial discovery. Instead, the myxospore life stage was considered a distinct and separate animal to the actinospore life stage. Wolf et. al (1986) were able to infect *T. tubifex* with *M. cerebralis* myxospores and observed actinospore (TAM) life stages within the posterior of the worms, combining the once separate life-cycle (Wolf & Markiw, 1984) and changing the taxonomy of the group Myxozoa.

The puzzle of the oligochaete host *T. tubifex*

Tubifex tubifex is a widespread freshwater oligochaete found within nutrient rich sediments and known for a resistance to pollutants and heavy metals (Chapman et al., 1982a, 1982b; Fargasova, 1994). Accurate morphological identification of the worm host requires the observation of mature sexual organs as well as presence, counts and morphology of various hair-like structures found on the body known as chaetae (R. O. Brinkhurst, 1986). The identification of these worms using morphology has proven to be inconsistent due to the phenotypic changes that *T. tubifex* chaetae undergo in response to variation in their environments (Chapman & Brinkhurst, 1987). These challenges have led to more current studies relying on molecular methods as a more suitable method of identification (Barry et al., 2021; Nehring et al., 2013; Beauchamp et al., 2001, 2002; James et al., 2021; Rasmussen et al., 2008).

M. cerebralis as an invasive species in North America: The United States

Whirling disease was first reported in Pennsylvania in the 1950s and was later found in multiple states in the Northeast but was considered mainly a hatchery concern (Hoffman et al., 1962). In the late 1960s and early 1970s whirling disease was discovered in Michigan, having spread outside of hatcheries and propagating within the wild Brook Trout population (Yoder, 1972). In the late 1980s to mid-1990s whirling disease was taking a foothold in the mid-west of the United States in Colorado and Montana (Barry. R. Nehring & Walker, 1996; Vincent, 1996). The results of this invasion were evident in the significant losses in the population of wild Rainbow Trout (Fetherman & Schisler, 2014; Barry. R. Nehring & Walker, 1996; R. B. Nehring, 2006;

Walker & Nehring, 1995). The invasion was of particular note in Colorado, with extensive research covering the impacts of whirling disease on fisheries and wild populations of Rainbow Trout (Nehring & Walker, 1996; R. B. Nehring, 2006; Walker & Nehring, 1995). Populations of wild Rainbow Trout were devastated in the upper Colorado river by *M. cerebralis*, with survivorship reported as 3.2% following a four-month monitoring period compared to 33.5% survivorship in Brown Trout (Walker & Nehring, 1995). Colorado River Rainbow Trout population density crashed in 1994 with recruitment of juvenile trout to mature adults failing (Walker & Nehring, 1995). The results of invasion within the Colorado River were seen in similar intensities throughout other waterbodies in the state such as South Platte River, Rio Grande, Cache la Poudre River, and others (R. B. Nehring, 2006).

In the early 2000s, a polymerase chain reaction (PCR) assay targeting the 16s rDNA gene of *T. tubifex* was developed to assess cadmium resistance in European *T. tubifex* (Sturmbauer et al., 1999). This assay separated *T. tubifex* into five different lineages based on the results of an end-point PCR, with the basepair length of the resulting bands on an agarose gel relating to the *T. tubifex* lineages which were categorized based on their respective resistance to cadmium (Sturmbauer et al., 1999). Given the challenges with morphologically identifying *T. tubifex,* the potential of a molecular assay that could differentiate these worms provided a channel for the future of whirling disease research. Two years after the initial assay was published, another assay was designed using the same region of 16s rDNA to differentiate lineages of *T. tubifex* with respect to whirling disease, with lineages varying in TAM production when infected with *M. cerebralis* (Beauchamp et al., 2002). These lineages were the focus of studies looking to assess population differences with respect to potential for whirling disease transmission in wild *T. tubifex* populations (Nehring et al., 2013; Beauchamp et al., 2002). This analysis also emerged as a useful tool for characterizing *T. tubifex* for in situ cultures to maximize TAM production and facilitate more controlled studies of *M. cerebralis* (Rasmussen et al., 2008). Many studies that utilized the lineage assay suggested that ‘lineage III’ worms were the primary drivers of TAM production and whirling disease propagation (Beauchamp et al., 2002, 2005; Rasmussen et al., 2008). This lineage assay continues to be used when analyzing populations of *T. tubifex* worms with regards to whirling disease (Baxa & Nehring, 2022), and has been expanded to be used as a measure of both transmission risk and disease fluctuations (Arsan, , Hallett, Bartholomew, 2007; Nehring et al., 2013).

M. cerebralis as an invasive species in North America: Canada

In late August 2016, the first observation of whirling disease-positive Rainbow Trout was noted in Johnson Lake in Banff National Park, Alberta, Canada. This was Canada's first whirling disease report, spurring investigations by the Canadian Food Inspection Agency (CFIA) that resulted in the declaration of four major watersheds positive within two years following the first detection(Canadian Food Inspection Agency, 2019).

Following the first detection, projects partnering researchers and government agencies were undertaken to understand the spread of the disease within the province and determine potential control measures or environmental barriers that might be used to prevent future spread. These provincial efforts and partnerships with researchers led to the development of a quantitative polymerase chain reaction (qPCR) assay that was specific for detecting *M. cerebralis* genetic material in sediment, extracted genomic DNA from aquatic oligochates and water, both quickly and efficiently (Barry et al., 2021). The established *T. tubifex* lineage assay ((Sturmbauer et al., 1999) was used for worms collected in Alberta, with results inconsistent with previous publications, suggesting a unique population structure (Barry et al., 2021). This led to a comprehensive *T. tubifex* population assessment utilizing DNA barcoding that targeted the cytochrome oxidase I (CoxI) gene, to identify oligochaete species collected within the province of Alberta (Barry et al., 2021). The results of this project suggest the possibility of five unique *T. tubifex* species within Alberta (Barry et al., 2021).

In southern Alberta, within the Crowsnest River region, an extensive study investigating the impact that *M. cerebralis* has had on Rainbow Trout found multiple indicators showing the deleterious effects already taking place only a few years after the initial detection in the province (James et. al, 2021). Densities of TAMs found within the southern waterbodies tested exceeded previously published thresholds known to cause dramatic losses seen in the Rainbow Trout populations of Colorado (James et al., 2021; R. B. Nehring, 2006). Temperature data from southern waterbodies found large overlaps between optimal temperatures for TAM shedding in *T. tubifex* and susceptibility to parasitism in young Rainbow Trout (James et al., 2021). Additionally, low juvenile recruitment to adult fish was noted and attributed to the invasion of *M. cerebralis,* suggesting the parasite is already taking hold within these wild populations (James et. al, 2021).

Aims of this thesis

Given the short time between the first detection of whirling disease positive fish and the declines seen in southern fish populations, it is crucial to investigate how long *M. cerebralis* had been present in the province. This information could provide context for how quickly the parasite gains a foothold within populations of wild salmonids, which could prove vital in target management and control methods should the parasite spread outside the province. Another important piece to improve our understanding of the parasite spread is to better examine the genetic groups of *Tubifex* found by Barry et al. (2021), as these groups could be important in the dynamics of both current infection and future transmission provincially and globally.

Figures:

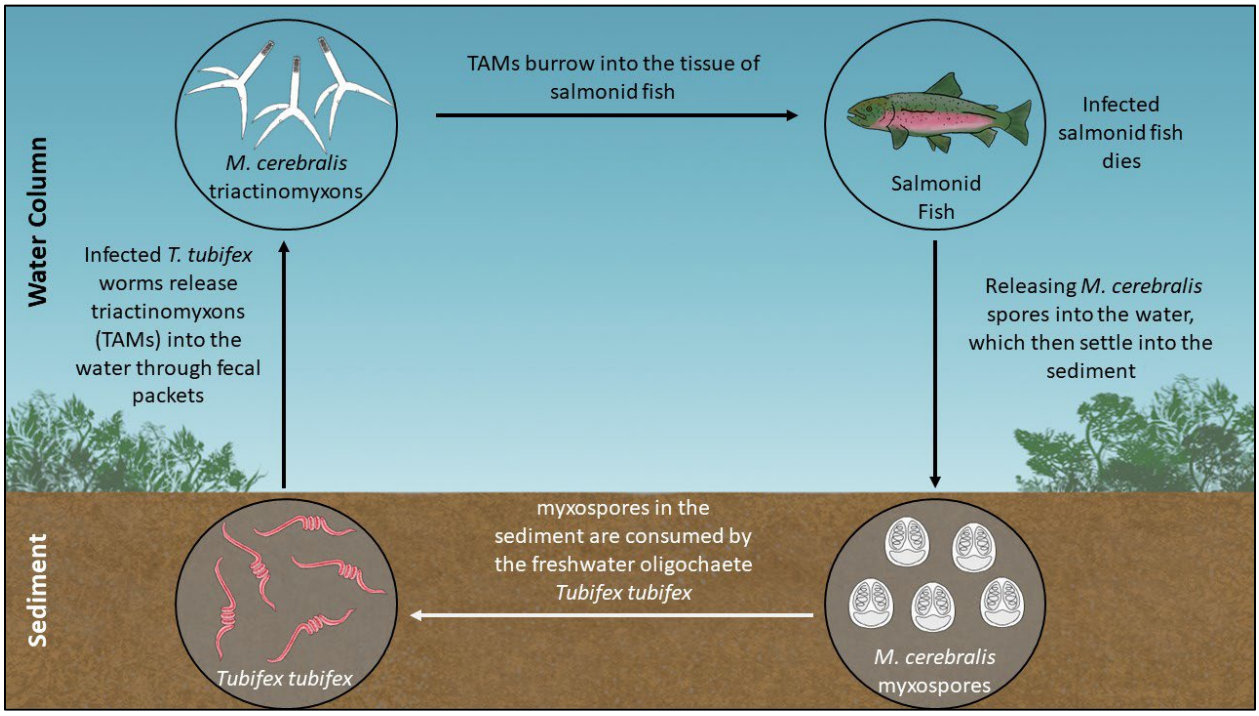

Figure 1-1: Life-cycle of *Myxobolus cerebralis*

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Chapter 2 Spatio-temporal assessment of whirling disease in Alberta using paleo-eDNA

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Abstract

Whirling disease is a detrimental parasitic infection of salmonid fish caused by the myxozoans *Myxobolus cerebralis*. It was first detected in Canada in Johnson Lake, Banff National Park, Alberta in 2016. The spread of this parasite across the province is of particular concern, as many salmonid species at risk of infection are imperative to the balance of aquatic ecosystems as well as to recreational and subsistence fishing. This study aims to expand on the understanding of *Myxobolus cerebralis* introduction in the province through the development, validation, and use of a method to reconstruct the presence/absence of *M. cerebralis* in dated sediment cores. Sediment cores were collected from Johnson Lake and two other lentic water bodies in the Bow River watershed where *M. cerebralis* is known to occur. The cores were extruded and sectioned with a procedure developed to prevent cross-contamination between/among sections, and subsamples from each section were analyzed for radioisotopes to determine sediment age and for *M. cerebralis* environmental DNA (eDNA). As a control, Johnson Lake sediments were also analyzed for eDNA of Rainbow Trout (*Oncorhynchus mykiss*) and Brook Trout (*Salvelinus fontinalis*), two introduced salmonid species with known stocking histories in the lake. Results illustrate that the method is useful for tracking the introduction/establishment and abundance of

M. cerebralis in lakes/reservoirs. However, there can be inhibition in extraction, amplification, or detection of eDNA in highly organic sediments, and sediment mixing (e.g., through bioturbation) can complicate interpretation. Assessment of core ages, mixing depths, and qPCR data support the introduction of *M. cerebralis* around 2016. Our study shows the utility and reliability of paleo-eDNA for understanding invasive species introduction events.

Introduction

Whirling disease is an infectious disease of fishes that can negatively impact fish behaviour, appearance, and health. Whirling disease is caused by *Myxobolus cerebralis*, a myxozoan parasite that cycles between a worm host (*Tubifex tubifex*, commonly found in the sediments of freshwater bodies) and impact fish in the Salmonidae family (salmon and trout species), which act as host. The cartilage damage due to *M. cerebralis* infection together with damage caused by the immune response of the fish leads to physical malformations and the whirling swimming behavior (Rose et al., 2000, Gilbert & Granath, 2003). The parasitic invasion of cartilage results in highest morbidities in young fry as skeletal features have not yet ossified into bone (Markiw, 1991; Ryce et al., 2005). Rainbow Trout have been shown to have the highest susceptibility of salmonids to *M. cerebralis* with the susceptibility of other members of the family differing between genera and species (O'Grodnick 1979; Hedrick et al.; 1999, El-Matbouli et al., 1999). This susceptibility is of particular importance to Canadian and Albertan recreational fishing industry as trout species are the second most caught species by anglers in Canada and third most caught by anglers in Alberta (Canada. Department of Fisheries and Oceans. & Nanos Research (firm), 2019). The recreational fishing industry attributed $7.9 billion to the Canadian economy and $1.137 billion to Alberta in 2015 (Canada. Department of Fisheries and Oceans. & Nanos Research (firm), 2019).

Whirling disease has been an important and well-studied parasitic infection since the first description of fish-illness in a German fishery in 1903 (Hofer, 1903). Imported Rainbow Trout from the United States were displaying abnormal swimming behavior, and spores were found upon dissection of the head and brain giving it the name *Myxobolus cerebralis* (Hofer, 1903). Since the first description, *M. cerebralis* has been detected across Europe and Asia but was largely considered a fisheries concern as wild populations of salmonids were predominantly that of Brown Trout which do not exhibit clinical signs of infection (Bartholomew & Reno, 2002).

The introduction of whirling disease in the United States began largely as a concern for fisheries in the eastern part of the country, with less focus on the wild populations (Bartholomew & Reno, 2002; Hoffman, 1962; Hoffman et al., 1962). In the mid to late 1980s, there were positive detections of whirling disease in wild populations of Rainbow Trout in Colorado and Oregon, spreading to Montana by the mid-1990s (Bartholomew & Reno, 2002; Vincent, 1996). The infection of wild Rainbow Trout in Colorado resulted in record low survival rates of juveniles and substantial reductions in adult fish year-over-year (Fetherman & Schisler, 2014; Nehring & Walker, 1996).

In August of 2016, fish exhibiting sign of whirling disease were detected in Canada for the first time in Johnson Lake, Banff National Park, Alberta (James et al., 2021). Confirmation that these fish were infected with *M.* cerebralis initiated a province-wide program to understand the extent of the disease spread and to attempt targeted control efforts. Studies focused on surveying fish and worm hosts across the province, with a species-specific quantitative PCR assay being developed to detect *M. cerebralis* DNA in different matrices such as soil and oligochaete whole genomic DNA (Barry et al., 2021). The Canadian Food Inspection Agency (CFIA) declared four of the major watersheds in Alberta positive for whirling disease: North Saskatchewan, Red Deer, Oldman, and Bow River (CFIA 2019). The lower Crowsnest River in Alberta has been highlighted as a key region for the spread of whirling disease within the province, with evidence suggesting low survival rates in juvenile fish (James et al., 2021).

Our study aims to understand how long whirling disease has been present in Alberta, as a proxy to better predict the rate of future spread. To accomplish this, we sampled sediment cores from lakes located within the Bow River where whirling disease has been observed. We then dated these cores using radioisotopes and analyzed the sediment samples for DNA evidence of *M. cerebralis.*

Materials and Methods

Core collection and preparation

Sediment cores were recovered from three lakes within the Bow River Watershed: one from Johnson Lake (core length of 32 cm), two from Ghost Lake (one core of 82.5cm and another of 35cm), and one from Glenmore Lake (core length of 75cm) (Figure 1). In late February of 2019,

sediment cores were collected from depositional basins with an HTH gravity corer (Pylonex) and a universal percussion corer (Aquatic Research Instruments). The latter, which is a gravity corer with a percussion hammer that allows the collection of longer cores in deeper waters, was used in (and necessary for) Ghost Reservoir only as the water depth reached 25m. For both corers, 7-cm diameter polycarbonate core tubes and plastic caps and bungs were used that were cleaned with a 3% bleach solution and rinsed thoroughly with DI water before use. Prior to sectioning, cores were visually inspected and noted for presence of invertebrates or color distinctions as early indicators of bioturbation. Other organic materials, disturbances and layer separations were noted in order to better understand radioisotope abnormalities. Cores were sectioned in the field and subsampled in 0.5-cm intervals up to 5 cm depth and 1-cm intervals below; depths >25 cm were sampled at 2.5-cm intervals. All cores were extruded and sectioned with the Pylonex extruding device, with an extension rod utilized for the Ghost Reservoir cores. Extruding (pushing up the sediment) causes smearing of the core against the wall of the core tube. To prevent cross-contamination of samples from smearing, we developed a procedure for sectioning the extruded sediment. First, a plastic scraper and metal spatula were cleaned before use with a 3% bleach solution and thoroughly rinsed with DI water. Then, an interval of sediment, e.g., 1 cm section, was extruded out of the top of the core tube and the plastic scraper was pushed under the bottom of the interval. The interval "sat" on the plastic scraper and the metal spatula was used to selectively subsample sediment that had not touched the core tube. This subsample was placed in a Falcon tube and designated for analysis of eDNA as well as dating. Subsamples were then frozen (-20C) until processing was undertaken. To understand whether it was necessary to prevent cross contamination to achieve valid results, we also collected, extruded, sectioned, and subsampled cores without these procedures.

Sediment core geochronology

For dating of cores and determination of mixing depth, subsamples were freeze-dried, disaggregated, and analyzed for Pb-210, Ra-226, Cs-137, and Be-7 with a high purity germanium coaxial well detector ("gamma counter"). Pb-210 is supplied to aquatic systems by the atmosphere; however, it is also produced in situ in sediments and soils. Ra-226 has a half-life of 1600 years and provides a measure of this in situ production. Pb-210 has a half-life of 22.3 years, and by measuring both Pb-210 and Ra-226 activities, sediment age up to ~150 years can

be estimated. This approach can be applied to natural systems, in which background Pb-210 can be reached with the constant rate of supply model. The data for Cs-137, an artificial radioisotope introduced to the environment with nuclear weapons testing that began in 1952 and peaked in 1963, can also be used for dating recent sediments, and is especially useful for reservoirs that pre-date 1963. For Glenmore Reservoir and Ghost Reservoir, both of which are reservoirs constructed in 1933 and 1929 respectively, the Cs-137 peak was assigned as 1963 and a linear model was used to calculate dates between the peak and the sediment-water interface (for which the date was the day of collection).

The data for Be-7 was used to determine the depth of sediment mixing. Be-7 is delivered to the Earth's surface via dry and wet deposition, and in aquatic environments sorbs to suspended particles. Upon deposition, Be-7 activity rapidly declines (to non-detection) with depth in undisturbed sediments because of burial, isolation from the atmospheric source, and a short half-life (53.3 days) (Fitzgerald et al., 2001). Mixing of sediments by physical and biological processes extends Be-7 activity deeper into the sediment column. Where sediment mixing (e.g., from *T. tubifex* worms, between 6-9 cm deep (Fisher et al., 1980)) is present, detection of *M. cerebralis* DNA below the base of detectable Be-7 activity can only be considered to pre-date the 2016 detections of whirling disease. The base of detectable Be-7 activity was defined as the depth of sediment mixing.

Sediment DNA extraction and M. cerebralis DNA quantification

DNA from sediment samples was extracted using DNA Isolation Plus Kit (Norgen Biotek) following Barry et al. (2021). Extracted DNA was run through the 18s targeted Click or tap here to enter text. qPCR assay in triplicate to detect *M. cerebralis* presence in the layers of sediment. DNA samples were run through the qPCR assay in order of increasing depth. When 10 or more sequential samples showed no qPCR amplification (a time span >7 years), the remaining sediment was assumed to be negative.

Inhibition analysis

Extracted DNA was run through United States EPA method 1611 (U.S. EPA, 2012) to determine whether samples were inhibited, due to organic compounds commonly found in sediment such as

humic acid (Sidstedt et al., 2020). Samples that were inhibited were diluted 2X and reassessed through the respective qPCR assays.

Rainbow Trout and Brook Trout control qPCR

DNA extacted from the Johnson Lake core sediment was assessed using the Rainbow trout (Wilcox et al., 2015) and Brook Trout (Schumer et al., 2019) specific qPCR assays as a control method for *M. cerebralis* detection, as fish stocking history is known at this lake to begin in the 20th century(Schindler, 2000). All samples were run in triplicate using 2X diluted DNA samples.

Results

Core properties and radioisotope analysis

Glenmore Lake core was not suitable for radioisotope analysis as presence of non-sediment, tree trunk material, disrupted the sections taken and as such was also not included in the final data. Johnson Lake and Ghost Lake Cores 2 and 3 returned with reasonable distributions for respective sediment ages (Table 1). The Johnson Lake core had invertebrates present in the top 3cm of sediment with vegetative organic material throughout the core, with the bottom layers being clay. The top layers of the core were coarse and brown-grey in color with a thin layer on the top of black organic matter. Deeper layers of the core were predominantly grey-brown and coarse in grit. Ghost Lake cores were predominantly brown-grey silt-clay material with a bottom layer of grey clay. The top 1 cm of Ghost Lake core 2 had oxidized and mixed sediment with indicators of animal presence and plant matter, this layer was less pronounced in core 3.

qPCR analysis

Johnson Lake had detections of *M. cerebralis* DNA in the top 1.5 cm of sediment cored. The deepest sediment with *M. cerebralis* detected was determined to be between 2009 and 2012. Ghost Lake core 2 had detections in the top 3.5 cm of sediment, with deepest detections in sediment aged around 2016. Ghost lake core 3 had detections of *M. cerebralis* DNA in the top 5 cm of sediment. Deepest sediment ranging from $2015_{.6}$-$2015_{.9}$ had *M. cerebralis* detections at very low copy number (Table 1). qPCR assays targeting Brook and Rainbow Trout did not amplify DNA from sediment older than 1967 (Table 2).

Discussion

Data from cores sampled in this study support that the introduction of whirling disease into Alberta was likely in 2016, however the Johnson Lake sediment section does age as early as 2012 (Table 1). It should be noted these samples from Johnson Lake that have detections of *M. cerebralis* DNA in samples older than 2016 overlap with layers where invertebrates were present, and given their mobility in the environment, could potentially contribute parasite DNA to these lower layers. Additionally, the calculated gene copy number for sediment section 3 from Johnson Lake is lower than the limit of quantitation (LOQ) and limit of detection (LOD) for the Barry qPCR assay (2021) of 22.5 and 7.4 copies respectively. As a further control for Johnson Lake both Rainbow Trout and Brook Trout qPCR assays were performed; the earliest stockings into Banff National Park for these species was 1916 and 1906 respectively(Donald, 1987). This alignment further supports the accuracy of the radioisotope dating, allowing it to be compared with the qPCR results more reliably. Ghost Core 3 had similarly small *M. cerebralis* gene copy numbers found in sediments dated as late 2015; however, these are also below the LOQ/LOD. Detections of *M. cerebralis* in both Ghost Lake cores in quantities within the LOD/LOQ all fall within sediments younger than 2016.

Focusing on eDNA analysis reduces the impact of detection and monitoring for species that are species at risk of extirpation as well as species that are less densely populated within their environment. Sediment core dating in combination with eDNA analysis has been shown to give historical insights into introduction events for aquatic organisms, allowing for more accurate understandings of populations over time (Nelson-Chorney et al., 2019). This study expands this method to better understand introduction events for an invasive species of interest (*M. cerebralis*). In this study we have utilized this method to detect more recent historic eDNA introduction events, which could prove invaluable in determining spread of this invasive species. Southern waterbodies within Alberta have already observed decreases in Rainbow Trout populations as well as lack of juvenile recruitment (James et al., 2021), making it vital to expand our understanding of how long this parasite takes to get a foothold in fish populations. Future studies should be completed targeting waterbodies surrounding the suggested introduction site of Johnson Lake, to expand on the knowledge around the speed of parasite invasion throughout the province. These efforts should also be expanded to other regions in the province as this could

elucidate whether the spread of whirling disease has been following one or multiple introduction events. Further information regarding how quickly *M. cerebralis* has spread within the province of Alberta could be used should the parasite spread outside of the province and allow for more targeted control measures to be implemented in a timely manner.

This study exemplifies the utility of combining eDNA analysis with sediment dating for biological monitoring. Data collected using these methods are shown here to be viable in determining introduction timelines for invasive species allowing for more informed conservation efforts to be employed. These methods could also be expanded for any target organism with sufficient sequencing data for qPCR assay development, allowing for a more extensive understanding of population changes over time.

Acknowledgements

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Tables and Figures

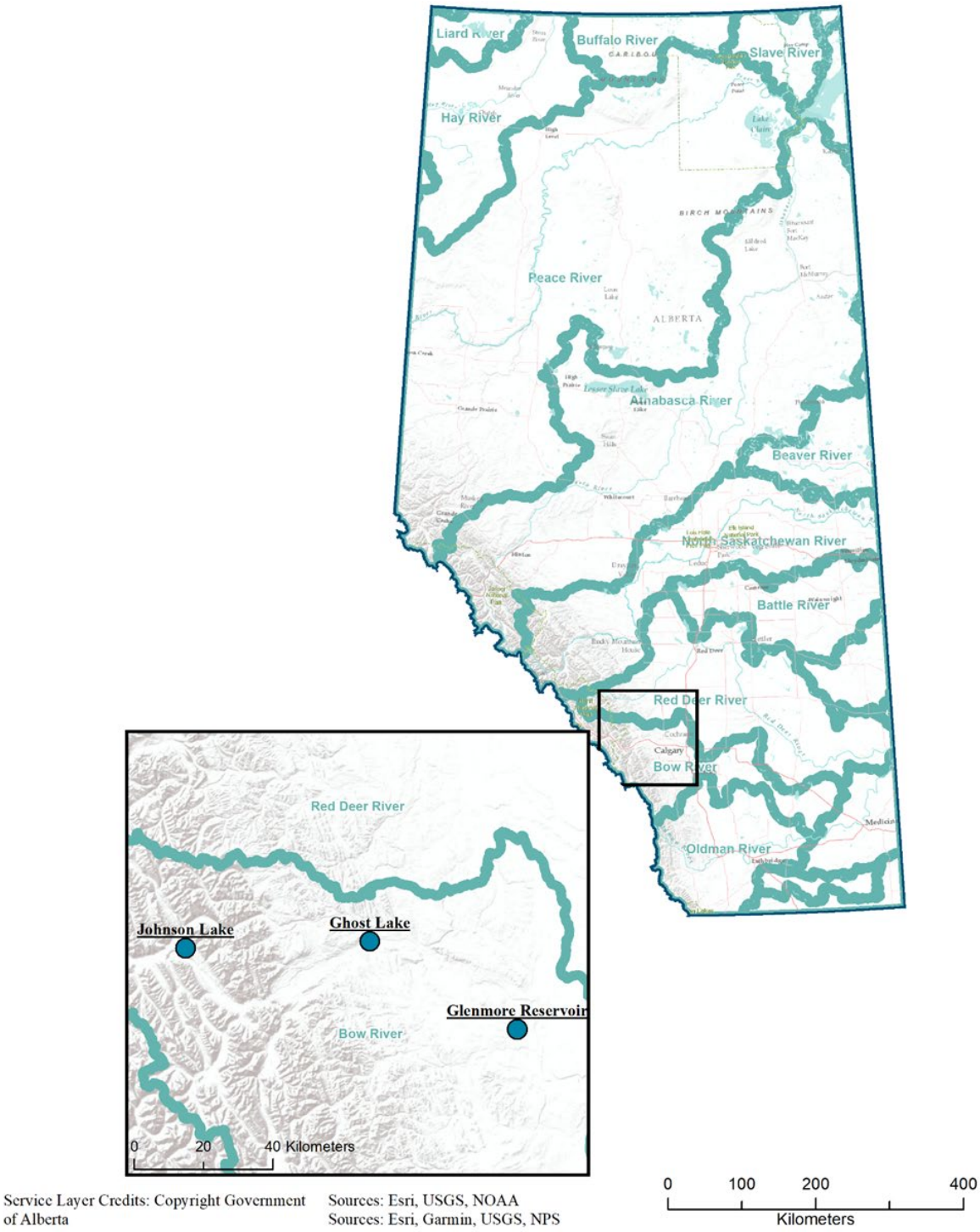

Figure 2-1: Map of Alberta with sample sites marked along the major watersheds.

Table 2-1: Depth and age of sediment comparisons with *M. cerebralis* qPCR detections. Johnson Lake Core DNA extracts include calculated 18s copy number from undiluted and diluted post-inhibition assay.

	Section ID	Estimated Top cm Date	Estimated Middle cm Date	Estimated Bottom cm Date	DNA Copy Number	DNA copy Number (Diluted)
Johnson Lake Core	1	2019.2	2017.5	2015.9	374.5	554.5
	2	2015.9	2014.2	2012.6	122.8	100.0
	3	2012.6	2010.9	2009.3	2.9	6.9
	4	2009.3	2007.6	2005.9	0.0	0.0
	5	2005.9	2004.3	2002.6	0.0	0.0
	6	2002.6	2001.0	1999.3	0.0	0.0
	7	1999.3	1997.7	1996.0	0.0	0.0
	8	1996.0	1994.4	1992.7	0.0	0.0
	9	1992.7	1991.1	1989.4	0.0	0.0
	10	1989.4	1987.8	1986.1	0.0	0.0
	11	1986.1	1982.8	1979.5	0.0	0.0
	12	1979.5	1976.2	1972.9	0.0	0.0
	13	1972.9	1969.6	1966.3	0.0	0.0
	14	1966.3	1963.0	1959.7	0.0	0.0
Ghost Core 2	1	2019.2	2019.0	2018.8	69.2	N/A
	2	2018.8	2018.6	2018.4	21.8	N/A
	3	2018.4	2018.3	2018.1	22.4	N/A
	4	2018.1	2017.9	2017.7	4.9	N/A
	5	2017.7	2017.6	2017.4	2.9	N/A
	6	2017.4	2017.2	2017.0	3.1	N/A
	7	2017.0	2016.8	2016.7	1.8	N/A
	8	2016.7	2016.5	2016.3	0.0	N/A
	9	2016.3	2016.1	2015.9	0.0	N/A
	10	2015.9	2015.8	2015.6	0.0	N/A
	11	2015.6	2015.2	2014.9	0.0	N/A
	12	2014.9	2014.5	2014.2	0.0	N/A
	13	2014.2	2013.8	2013.5	0.0	N/A
	14	2013.5	2013.1	2012.7	0.0	N/A
	15	2012.7	2012.4	2012.0	0.0	N/A
	16	2012.0	2011.7	2011.3	0.0	N/A
	17	2011.3	2011.0	2010.6	0.0	N/A
Ghost Core 3	1	2019.2	2019.0	2018.8	118.0	N/A
	2	2018.8	2018.6	2018.4	38.1	N/A
	3	2018.4	2018.3	2018.1	40.8	N/A
	4	2018.1	2017.9	2017.7	9.7	N/A
	5	2017.7	2017.6	2017.4	6.1	N/A
	6	2017.4	2017.2	2017.0	4.0	N/A
	7	2017.0	2016.8	2016.7	3.7	N/A
	8	2016.7	2016.5	2016.3	2.6	N/A
	9	2016.3	2016.1	2015.9	2.0	N/A
	10	2015.9	2015.8	2015.6	1.7	N/A
	11	2015.6	2015.2	2014.9	0.0	N/A
	12	2014.9	2014.5	2014.2	0.0	N/A
	13	2014.2	2013.8	2013.5	0.0	N/A
	14	2013.5	2013.1	2012.7	0.0	N/A
	15	2012.7	2012.4	2012.0	0.0	N/A
	16	2012.0	2011.7	2011.3	0.0	N/A
	17	2011.3	2011.0	2010.6	0.0	N/A
	18	2010.6	2010.2	2009.9	0.0	N/A
	19	2009.9	2009.5	2009.2	0.0	N/A
	20	2009.2	2008.8	2008.5	0.0	N/A

Table 2-2: Average DNA copy number for Rainbow Trout and Brook Trout qPCR assays

	Section ID	Estimated Top cm Date	Estimated Middle cm Date	Estimated Bottom cm Date	Rainbow Trout Average DNA Copy #	Brook Trout Average DNA Copy #
Johnson Lake Core	1	2019.2	2017.5	2015.9	2.8	3.1
	2	2015.9	2014.2	2012.6	4.3	29.5
	3	2012.6	2010.9	2009.3	5.6	25.9
	4	2009.3	2007.6	2005.9	5.6	23.1
	5	2005.9	2004.3	2002.6	3.5	11.9
	6	2002.6	2001.0	1999.3	6.2	16.1
	7	1999.3	1997.7	1996.0	4.3	6.9
	8	1996.0	1994.4	1992.7	1.4	10.9
	9	1992.7	1991.1	1989.4	3.1	7.9
	10	1989.4	1987.8	1986.1	1.5	4.0
	11	1986.1	1982.8	1979.5	2.4	5.1
	12	1979.5	1976.2	1972.9	0.0	3.2
	13	1972.9	1969.6	1966.3	0.0	0.0
	14	1966.3	1963.0	1959.7	0.0	0.0

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Chapter 3 Taxonomic differentiation of *Tubifex* spp. that defines *Myxobolus cerebralis* transmission in Alberta, Canada

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Abstract

Following the first detection of the whirling disease-causing parasite *Myxobolus cerebralis* in 2016, the parasite has been detected throughout the South and Central regions of Alberta, Canada's. Since this discovery, research has focused on understanding the current and potential future spread throughout the province. Early efforts to understand how the oligochaete host, *Tubifex tubifex,* influenced transmission in Alberta described novel phylogenetic groupings that suggested that there may be oligochaete host specificity in Alberta that underpinned *M. cerebralis* spread. This study focuses on better understanding the relationship between *Myxobolus cerebralis* infection and these *T. tubifex* phylogenetic groupings through combined CoxI DNA barcoding of oligochaetes, oligochaete mitochondrial genome sequencing and qPCR assays targeting *M. cerebralis* DNA. Phylogenetic group 3 of the *Tubifex* consistently displayed high *M. cerebralis* DNA copy numbers, while groups 1 and 2 consistently yielded low *M. cerebralis* DNA copy numbers or possessed no detectable *M. cerebralis* DNA. These results suggest that phylogenetic group 3 *T. tubifex* primarily contributes to *M. cerebralis* propagation in South and Central Alberta. This data also provides an improved framework for reliable *T. tubifex* analyses in future studies.

Background

Whirling disease is a debilitating and often fatal parasitic infection of trout caused by the myxozoan *Myxobolus cerebralis* (Hofer, 1903). *Myxobolus cerebralis* is released from infected salmonid fish as myxospores following fish death, entering the definitive host *Tubifex tubifex*

(Müller, 1773; WOLF et al., 1986), through ingestion of myxospores. Following ingestion, the parasite migrates to the gut and intestinal lumen of the *T. tubifex* worm and matures into the triactinomyxon (TAM) stage (El-Matbouli and Hoffmann 1998; Wolf and Markiw 1984). The TAMs are released into the water column along with *T. tubifex* fecal packets (Gilbert & Granath, 2001), where the life cycle continues. The effects of infection on the intermediate fish host vary among species of salmonids as well as the age at which they are infected (Baldwin et al., 2000; R. Hedrick et al., 1999; R. P. Hedrick et al., 1999; Hoffman & Byrne, 1974; Markiw, 1991; O'Grodnick, 1979; Ryce et al., 2005). Younger salmonids have higher cartilage content relative to adult salmonids, making them more suitable for *M. cerebralis* proliferation (Hoffman & Byrne, 1974; Markiw, 1991; Putz & Hoffman, 1966; Ryce et al., 2005). Brown trout, *Salmo trutta* (Linnaeus, 1758), show lower susceptibility to disease compared to Rainbow Trout, *Oncorhynchus mykiss* (Artedi & Walbaum, 1788), with brown trout showing few to no clinical signs of whirling disease and infected tissues showing smaller and less severe lesions (Baldwin et al., 2000; R. Hedrick et al., 1999).

The definitive host of *M. cerebralis*: the freshwater oligochaete *T. tubifex,* is broadly distributed throughout freshwater habitats worldwide. *Tubifex tubifex* has been studied because of its tolerance for poor environmental conditions, including heavy metal pollution (Chapman, Farrel, et al., 1982; Chapman, Farrell, et al., 1982; Fargasova, 1994). A study conducted by Sturmbauer et al. (1999) described different genetic lineages of *T. tubifex* in Europe that had varying resistances to cadmium, a freshwater pollutant, based on the 16S rRNA region. The Sturmbauer et al. (1999) assay was then modified by Beauchamp et al. (2002) to describe *T. tubifex* genetic lineages with varying susceptibility to *M. cerebralis.* This lineage assay defined lineage III *T. tubifex* worms as a critical piece in the whirling disease puzzle, as lineage III worms produced the largest quantity of TAMs. The lineage assay provided a tool to characterize *T. tubifex* populations in regions where whirling disease was an expanding threat (Nehring et al., 2013; Rasmussen et al., 2008), as well as determine the risk of future spread of the disease (Arsan, Hallett., Bartholomew, 2007; Beauchamp et al., 2005).

In August 2016, *M cerebralis* was first detected in Alberta, Canada at Johnson Lake in Banff National Park. This detection sparked an ongoing province-wide monitoring effort resulting in the formation of partnerships between researchers and government bodies. Studies were

undertaken to understand the current distribution of *M. cerebralis* and evaluate potential future spread. These projects were able to expand on the understanding of genetic lineages of *Tubifex* through large-scale barcoding and phylogenetics (Barry et al., 2021). A new rapid qPCR assay was developed to detect *M. cerebralis* DNA more accurately in sediment as well as aquatic oligochaetes and expanded on the known freshwater oligochaete populations in the province (Barry et al., 2021). Finally, a study focusing on the southern Crowsnest region of Alberta, Canada, elucidated a key relationship between water body temperature and TAM release within the *T. tubifex* population (James et al., 2021). James et al. (2021) described a considerable overlap between temperatures required for TAM release from host *T. tubifex* and the period when juvenile Rainbow Trout are most susceptible to *M. cerebralis* infection. James et al. (2021) also studied the distribution of different aquatic oligochaete species within their study region through extensive CoxI barcoding.

This study aims to clarify the relationship between these *T. tubifex* phylogenetic groupings reported by Barry et al. (2021) concerning their potential for *M. cerebralis* propagation using a combination of new DNA barcoding data and existing data from James et al. (2021), as well as annelid mitochondrial genome data. For clarity, this paper will differentiate the Beauchamp et al. (2002) and Sturmbauer et al. (1999) worm lineages from the Barry et al. (2021) phylogenetic groups through different numeric systems. When referring to Beauchamp et al. (2002) and Sturmbauer et al. (1999), Roman numerals will be utilized, whereas Arabic numerals will be used when discussing Barry et al. (2021) groupings.

Methods

Site selection

Six sites were selected along the Crowsnest River and one site along the Old Man River watershed, all within southern Alberta, Canada. The northernmost site sampled, the Old Man River, was negative for *M. cerebralis*, with sites along the Crowsnest River increasing in positivity for *M. cerebralis* further south, as described in James et al. (2021). Worms were collected via kick-sampling using 200-µm mesh nets between July 17th and August 29th of 2019. Oligochaetes collected were split as described James et al. (2021) and transported to the University of Alberta for sorting, preservation in 95% ethanol and storing at -80°c.

Oligochaete qPCR testing

Oligochaete DNA was extracted using Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Hildon Germany) as described in James et al. 2021. Samples were run in triplicate through the 18S rDNA qPCR assay described in Barry et al. (2021) to detect *M. cerebralis* DNA, with replicates performed as described in James et al. (2021). Copy numbers of 18S rDNA reported are not adjusted for DNA extraction efficiency and are the mean quantity detected through qPCR.

Oligochaete barcoding

Initial barcoding of the CoxI gene was completed on a subsample of 350 oligochaetes, apportioned evenly among sites, with high-quality sequences returning for 330 individuals. After qPCR, further worm specimens were barcoded, focusing on worms with qPCR detections of *M. cerebralis* DNA, to a total 409 worms, following Barry et al. (2021). PCR volumes were doubled to allow amplified DNA to be run through Truin Science PCR cleanup kit (Truin Science, Edmonton, Alberta, Canada) following 1% agarose gel analysis. Forward and reverse sequences were trimmed of primer regions using SnapGene Viewer (https://www.snapgene.com/snapgene-viewer) software before being transferred and aligned in Geneious prime (https://www.geneious.com) software to extract the consensus sequence. Extracted consensus sequences were then compared to the online NCBI Genbank BLASTn database to find the closest matches. Extracted consensus sequences that matched ≥90% to any *T. tubifex* sequence on Genbank were then compared to our lab database of *Tubifex* groups from Barry et al. 2021 to determine the closest relationships.

Oligochaete genomics

Representative worms from the five *Tubifex* groups described in Barry et al. (2021) were sent to Psomagen, Rockville, MD, USA, for genome sequencing. I then assembled the returned reads into mitochondrial genomes using GetOrganelle (Jin et al., 2020). All GetOrganelle runs were seeded with a representative *T. tubifex* mitochondrial genome (accession MW960579.1, Lee and Jung 2022) to reduce required input reads and increase run efficiency. Mitochondrial genomes or largest scaffolded region of the respective mitochondrial genomes were utilized for downstream phylogenetic analyses of 16s, CoxI and 12s. Additionally, extracted 16s rRNA sequences were

aligned to published lineage assay sequences (Beauchamp et al., 2002; Sturmbauer et al., 1999) to clarify of potential relationships between these lineages and CoxI taxonomic groups from Barry et al. (2021). A database of annotations was created within Geneious prime (https://www.geneious .com) using annotations from the *T. tubifex* reference mitochondrial genome (MW960579.1), which was then used to annotate the sequenced mitochondrial genomes and scaffolds using >50% similarity limitations.

Phylogenetic analysis

Three target regions from the assembled genomes were extracted for phylogenetic analysis: CoxI, 12s and 16s. Target regions for all taxonomic groups were aligned in Geneious Prime (2023.0.4) along with the mitochondrial genome of a distantly related outgroup, the leech *Hirudo medicinalis* (EU100093 and KU672396). Alignments were then exported to MEGA-X version 10.2.6 (Tamura et al., 2021) to determine the best phylogenetic models using Bayesian Information Criterion (BIC), models with the lowest BIC available in the MrBayes plugin for Geneious Prime were used. GTR +gamma was the best match for 16s and 12s, whereas GTR +invgamma was the best match for the CoxI data. Phylogenetic trees were generated using the MrBayes extension for Geneious Prime with the respective models and parameters as follows: Chain Length: 10 000 000, subsampling frequency: 100 000, heated chains: 4, Burn-in length: 1 000 000 and heated chain temp and random seed as defaults.

Results

Barcoding results

Initial random barcoding returned good quality sequences for 330 worms (Figure 1). Further positive qPCR detection focused barcoding added 79 additional worms for a total of 409 worms successfully barcoded. Of these, 352 matched GenBank sequences identified as *Tubifex* species, 18 matched sequences identified as members of the genus *Limnodrilus,* 36 identified as belonging to Naidinae, two identified as *Ilyodrilus* spp. and one identified as Lumbriculidae. Only worms characterized as *Tubifex* taxonomic groups 1-3 of Barry et al. (2021) were found in this study, 125 from group 1, 159 from group 3, and 68 from group 2.

qPCR results

Of the total barcoded worms, 156 were positive for *M. cerebralis* based on DNA copy number thresholds established by James et al. (2021). Of the positive worms detected, 26% of site CRR-2 worms and 28% of site CRR-3 worms had qPCR results >50 copies of the target 18s (Table 1), with only two worms from site CRR-2 and three from site CRR-3 having copies >1000 (Table 1) all of which belonged to taxonomic group 3. All qPCR positive results for *M. cerebralis* were found in *Tubifex*-identified worms, except a single *Limnodrilus* worm returning a low positive result of 21.274 copies of 18s. Group 3 was the only group with qPCR-assessed *M. cerebralis* DNA copy number counts over 1000, with group 1 and group 2 having maximum DNA copy counts of 663.7 and 476.3, respectively.

Genomics results

Circular mitochondrial genomes of varying complexities were successfully assembled for *Tubifex* taxonomic groups 1, 2, 3 and 5 with 1453x, 1073x, 86.6x and 1939x coverage, respectively. Taxonomic group 4 could not be circularized; however, the largest scaffold assembled contained all target regions for downstream phylogenetic analyses with a length of 15,360 bp and 117x coverage. Taxonomic group 5 did have a small fragment outside of the circular genome containing a sequence of DNA mapping to CoxIII; however, this was discarded from downstream analyses due to very low coverage of 0.725x and a complete CoxIII gene still being contained within the high coverage (1939x) circular mitochondrial genome assembled.

Genetic variation between the representative taxonomic groups varied in similarity to the reference *T. tubifex* mitochondrial genome data (Figure 2). The taxonomic group with the highest similarity to the reference was 4, with all genes matching >95%, whereas the remaining taxonomic groups showed lower similarities, ≤91% similarity for all regions with the exception of 12s which had a maximum similarity of 95%, to the reference (Figure 2).

16s rRNA regions from the described CoxI taxonomic groups did not uniformly align with previously published 16s rRNA regions (Table 2), with group 4 aligning over 99% with both lineage III and lineage V.

Phylogenetic results

All three trees varied in the ordering of branching for *Ilyodrilus templetoni* and *Limnodrilus hoffmeisteri*; however, all iterations grouped these species basally to *T. tubifex*. The five *Tubifex* groups produced distinct branches in all the trees with a high posterior probability supporting this separation; however, a lower posterior probability was present in the CoxI tree for separating taxonomic group 5 and 4 at 0.62. Lineage assay results do not group consistently to taxonomic groups concerning transmission potential. This is evident in the grouping of 3 closely with lineage VI and lineage III grouping with 4.

Discussion

The findings of this study can be separated into two key discussion points. Our results demonstrate novel *Tubifex* species exist within Alberta, Canada oligochaete populations. These unique species *Tubifex* may vary in their ability to propagate *M. cerebralis*, and sp. 3 appears most compatible with *M. cerebralis* in Southern Alberta. This should be considered as conservation biologists and wildlife managers consider the threat of whirling disease to salmonid populations within the province and beyond.

The mitochondrial genomes and scaffolds assembled in this study can be used as groundwork for future comparative studies between these species and other global species of *Tubifex* to further elucidate geographic differences between species. As well, these mitogenomes can be utilized as reference data for transcription studies to evaluate *M. cerebralis* infection impact on mitochondrial gene expression of *T. tubifex,* which could lead to better understanding these differences in host compatibility. These mitochondrial genomes expand the available reference data and will be valuable for future genome sequencing studies globally.

Previous studies published single-gene phylogenies that separated *T. tubifex* worms from Alberta, Canada, into five genetic groups (Barry et al., 2021). Further multi-gene phylogenetic analyses comparing these taxonomic groups completed here suggest these groups are different species with an overall high posterior probability to support this claim. Each taxonomic group has three separate gene phylogenies (12s, 16s, and CoxI). These trees do vary in the basal positioning of *I. templetoni* and *L. hoffmeisteri*; however, these are likely due to the low quantity of reference sequences available online as well as the previously published (Barry et al., 2021) variation within the ‘species’ *L. hoffmeisteri*.

One of these species, *Tubifex* sp. 3 is the only one assessed in this study that exceeds the previously established *M. cerebralis* DNA copy number threshold that signifies infection. This suggests that *Tubifex* sp. 3 primarily underpins *M. cerebralis* transmission in Southern Alberta. Comparing 16s rRNA regions to published sequences from Beauchamp et al. (2002), taxonomic groups from Alberta do not align consistently with previously published lineage assay sequences, and lineages that have been previously described as being the main propagators of *M. cerebralis* group with Alberta representatives that have not shown the same potential. This is of note with Lineage III grouping with Lineage V and *Tubifex* sp. 4; Lineage III has been previously attributed as one of the two main genetic groups responsible for releasing TAMs when infected (Nehring et al., 2013; Beauchamp et al., 2002, 2005) but the same is not true for Lineage V and previous qPCR detections for *Tubifex* sp. 4 worms. A similar pairing is seen with the *Tubifex* sp. 3 representative, the group in this study that seems most important in propagating *M. cerebralis*, grouping with the resistant lineage VI. This further illustrates the difficulty experienced when applying the lineage assay in the province (Barry et al., 2021). It appears likely that *T. tubifex* lineage assessment is incompatible with the Albertan *Tubifex* species.

This study further elucidates the relationship between *M. cerebralis* infection and CoxI delineated *Tubifex* spp., showing *M. cerebralis*-positive *Tubifex* sp. 3 worms consistently having the highest quantity of *M. cerebralis* DNA copy number. *Tubifex* sp. 1 and sp. 2 did display positive results but possessed a lower average *M. cerebralis* 18S copy number that never exceeded 1000 copies per worm. As discussed by Barry et al. 2021; differentiating between a patent infection, where the worm is actively shedding TAMs into the water, and a pre-patent infection or a worm that has consumed a myxospore can be quite challenging given the sensitivity of qPCR methods and the low efficiencies of DNA extractions. Since *M. cerebralis* has an estimated 104 copies of 18s per cell (Kelley et al., 2004), Barry et al. (2021) calculated TAMs to have between 7200-8100 copies of 18s, and myxospores have between 600-712. With these numbers, we can conservatively estimate that in this study, *Tubifex* sp. 3 is the only species of *Tubifex* worms with the potential tobe shedding TAMs into the water. In contrast, *Tubifex* sp. 1 and sp. 2, due to the low mean *M. cerebralis* DNA copy number, may be consuming spores within the sediment but not reaching patent infection. *Tubifex* sp. 4 and sp. 5 were not found within the sample sites in this study. This coincides with populations described in Barry et al.

(2021), with *Tubifex* sp. 4 worms found in more northern watersheds and sp. 5 worms only rarely found within the Bow River watershed.

Barry et al. (2021) did not find any *M. cerebralis*-positive representatives from these two species; overall, they were quite rare within the assessed freshwater oligochaetes in Alberta. Given the low representation of these species in past population analyses, as well as the prevalence of whirling disease in southern Alberta, it is unlikely that *Tubfiex* sp. 4 and sp. 5 worms are contributing significantly to the spread of *M. cerebralis* in these waterbodies. Future studies should target assessing live populations of each of these 5 respective *Tubifex* subspecies and their compatibility with *M. cerebralis* to extrapolate on these initial findings, as well as acquire morphological data to better describe and name these species.

Mapping of initial discrete barcoding results detail the complex relationship between *M. cerebralis* detections and *Tubifex* species present at these sample sites (Figure 1). Sample sites CRR-2, CRR-3 and CRR-4 are exclusively populated by *Tubifex* species according to initial random barcoding, these same sites have also found to have high overlap of thermal regimes for TAM release and Rainbow Trout susceptibility (James et al., 2021). Site CRR-1 was found to have presence of high transmitting species of *Tubifex* but has been found with lower thermal regime overlap than other sites and is downstream of the fish barrier, Lundbreck falls (James et al., 2021), whereas CRR-5 and CRR-6 were found with lower proportions of *Tubifex* spp. and particularly low sp. 3 detections. Site OMR-1 is of particular interest as it was found to only contain *Tubifex* spp. postulated here to transmit *M. cerebralis*, but no positive detections were recorded. This observation could be due to the geographic location of this site, with increased positive detections in the future as water flows downstream and whirling disease progresses in the province. Site OMR-1 has also previously been shown to have the lowest thermal regime overlap within these study sites (James et al., 2021), reducing possibility of disease propagation. Future research should focus on this site and elucidating the underlying causes of this relationship.

It is unknown whether these described *Tubifex* species' representatives exist elsewhere and with the same host-parasite compatibility phenotype. There are different population dynamics within the provincial watersheds, so this relationship is likely more complex than initially understood. We suggest that future assessments of *Tubifex* spp. populations within and outside the province

of Alberta focus on CoxI gene sequencing as a more reliable method of *Tubifex* spp. identification and determination of potential *M. cerebralis* transmission. Further, populations of *Tubifex* in other high *M. cerebralis* transmission locations require additional investigation to determine whether the *M. cerebralis* propagation potential holds consistent across the same species in different geographical locales.

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Tables and Figures

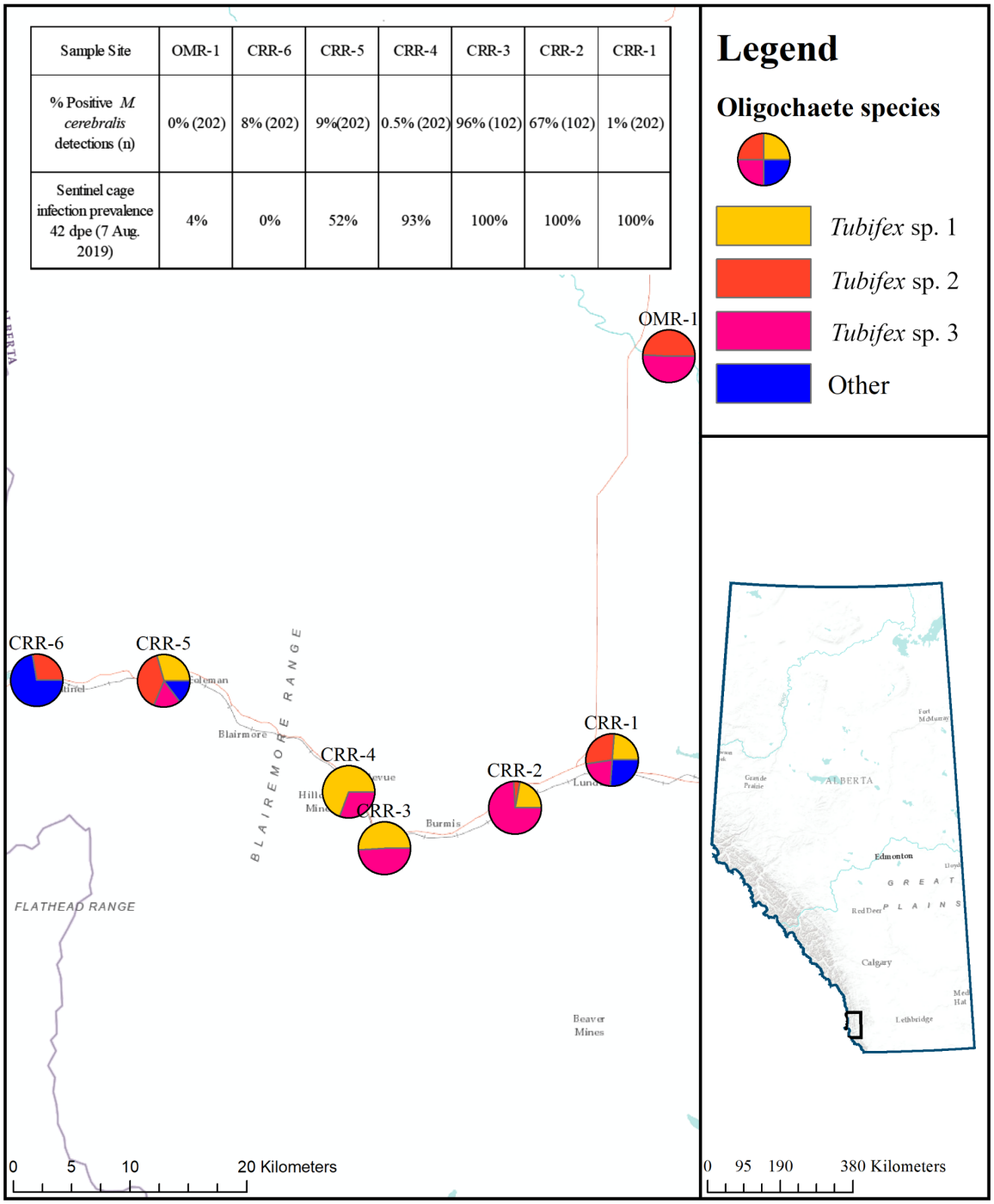

Figure 3-1: Distribution of aquatic oligochaete species from initial indiscriminate sampling for each study location. Chart proportions labelled “other” indicate all oligochaetes not matching to *Tubifex* spp. The overlayed infection prevalence table is select data from James et. al 2021 (27).

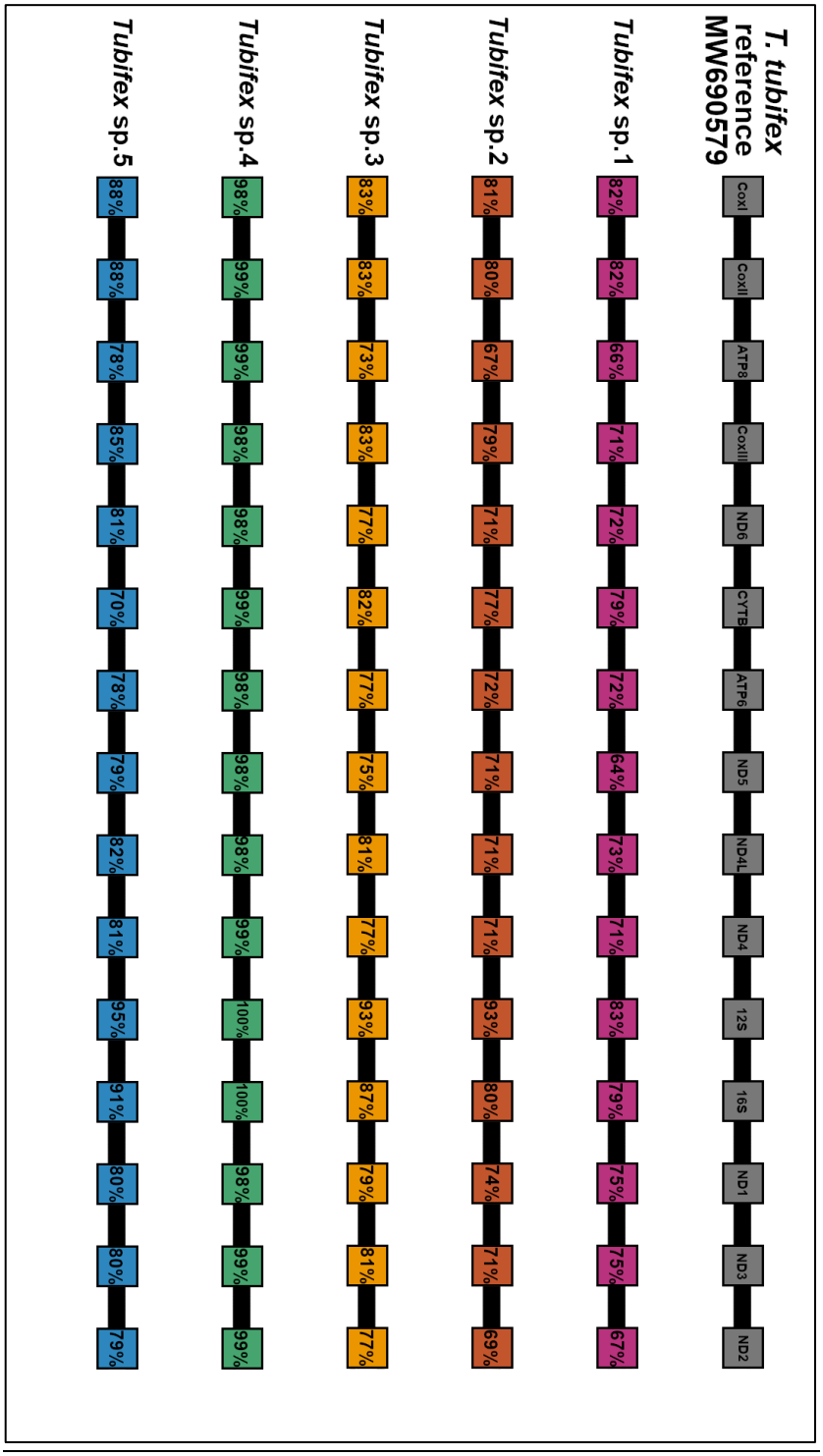

Figure 3-2: Percent identity comparisons for the respective *Tubifex* spp. found in Alberta, CA, to the published regions found in the reference mitochondrial genome of *T. tubifex.* Percentages are between the respective species and the reference exclusively, not between the species themselves.

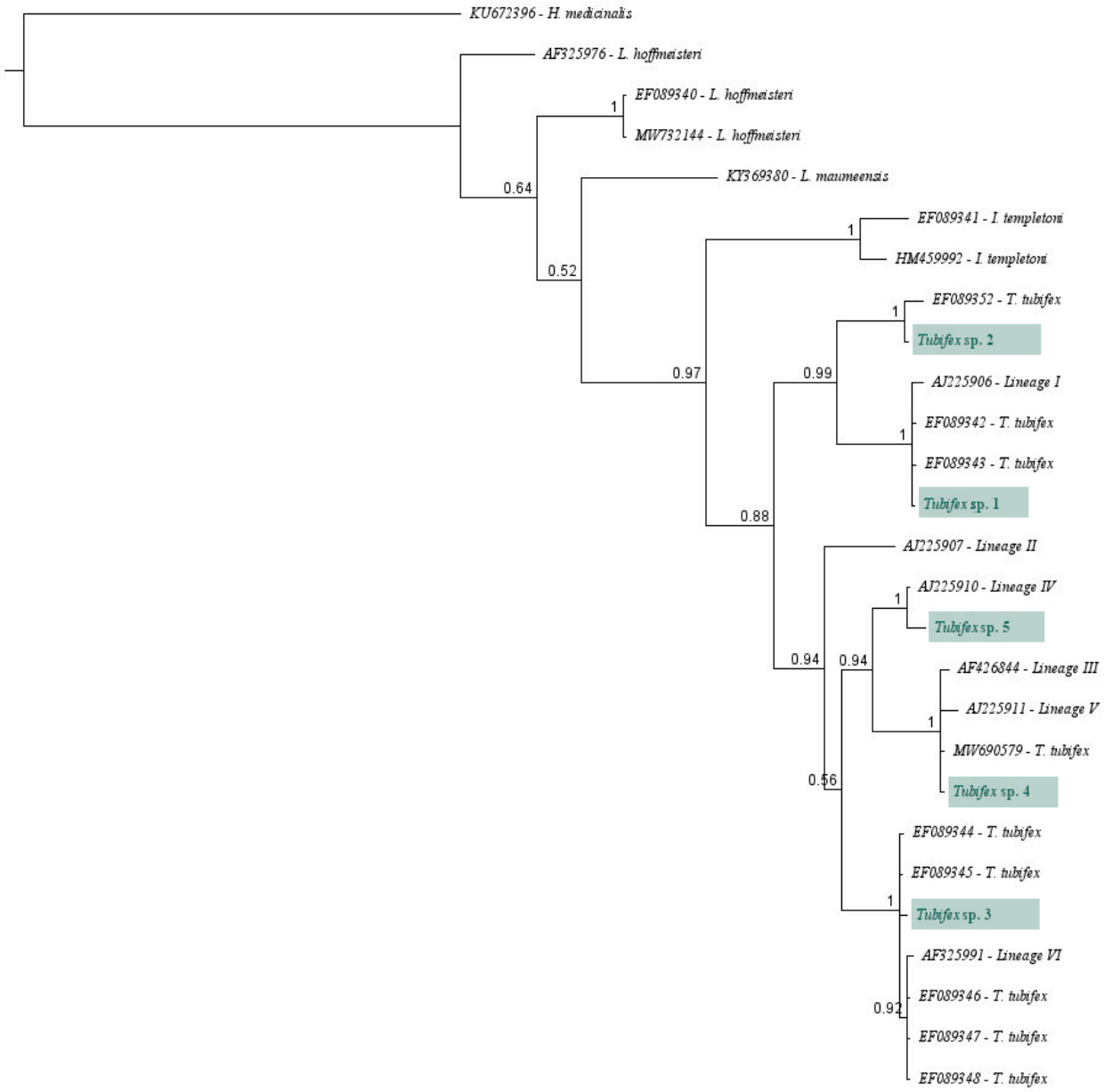

Figure 3-3: Phylogenetic tree of 16s rRNA region extracted from mitochondrial genomes of *Tubifex* spp. shown in green. Representative sequences for the previously used lineage assay are noted with their respective roman numerals, as well as additional freshwater aquatic oligochaetes sequences available from NCBI. Labels included on branches indicate the calculated posterior probability.

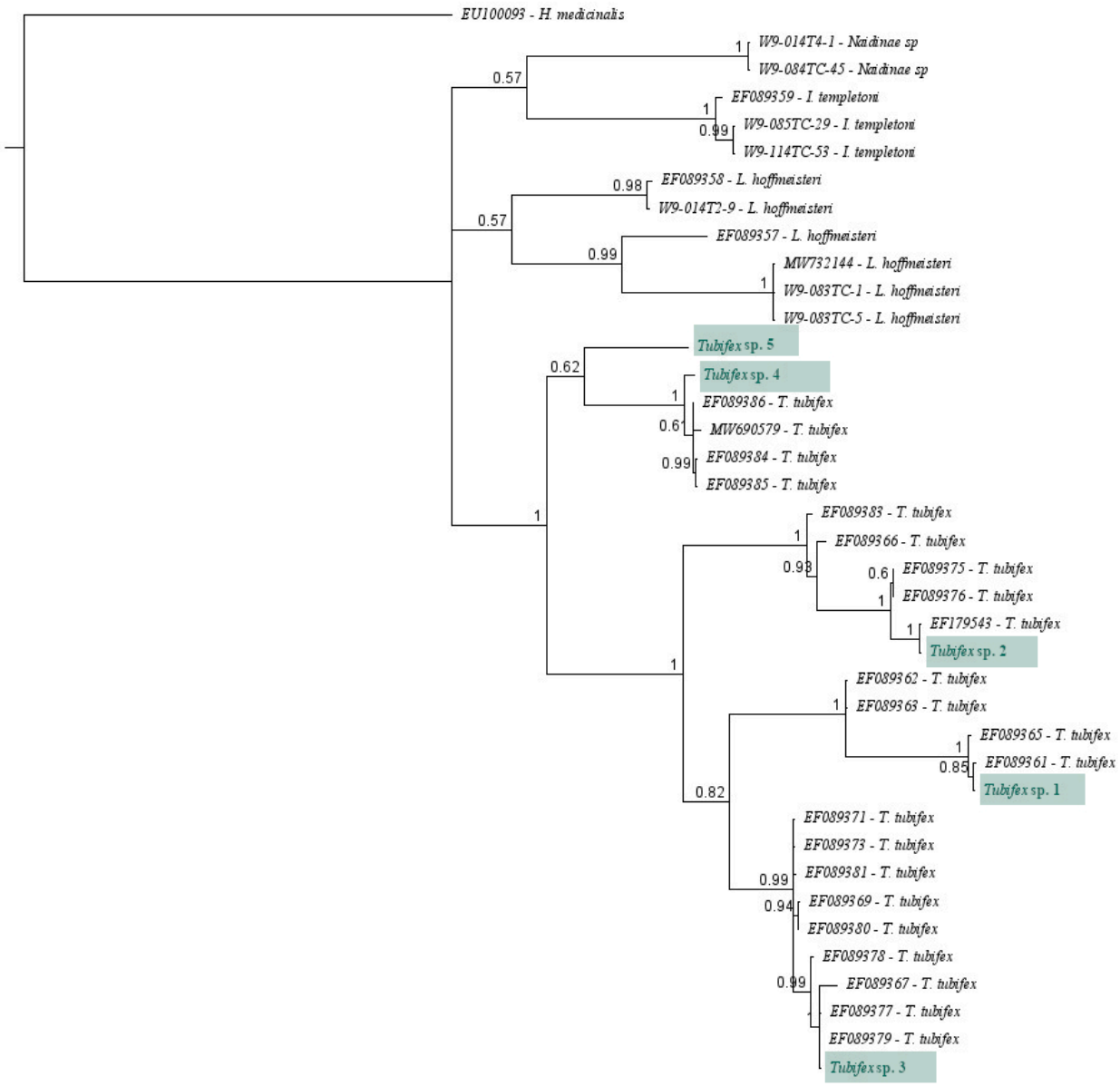

Figure 3-4: CoxI phylogenetic tree of reference NCBI sequences and representative Alberta aquatic oligochaete species, emphasizing *Tubifex* sequence distinctions. Branches labelled to include the calculated posterior probability. Extracted mitochondrial sequences for *Tubifex* spp. are noted in green.

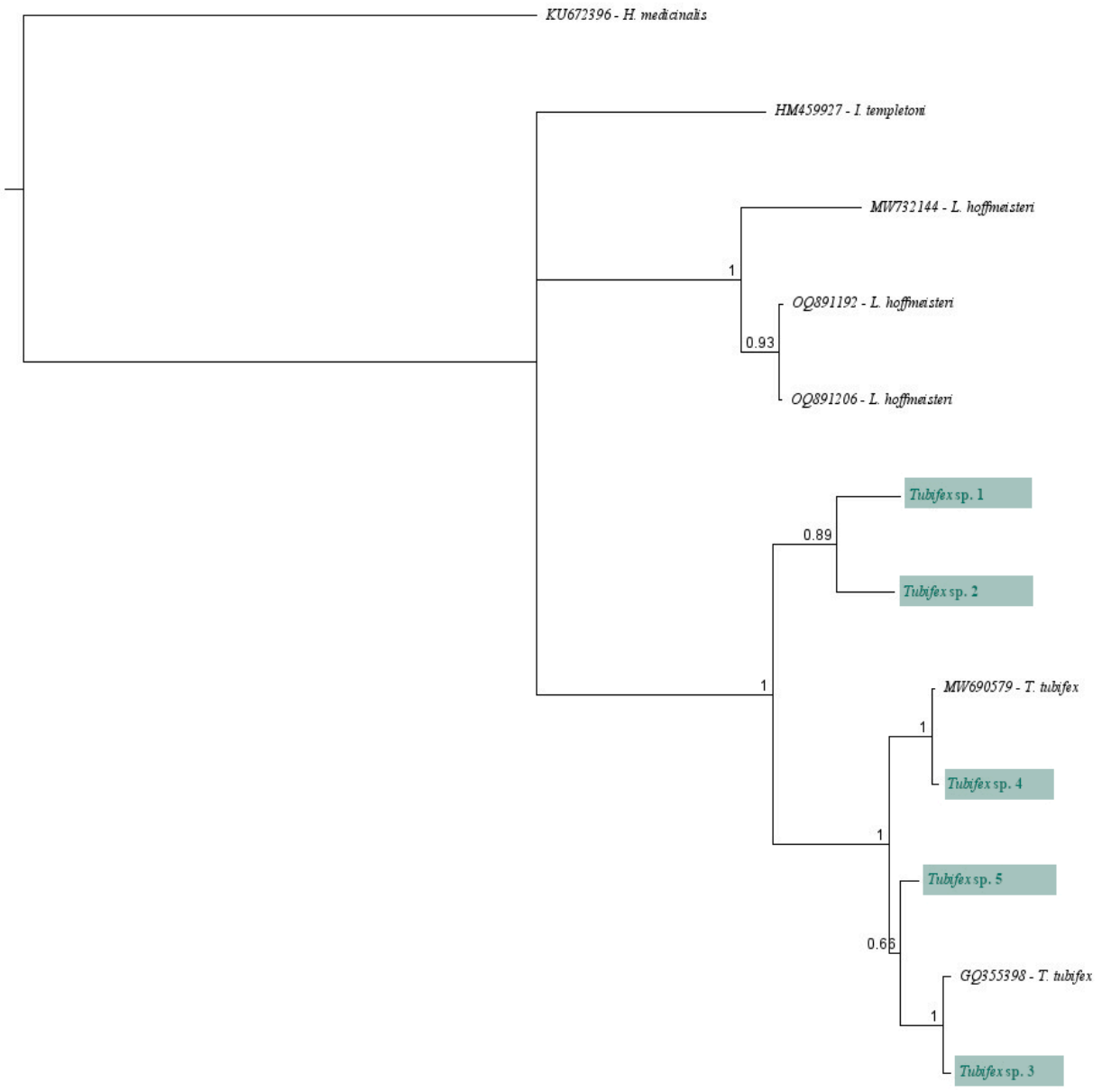

Figure 3-5: Phylogenetic tree using 12S rRNA for species of *Tubifex* from Alberta, Canada and reference NCBI sequences. Green notation has been used for *Tubifex* spp. found in Alberta., Canada. Branches labelled to indicate the posterior probability calculated.

Table 3-1: Sequenced oligochaete (CoxI) samples with >50 copies of *M. cerebralis* DNA (18s) detected by qPCR shown by site.

Site ID	Processing ID	Ct Mean	Quantity Mean	*Tubifex* spp.
CRR-2	W9-016TC-100	28.092	433.847	3
CRR-2	W9-016TC-23	29.622	187.831	3
CRR-2	W9-016TC-48	19.288	159,529.313	3
CRR-2	W9-016TC-57	20.594	67,078.867	3
CRR-2	W9-016TC-58	30.923	70.714	1
CRR-2	W9-016TC-59	30.995	67.249	1
CRR-2	W9-016TC-78	28.846	290.877	1
CRR-2	W9-016TC-84	29.555	183.526	3
CRR-2	W9-016TC-86	29.000	263.395	3
CRR-2	W9-016TC-87	31.333	57.936	3
CRR-2	W9-016TC-89	29.133	242.135	3
CRR-2	W9-016TC-90	30.543	96.532	1
CRR-2	W9-016TC-91	30.321	111.511	3
CRR-2	W9-016TC-93	29.092	248.064	3
CRR-2	W9-016TC-94	27.578	663.714	1
CRR-2	W9-016TC-95	27.955	476.341	2
CRR-2	W9-016TC-96	30.586	78.817	1
CRR-2	W9-016TC-97	29.425	174.635	1
CRR-2	W9-016TC-99	27.509	648.215	3
CRR-3	W9-017TC-13	25.552	2,771.043	3
CRR-3	W9-017TC-16	29.801	159.601	3
CRR-3	W9-017TC-18	29.947	145.007	3
CRR-3	W9-017TC-20	30.639	91.515	1
CRR-3	W9-017TC-24	31.491	51.550	3
CRR-3	W9-017TC-31	30.928	75.158	3
CRR-3	W9-017TC-32	31.335	57.385	3
CRR-3	W9-017TC-35	30.021	137.867	1
CRR-3	W9-017TC-38	16.794	1,006,685.750	3
CRR-3	W9-017TC-39	27.772	627.409	3
CRR-3	W9-017TC-44	28.043	522.783	1
CRR-3	W9-017TC-46	30.569	95.766	3
CRR-3	W9-017TC-47	29.958	145.321	3
CRR-3	W9-017TC-49	29.572	186.854	1
CRR-3	W9-017TC-52	30.964	73.358	3
CRR-3	W9-017TC-55	30.002	140.270	3
CRR-3	W9-017TC-58	31.787	52.388	3
CRR-3	W9-017TC-65	30.976	88.637	1
CRR-3	W9-017TC-74	30.537	118.584	3
CRR-3	W9-017TC-8	30.935	74.728	3
CRR-3	W9-017TC-80	30.225	144.638	3
CRR-3	W9-017TC-85	29.915	131.204	1
CRR-3	W9-017TC-86	30.688	77.089	3
CRR-3	W9-017TC-90	30.646	79.494	3
CRR-3	W9-017TC-91	31.051	60.423	3
CRR-3	W9-017TC-96	27.832	563.154	1
CRR-3	W9-017TC-99	24.411	5,864.696	3

Table 3-2: Distance matrix of 16s rRNA alignment depicting percent similarity between sequences

	Tubifex sp.5	*Tubifex* sp.4	*Tubifex* sp.3	*Tubifex* sp.2	*Tubifex* sp.1	AJ225911 Lineage V	AJ225910 Lineage IV	AF426844 Lineage III	AJ225907 Lineage II	AJ225906 Lineage I	AF325991 Lineage VI
Tubifex sp.5		94.054	92.992	87.634	87.097	93.243	98.919	93.784	91.935	86.828	26.665
Tubifex sp.4	94.054		91.644	87.097	86.828	99.189	94.865	99.73	92.204	86.559	26.264
Tubifex sp.3	92.992	91.644		88.172	88.978	90.836	93.801	91.375	92.204	88.71	28.363
Tubifex sp.2	87.634	87.097	88.172		92.162	87.366	87.634	86.828	89.189	91.892	24.96
Tubifex sp.1	87.097	86.828	88.978	92.162		87.634	87.366	86.559	88.649	99.458	25.26
AJ225911 Lineage V	93.243	99.189	90.836	87.366	87.634		94.054	98.919	91.398	87.097	87.702
AJ225910 Lineage IV	98.919	94.865	93.801	87.634	87.366	94.054		94.595	92.473	87.097	90.659
AF426844 Lineage III	93.784	99.73	91.375	86.828	86.559	98.919	94.595		91.935	86.29	67.687
AJ225907 Lineage II	91.935	92.204	92.204	89.189	88.649	91.398	92.473	91.935		88.649	88.837
AJ225906 Lineage I	86.828	86.559	88.71	91.892	99.458	87.097	87.097	86.29	88.649		85.852
AF325991 Lineage VI	26.665	26.264	28.363	24.96	25.26	87.702	90.659	67.687	88.837	85.82	

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Chapter 4 Synthesis and future directions

This thesis can be split into two main foci within the field of whirling disease: (1) the history of the disease's introduction in the province of Alberta, and (2) the potential future spread of the disease. When discussing invasive species, there are many pieces that need to be addressed to understand the impact on the ecosystem being invaded. To truly grasp the threat *M. cerebralis* poses to the provincial aquatic ecosystems we must understand all contributing factors to the success and proliferation of the parasite. This research has started parsing apart the story of how *M. cerebralis* was introduced to the province, though we may never truly know the exact introduction timeline. We now know that the year the first whirling disease-positive fish were detected at Johnson Lake, 2016, is likely the first-time whirling disease was present in that waterbody. I think it is important that future studies expand on this paleo eDNA project to more southern locations in Alberta, such as the Crowsnest River region, where this disease is widespread. With proximity to the state of Montana, where whirling disease has been present for decades, the possibility for multiple introduction events is possible. Additionally, larger sample sizes within the same waterbody would be beneficial to allow for a more robust dataset and would allow for additional data about sediment deposition and age depths within the waterbody. Knowing how long a parasite has been present in an invaded ecosystem is crucial in directing efforts towards preventing future spread and preparing infected areas for the impact of the parasite over time. This project has given insight into the timeline from initial parasite introduction to parasite establishment within Alberta, Canada.

The oligochaete host, *T. tubifex,* has been the focus of many studies aiming to find an ecological barrier to whirling disease spread. Previous methods focusing on end-point PCR analysis of *T. tubifex* have been the focus for many years within the United States following the introduction of *M. cerebralis.* Given the continuously growing sequencing data available, and the decrease in cost for targeted gene sequencing, genetic barcoding is being proven as a more reliable method of species identification. This study has expanded the knowledge regarding the genetic populations of *T. tubifex* present in Alberta, but also in relation to the populations outside the province. There is growing evidence for a relationship between parasite compatibility with specific groups of *T. tubifex* varying in their mitochondrial genetics (figure 1). From the samples analyzed in this project taxonomic group 3 worms could be a future target for determining waterbodies at risk for future invasion in the province, as they are the only subspecies with qPCR

M. cerebralis gene copies over 1000 which is indicative of a patent infection. This is particularly important when considering northern Alberta watersheds, which have yet to be declared positive by the CFIA. This project has generated important genetic information on five *T. tubifex* representatives from Alberta, Canada that support the separation of these organisms into subspecies. All mitochondrial genes for these five respective subspecies have been sequenced providing a glimpse into how different these subspecies are. This study is the first to examine multiple gene targets in *T. tubifex* phylogenetically with respect to potential susceptibility to infection by *M. cerebralis*. The results of this study provide the foundation for future research into the genetic diversity of the *T. tubifex* worm. Future studies should direct efforts at sequencing populations of northern *T. tubifex* and look at any relationships found between parasite compatibility and worm host. Further, studies should be completed targeting immunological responses to *M. cerebralis* infection within these subspecies of *T. tubifex* and examine the potential genetic differences that are responsible.

The techniques used throughout this research, however, are not bound to whirling disease research. The first half of this project, surrounding paleo eDNA can be widely applied to learn about the introduction of other invasive species such as Zebra mussels (*Dreissena polymorpha*), Mystery Snails (*Pomacea bridgesii*) and others in any location of interest. The potential data generated could prove invaluable with regards to understanding different invasive species spread and targeting control efforts to ensure ecological stability. This project has also contributed large datasets to the field of whirling disease research, supporting previous provincial research in the need for updating the methods used to detect and assess ecological barriers to disease spread both provincially and worldwide.

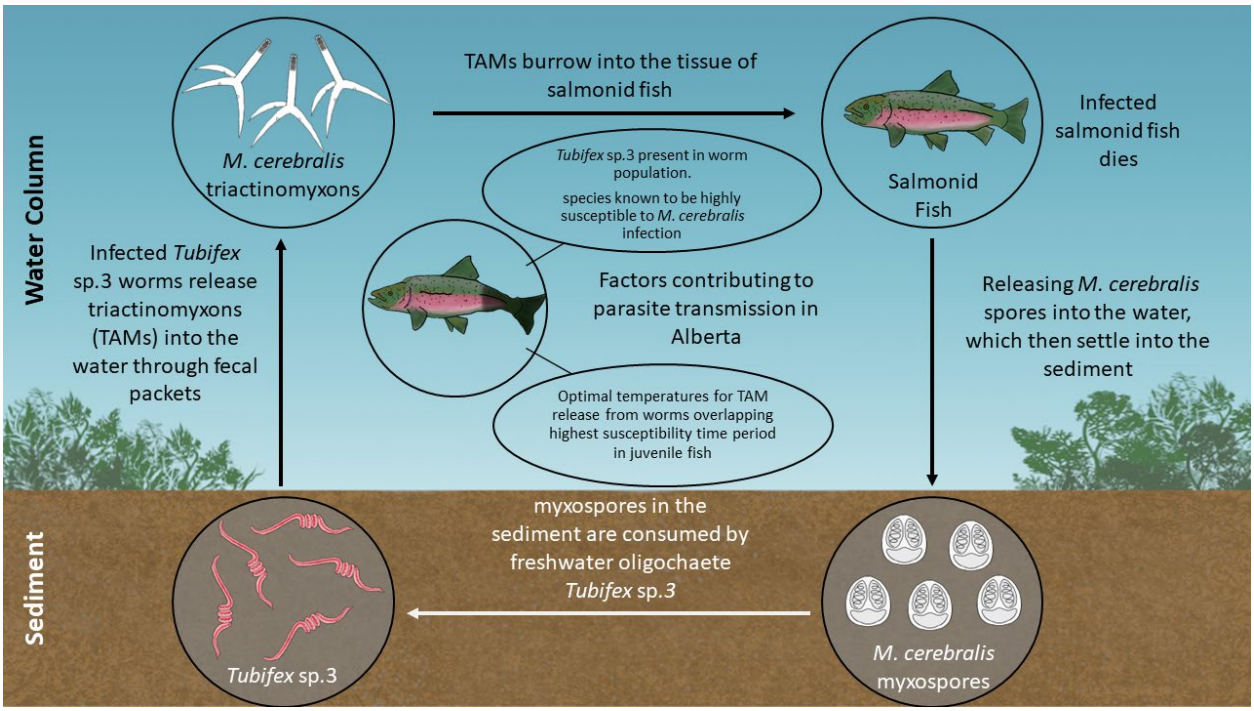

Figure 4-1: Life-cycle of *M. cerebralis*, updated with research completed within Alberta, Canada

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